Curtis D. Klaassen
John B. Watkins III

CASARETT & DOULL'S ESSENTIALS of TOXICOLOGY

Fourth Edition

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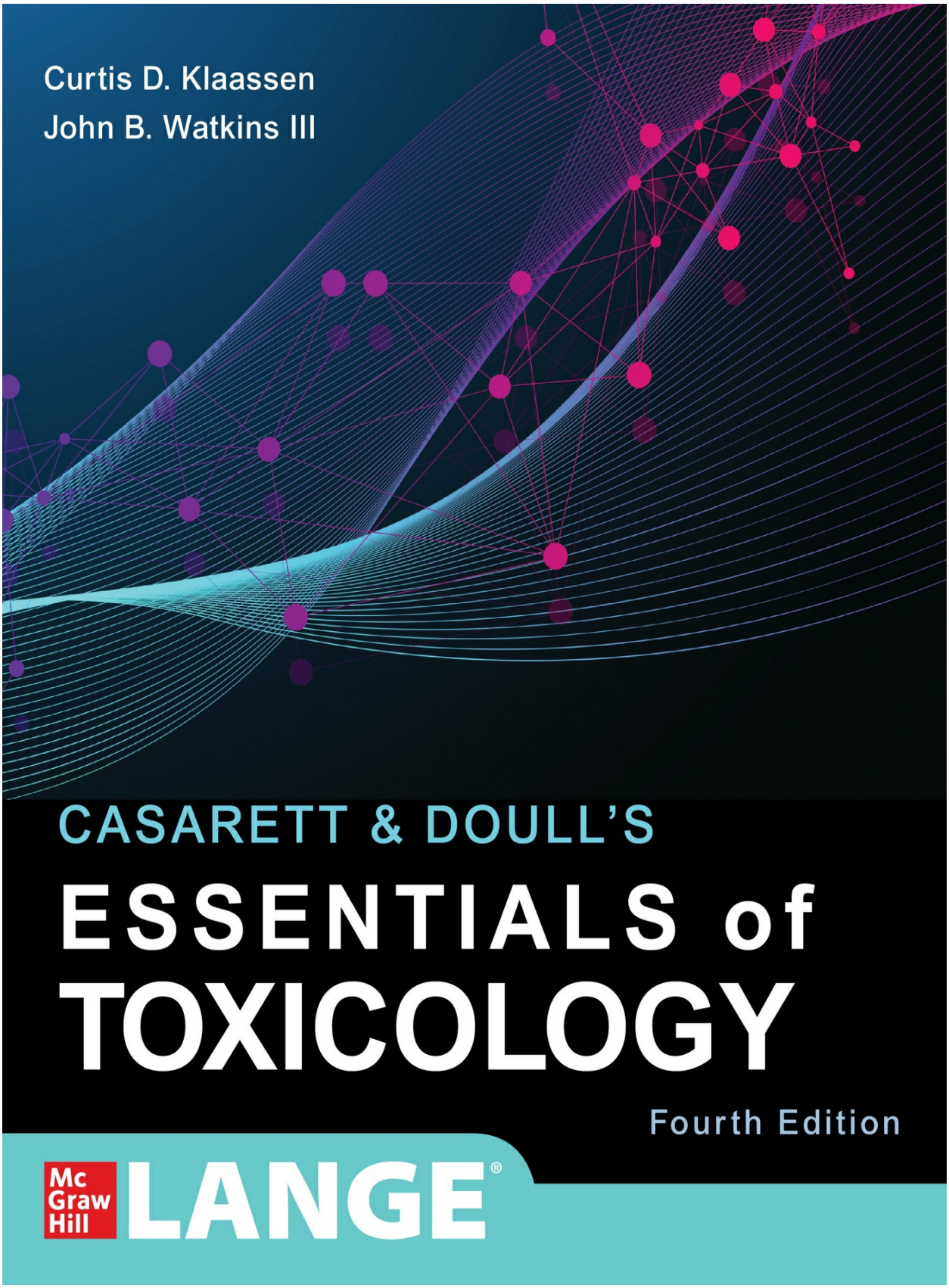

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Casarett & Doull's Essentials of Toxicology

Fourth Edition

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Preface

This fourth edition of *Essentials of Toxicology* condenses the principles and concepts of toxicology that were described in the ninth edition of *Casarett & Doull's Toxicology: The Basic Science of Poisons*. *Essentials of Toxicology* succinctly defines the expansive science of toxicology, and includes important concepts from anatomy, physiology, and biochemistry to facilitate the understanding of the principles and mechanisms of toxicant action.

We greatly appreciate the authors who contributed to the ninth edition of *Casarett & Doull's Toxicology: The Basic Science of Poisons* because their chapters in the parent text provided the foundation for this edition of *Essentials of Toxicology.*

The book is organized into seven units: (1) General Principles of Toxicology, (2) Disposition of Toxicants, (3) Non-organ-directed Toxicity, (4) Target Organ Toxicity, (5) Toxic Agents, (6) Environmental Toxicology, and (7) Applications of Toxicology. Key points and review questions are provided for each chapter.

We trust that this book will assist students in undergraduate and graduate courses in toxicology, as well as students from other disciplines, to develop a strong foundation in the concepts and principles of toxicology. We invite readers to send us suggestions of ways to improve this text and we appreciate the thoughtful recommendations that we received on the last edition.

We particularly give a heartfelt and sincere thanks to our families for their love, patience, and support during the preparation of this book. The capable guidance and assistance of the McGraw-Hill staff are gratefully acknowledged. Finally, we thank our students for their enthusiasm for learning and what they have taught us during their time with us.

Curtis D. Klaassen
John B. Watkins III

UNIT 1 GENERAL PRINCIPLES OF TOXICOLOGY

CHAPTER 1

The Evolving Journey of Toxicology: A Historical Glimpse

Philip Wexler and Antoinette N. Hayes

KEY POINTS

- Toxicology is the study of the adverse effects of xenobiotics on living systems.
- Toxicology assimilates knowledge and techniques from biochemistry, biology, chemistry, genetics, mathematics, medicine, pharmacology, physiology, and physics.
- Toxicology applies safety evaluation and risk assessment to the discipline.

INTRODUCTION

The word *toxicology* is derived from the Latinized form of the Greek word *toxicon*, meaning "arrow poison." *Poison*, as a noun, dates back to the Old French *poison* or *puison*, meaning, originally a drink, especially a medical drink, but later signifying more of a magical potion or poisonous drink. The commonly misused term *toxin* formally should be used to refer to toxic substances produced biologically. Other terms, *toxicant*, *toxic agent*, and *toxic substance*, could be used to delineate the broader category of substances that are toxic, regardless of origin. *Xenobiotics* is a term referring to substances, whether toxic or not, foreign to a given organism.

ABOUT HISTORY

History is *about* the past; it is not the past. The past is passive, objective, all encompassing. History is active, subjective, and selective. The further back in time that we look, the more problematic it is for us to reach, in the present, conclusions about what happened in the past.

Science begins with observation. In the distant past, our observational skills did not extend beyond our senses in assessing toxicity and safety. Our hominin ancestors used the process of trial and error making careful note of which substances, particularly potential food sources, were safe and which were hazardous. Although it might very well be after the damage was done, they and their tribe and descendants would quickly learn to differentiate between the safe and toxic. Toxic substances were to be avoided, unless used against enemies.

TOXICOLOGY IN ANTIQUITY

Ancient China

Shen Nong, the legendary founder of Chinese Herbal Medicine, also known as the farmer god, and said to live circa 2800 B.C., was said to have tasted hundreds of herbs daily to differentiate the poisonous from the medicinal or just plain edible. He is considered the compiler of perhaps the world's first pharmacological compendium, *Divine Farmer's Classic of Materia Medica*.

Du (毒) is the standard word for poison or toxicity in Chinese. It was understood by the ancient Chinese that herbals were potentially toxic and dose played a role. Aconite, derived from the plant wolfsbane and possessing extreme potential toxicity, was widely used medicinally in

small doses in China over 2000 years ago—usually applied externally to treat various wounds or ingested as a tonic to restore qi (the vital energy defined by Chinese medicine) and extend life. Unadulterated aconite in larger doses was often used to murder. Today we know that the alkaloids in aconite have a narrow therapeutic index and their use is not generally recommended.

Ancient India

Ancient India was no stranger to the knowledge and uses of poisons. Indian surgeon Sushruta prepared *Suśrutasam.hitā*, a foundational medical/surgical compendium for Ayurveda (traditional Indian medicine). Volume 5 contains several chapters related to poisons and poisoning, including descriptions of vegetable and mineral poisons and animal poisons, as well as advice on medical treatment of snake bites and insect bites. *Agada Tantra*, one of the eight clinical specialties of Ayurvedic medicine, is specifically associated with toxicology.

India has a long tradition of tales about the so-called "venomous virgin" who would, as a young girl, be fed "tolerably minute, but gradually increasing, amounts of poison or snake venom, so that by the time she was an attractive young woman, the level of toxin in her body would be so high that she could be sent to an enemy king as a gift. Upon kissing her, making love to her, or even just sharing glass of wine with her, he would instantly fall dead."

Ancient Egypt

Venomous snakes and insects were well known and the focus of toxicology as it existed in ancient Egypt. One of the major documents examining snakebite is the Brooklyn Papyrus (held by the Brooklyn Museum), 525–600 b.c. For example, Paragraph 15 of the Papyrus describes the snake known by the Egyptians as Apophis which, mythologically, personified evil. Scholars believe this may be the Boomslang (*Dyspholidus typhus*) in the Colubridae family. Symptoms and signs of snake envenomation are presented in the Papyrus. The treatments offered could be general, for any snakebite, or specific. Bites by snakes known to be lethal generally received no treatment. Therapeutic measures, overall, were largely symptomatic. Toxicity is addressed to a lesser extent in the Berlin, Edwin Smith, and Ebers papyri.

The death of Cleopatra VII, born in 69 b.c. and one of the most fascinating personalities in Egypt when Greece and Rome held sway, holds toxicological interest for us. After learning that Marc Anthony killed himself by a self-inflicted sword wound, Cleopatra decided to follow suit by supposedly holding an asp (Egyptian cobra) to her breast and succumbing to its venomous bite. However, she may have been murdered perhaps with a poisonous draught by Octavian, the victor in their battle, who then spread the rumor of her suicide to avoid retribution by her adoring subjects.

Pontus, Mithridates, and Theriacas

Mithridates VI, ruler of Pontus in northeastern Turkey beginning in 120 b.c., experimented with poisons and antidotes, even on himself. Son of a father who was murdered with poison and a mother who would have poisoned him in order to ascend to the throne, he went into hiding for a period of years. He returned to capture his rightful position by likewise using poison, probably arsenic. He feared assassination by poison and took precautions to avoid it.

His approach was to ingest small doses of toxicants to become tolerant to them. His lifelong pursuit was to create a universal antidote, which came to be known as a theriac. His Mithridatium was a concoction of tiny amounts of deadly poisons and antidotes. When Mithridates wanted to end his own life, consumption of poison weakened him, but he did not die. In one version of his actual death, he appealed to his bodyguard to impale him with a sword.

Ancient Greece

Nicander of Colophon (fl. 130 b.c.), a Greek poet and physician, is the author of two of the oldest extant works on poisons—*Theriaka* and *Alexipharmaka*. The *Theriaka* concerns venomous animals, including snakes, spiders, scorpions, insects, lizards, and fish. His *Alexipharmaka* deals with 21 poisons from the vegetable, mineral, and animal kingdoms. Among them are aconite, white lead, and hemlock. In both works, Nicander describes the poison, its symptoms, and antidotes.

The Greek philosopher, Socrates (469–399 B.C.), became an iconic figure in the history of toxicology through his death. Convicted of corrupting the youth of Athens and disrespecting the gods, he was sentenced to death. His execution was supposedly carried out in suicidal fashion, with Socrates condemned to drink an extract of hemlock (*Conium maculatum*). This story has been questioned largely because the account provided in Plato's *Phaedo* describes a clinical disorder not caused by hemlock poisoning, but rather a possible mixture of hemlock and opium.

Alexander the Great (born in 356 b.c.) plays a role in the history of toxicology in that the cause of his death is an unsolved mystery. He is said to have drunk vast quantities of wine at a banquet in Babylon, after which he suffered severe abdominal pain. Over days, things went from bad to worse and he developed partial paralysis finally dying two weeks later. Rumors of poisoning began circulating in no time. Though speculation is widespread, the true cause of Alexander's death has never been confirmed.

The Oracle at Delphi, perhaps the most important and sacred shrine in ancient Greece, is associated with the Greek god Apollo. Pilgrims to Delphi would address their questions to the Pythia, a role filled by various women at different times. Plutarch, the celebrated Greek biographer and essayist, served as one of the priests at the temple of Apollo at Delphi. He noted that *pneuma* (a kind of gas or vapor) was emitted in the *adyton*, a small inner sanctum type area. The Pythia would inhale the *pneuma* and go into a trance, after which a priest would ask her the questions raised by the petitioners. It may be that the trancelike state of the Pythia was induced by inhaling ethylene gas or a mixture of ethylene and ethane from a naturally occurring vent of geological origin.

Toxicology is also heir to a rich mythological tradition. After Hercules killed the nine-headed sea monster known as the Hydra, as part of his second labor, he cut it open and dipped his arrows in its venom, providing him with what may have been the first biological weapon for use in battle. Achilles, one of the prominent heroes in Homer's *Iliad* was a victim of just such a poison. Immersed as an infant in the river Styx by his mother to make him immortal, she failed to realize that in holding him by the heel, that very part of the body would make him susceptible to future danger. In the final battle of the Trojan War, he was killed by a poisoned arrow shot into this heel.

Ancient Rome

The Romans of antiquity were also knowledgeable in the principles and practice of toxicology. Dioscorides (born in 40 a.d.), a native of Anazardus, Cilicia, Asia Minor, was a physician who traveled throughout the Roman Empire and collected samples of local medicinal herbs. The information he gleaned was compiled in the encyclopedic *De materia medica* in the first century a.d. In it he classified poisons as animal, plant, or mineral. More specifically, *De Venenis* and *De venenosis animalibus*, ascribed to Dioscorides but probably not written by him, covered poisons in general and animal venoms, respectively.

Galen, born (129 a.d.) in Pergamon, was a firm subscriber to the theory of the humors (blood, yellow bile, black bile, and phlegm), the origins of which may go back to ancient Egypt but which were first articulated about medicine by Hippocrates. Galen formulated his own Galeni Theriaca and claimed it improved upon the one concocted by Mithridates. He wrote about assorted theriac compounds in his books *De Antidotis* I and II and *De Theriaca ad Pisonem*.

The legal framework of toxicology is sometimes dated back to the age of the Roman military and political leader Sulla. Under the *lex Cornelia de sicariis et veneficis* (81 b.c.), punishment was imposed for anyone who prepared, sold, bought, kept, or administered a noxious poison (*venenum malum*).

In 1983, Jerome Nriagu popularized the idea that the metal lead was responsible for the fall of the Roman Empire. It has been stated that lead contamination in water supplies, cooking, and the production of wine, ultimately decreased fertility and reproductive capacity. Recent archaeological investigations have found that the mean skeletal lead content of populations at the time was less than half that of present-day Europeans in the same regions. The assertion that lead was the primary culprit in Rome's decline and fall has been largely refuted.

THE MIDDLE AGES AND RENAISSANCE

The Venetian Council of Ten was a governing body in Venice from around 1310 until 1797. They were known for conducting secret tribunals whereby figures perceived as a threat to the state were ordered to be executed. Many of these executions were carried out by poisoning.

Poisoning as an assassination method was widespread during the fourteenth to sixteenth centuries in Europe. Letters to Grand Duke Cosimo I de'Medici affirm as much. Animal venoms, phytotoxins, and mineral poisons were all employed. Poisoning was clearly a family affair with the Medicis, and Cosimo's sons Ferdinando and Francesco were equally complicit in it. Many legends surround Catherine de'Medici who moved to France to marry the future King Henry II. Despite multiple purported victims, there is no definitive evidence that she poisoned anyone. Developing and testing antidotes was also part of the Medicis' stock-in-trade.

Another powerful and infamous Italian family, originally from Spain, and on whom were pinned numerous heinous crimes, poisoning among them, were the Borgias. There were claims, for example, that Cesare murdered a servant who was a lover of his sister, Lucretia, in front of their father Pope Alexander. Cesare was also said to have poisoned Cardinal Juan Borgia. The reputation of Lucretia herself was stained with allegations that she was a poisoner. Documents uncovered recently in the Vatican archives refute these and other claims that the Borgias' reputation for extensive poisonings and murders stems from rumors spread by their enemies.

In seventeenth century France, during the reign of Louis XIV, a series of poisonings became known as *L'affaire des poisons* (the Affair of the Poisons). Madame de Brinvilliers was

convicted of poisoning her father and two brothers and attempting to poison other family members. Prior to her execution she implicated, without specifically naming them, many others, who were subsequently prosecuted and sentenced to death. The notorious Catherine Deshayes, also known as La Voisin, an acknowledged sorceress, was burned at the stake in 1680 for her crimes.

It is thought that two women in Palermo, Francesca la Sarda and Teofania di Adamo, jointly concocted and marked a poison known as "Acqua Tufania" for which they were executed. Under the leadership of Giulia Tofana, possibly Teofania's daughter, they carried on the business. The poison became known as Aqua Tofana. Arsenic was likely a primary ingredient.

An increasingly sophisticated understanding of toxicology developed during the Middle Ages and Renaissance. Moses Maimonides, the great Jewish philosopher, theologian, and scientist, wrote his *Treatise on Poisons and their Antidotes*, originally in Arabic, in 1198. Part I was concerned with bites from snakes and rabid dogs, and stings of scorpions and insects. Part II dealt with poisons in food and minerals, as well as remedies. He made a distinction between "hot" and "cold" poisons which, it has been claimed, may be equivalent to modern-day hemolysins and neurotoxins. Maimonides also emphasized preventive measures.

The study of toxicants was widespread in Persian and Arabic countries. Known by his Latin name of Avicenna in the West, Abū ʻAlīAal-H.usayn ibn Abd Allāh ibn Sīnā was perhaps the most noteworthy physician/scientist/philosopher of the Islamic world. His celebrated "Canon of Medicine" remained the most popular medical textbook for some six centuries, and it included detailed descriptions of venoms and other poisons, as well as instructions related to antidotes.

On a very practical level, it became clear to ordinary people that their very occupations could be harmful. Georgius Agricola (1494–1555), born in Saxony, currently part of Germany, is known as "the father of mineralogy" largely as a result of his best known monograph, *De Re Metallica*, published in 1556.

The unorthodox medical revolutionary, Theophrastus von Hohenheim, called Paracelsus (1493/94–1541) was born in Einsiedeln, a municipality now in modern-day Switzerland. He theorized that there were four pillars of medicine: natural philosophy, astronomy, alchemy, and medical virtue. In addition to his medical works, he was a keen observer and investigator of toxic effects of various agents and wrote a treatise about their effects upon miners. He concludes this work with a discussion of metallic mercury and criticizes its use at the time as therapy for people afflicted with syphilis. The most famous toxicological adage associated with Paracelsus is "The dose makes the poison," which is a distillation of what he wrote in his *Seven Defenses*:

> *Wenn jhr jedes Gifft recht wolt außlegen/ Was ist das nit Gifft ist? alle ding sind Gifft/ vnd nichts ohn Gifft/ allein die Dosis macht/ dz ein ding kein Gifft ist.*
>
> *When you want to correctly evaluate a poison, what is there that is not poison? All things are poison and nothing is without poison; only the dose determines that something is not a poison.*

Paracelsus deserves the laurel crown and the oft-cited appellation, "Father of toxicology." An understanding of the dose–response relationship is no less significant to our understanding of toxicology today than it was 500 years ago.

18TH AND 19TH CENTURIES

The scientific method gained increasing prominence in the eighteenth and nineteenth centuries as a way of understanding our universe, and toxicology benefited from this more sophisticated and methodical approach.

Richard Mead (1673–1754) authored the first book in English devoted solely to poisons, *A Mechanical Account of Poisons in Several Essays*. He described the signs and symptoms of snake envenomation, performed chemical tests on venom, and experimented on snakes (to study their venom delivery system) and other animals.

Bernardino Ramazzini was a physician whose seminal achievements have earned him the moniker Father of Occupational Medicine. The first edition of his most famous book, *De Morbis Artificum Diatriba* (*A Treatise on the Diseases of Workers*), published in 1700, is the first comprehensive and systematic work on occupational diseases. It outlined the health hazards of chemicals and other substances, including repetitive motions, encountered by workers in over 50 occupations.

Percivall Pott (1714–1788) published in 1774 an essay, *Chirurgical Observations Relative to the Cataract, the Polypus of the Nose, the Cancer of the Scrotum* in which he made the link between the profession of chimney sweeps (regarding soot lodging in the folds of scrotal skin) and scrotal cancer. This was the first occupational link to cancer and Pott's investigations contributed to the science of epidemiology. It wasn't until the 1920s that benzo[a]pyrene was identified as the actual chemical responsible.

Four scientists who made remarkable advances in the chemical detection of poisons were Karl Wilhelm Scheele, Christian Friedrich Samuel Hahnemman, Johann Daniel Metzger, and Valentine Rose. Scheele discovered oxygen before Joseph Priestley, although he published his results later. He is also credited with the discovery of hydrofluoric, hydrocyanic, and arsenic acids, and devised methods for detecting arsenic in body fluids and corpses. Hahnemman discovered a test for arsenic oxide. Rose and Metzger discovered the first methods for detecting elemental arsenic and arsenic oxides in fluids and tissues. In 1836, the English chemist James Marsh developed what came to be known as the Marsh test, a groundbreaking method for detecting arsenic.

Mathieu Joseph Bonaventure Orfila (1787–1853) experimented widely with dogs, varying the amount of poison (such as arsenic) administered and the route of administration, and tested antidotes and treatments. He authored *Traite des poisons* in 1814/5. He subsequently extracted the sections on antidotes and treatments and published them in a compact free-standing volume designed for physicians and for lay audiences that may not have access to medical care but need to know what to do in the event of a poisoning emergency.

As a medical expert, Orfila is best known for a case involving Marie Lafarge, charged with poisoning her husband. Eyewitnesses had seen her buying arsenic (used to exterminate rats) and stirring a white powder into her husband's food. Upon his exhumation, Orfila found definite traces of arsenic in the body and demonstrated that it did not come from the surrounding soil. Marie Lafarge was found guilty of murder and received a death sentence, later commuted to life in prison. The case cemented Orfila's reputation as the greatest toxicologist of the day. And like Paracelsus, Orfila has been called "Father of Toxicology," but of course representing a different era, and for different reasons.

In France, Francois Magendie (1783–1855) was best known for his pioneering contributions in neuroscience and neurosurgery, and experimental physiology. His studies on the effects of drugs on different parts of the body led to the introduction of compounds such as strychnine and morphine into medical practice. His research into the mechanisms of toxicity of these and other

alkaloids furthered the science of toxicology.

Claude Bernard (1813–1878), Magendie's most celebrated pupil, discovered the role of the pancreas in digestion, the regulation of the blood supply by vasomotor nerves, and the glycogenic function of the liver. His work also led to an understanding of the self-regulating process of living organisms we now refer to as homeostasis. He won acclaim for his book *Introduction à l'Etude de la Médecine Expérimentale* (*An Introduction to the Study of Experimental Medicine*). His approach of starting with a hypothesis and having results, which are reproducible, furthered the paradigm of the modern scientific method. In the realm of toxicology, Bernard demonstrated that the mechanism of action of curare resulted from its interference in the conduction of nerve impulses from the motor nerve to skeletal muscle. In addition to curare, he studied the toxicological properties of other neuroactive compounds such as opium, atropine, strychnine, and nicotine. He was the first to describe the hypoxic effects of carbon monoxide.

Greatly influenced by Orfila, Robert Christison (1797–1882), a Scottish physician, was interested in underpinning medical jurisprudence, especially toxicology, with a scientific foundation. Early on, he investigated the detection and treatment of oxalic acid poisoning and followed this up with investigations on arsenic, lead, opium, and hemlock. His celebrated book, *Treatise on Poisons,* first published in 1829, went through four editions.

THE MODERN ERA

Radiation

The late nineteenth century is about the time when an understanding of radiation and its potentially hazardous effects began to surface. In 1895, Wilhelm Röntgen discovered that x-rays could penetrate human flesh. In 1896, Nikola Tesla intentionally exposed his fingers to x-rays and reported burns. In that same year, Henri Becquerel discovered that uranium salts naturally emitted similar rays. Marie Curie, a student of Becquerel, named the phenomenon "radioactivity." She went on to discover thorium, polonium, and radium.

Soon after radium's discovery, it was manufactured synthetically and appeared in food products such as bread, chocolate, toys (because of its luminescence), toothpaste, cosmetics, suppositories, and products to treat impotence. One of the first revelations about the scope of its potential danger concerned the unfortunate girls who became radium watch dial painters in the early 1900s. These "radium girls" applied radium paint to watch and clock faces so they would glow in the dark. They were instructed to use their lips to shape the brushes to a fine point. By 1927, over 50 women died due to radium paint poisoning, and many survivors suffered significant health problems.

The detonation of atomic bombs over the cities of Hiroshima and Nagasaki in World War II killed a couple hundred thousand almost immediately. Tens of thousands of people in both cities would later die of radiation exposure or otherwise suffer devastating injuries.

Food and Drugs

Toxicology has developed and continues, to some extent, to develop as a reactive (rather than

proactive) field. Chemical laws and regulations often are enacted in reaction to major or widespread exposure incidents. Lewis Caleb Beck, an American physician and chemist, published in 1846 *Adulterations of Various Substances Used in Medicine and the Arts with Means of Detecting Them: Intended as a Manual for the Physician, the Apothecary, and the Artisan*. His publication helped promote the Drug Importation Act of 1848, which required the U.S. Customs Service to inspect and stop any adulterated drugs from entering the U.S. market. Inspectors could conduct qualitative tests, such as those detailed in Beck's publication, to determine if a drug was adulterated.

In 1902, Harvey W. Wiley administered the so-called "Poison Squad" experiments, asking healthy volunteers to consume measured amounts of preservatives routinely added to food items to determine whether they were safe for human consumption. In time, hundreds of patent medicines were identified as misleading, harmful, and sometimes deadly. In 1905, Samuel Hopkins Adams published, in *Collier's Weekly*, "The Great American Fraud," a sensational article exposing the hoax of patent medicines. Upton Sinclair's 1906 book, *The Jungle*, detailed unsanitary conditions of workers in the meat packing industry. The Pure Food and Drugs Act and the Meat Inspection Act were passed on the very same day in 1906.

England's attention to the adulteration of food and drugs actually preceded that of the United States. Friedrich Accum published a book in the 1820s titled *A Treatise on the Adulterations of Food, and Culinary Poisons* with the subtitle *There Is Death in the Pot*. Accum wrote about hundreds of poisonous additives commonly used in food products to either sweeten, color, or bulk up foods. His and others' campaign to prevent food adulteration eventually resulted in food and drug legislation in the United Kingdom.

The 1906 Pure Food and Drug Act in the United States did not have the broad impact that was intended. Its main purpose was to ban foreign and interstate traffic of adulterated, falsely advertised, or mislabeled food and drug products. It empowered the U.S. Bureau of Chemistry to inspect products and refer offenders to prosecutors, but gave no prosecutorial power to the agency itself. The United States Pharmacopeia (USP) and the National Formulary served as a foundation for the Pure Food and Drugs Act. Although the law was popular, it was virtually impossible to enforce. The 1906 law prevented the manufacture, sale, or transportation of adulterated, misbranded, poisonous, or deleterious foods, drugs, medicines, and liquors.

Prohibition in the United States ran from 1920 to 1933. During this time, there were very few legal means for obtaining alcohol. One infamous concoction was Jamaica Ginger, which contained between 70% and 80% alcohol by weight. The U.S. Treasury Department required changes to the ingredients of Jamaica Ginger to discourage its abuse. The minimum requirement of ginger solids per cubic centimeter of alcohol resulted in a bitter concoction that was not palatable. An alternative recipe that could pass the inspection and taste well enough to sell was prepared by adding tri-ortho-cresyl phosphate (TOCP) to the mixture. In early 1930, reports detailed a strange paralysis of the legs, arms, and wrists with little to no recovery in large numbers of people. By 1931, the disease, known colloquially as Ginger Jake paralysis, had reached epidemic proportions affecting an estimated 10,000 people and the adulteration with tri-ortho-cresyl phosphate was discovered.

Sulfa drugs were a twentieth century miracle for the treatment of bacterial and fungal infections. The first sulfa drug, Protonsil, showed no effect in vitro with bacterial assays but was extremely effective in vivo. It was later discovered that Protonsil is metabolized to sulfanilamide in vivo and the science of the bioactivation of drugs was revealed. However, for a drug to be effective there needed to be an effective delivery system. Sulfanilamide is highly insoluble in an

aqueous solution. Originally prepared as an elixir in ethanol, chemists discovered that the drug was more soluble in diethylene glycol. Many patients, most of whom were children, died of acute kidney failure resulting from metabolism of the glycol to metabolites that crystallized in the kidney tubules. This tragedy led to the passage of the 1938 Food, Drug, and Cosmetic (FD&C) Act. It contained provisions for both misbranding and adulteration. The law also required that a package's ingredients and their amounts, as well as the name and address of the manufacturer, packer, or distributor, be clearly displayed on the label. To enforce the statute, the FDA was given search, seizure, and prosecutorial powers.

In 1960, a new drug thalidomide (Kevadon), an anti-nausea medication also used to alleviate morning sickness in pregnant women, was distributed by the Merrell drug company to over 1200 U.S. doctors with the expectation that it would be approved quickly. By 1961, it became clear that thalidomide posed a serious safety risk. Infant deaths and deformities occurred at an alarming rate across Europe and the German manufacturer began pulling the drug from the market in the late 1961. By 1962, the application for approval in the United States was withdrawn completely. Though never licensed in the United States, physicians distributed drug samples to around 20,000 patients in the United States. By late 1962, there were at least 10,000 babies born with thalidomide-related defects and countless pregnancies that ended in miscarriage worldwide. The thalidomide tragedy led to the 1962 Kefauver-Harris Amendments, which gave the FDA the authority to require proof of efficacy (rather than just safety) before a new drug could gain approval. The amendments created the groundwork for the multi-phased approval process involving clinical trials, which is still very much in use today.

Even with the current laws in place, occasionally a drug must be highly regulated, recalled, or removed from the open market for reasons such as toxicity, impurities, lack of efficacy, or abuse potential. Mylotarg (gemtuzumab ozogamicin), for example, was approved under an accelerated approval process in 2000 for the treatment of acute myelogenous leukemia. In 2010, the drug was voluntarily withdrawn from the market because a phase 3 comparative controlled clinical trial demonstrated an increase in mortality. The nonsteroidal, anti-inflammatory medication for arthritis, Vioxx (refecoxib), was responsible for perhaps over 27,000 heart attacks and cardiac deaths. These effects did not emerge in the original clinical trials.

From around 1938 to 1971, millions of pregnant women were prescribed diethylstilbestrol (DES) as a hormone-replacement therapy and to prevent miscarriages and premature births. It was discovered that DES caused a rare vaginal cancer (clear cell adenocarcinoma) in girls and young women who had been exposed to DES in the womb. It was recalled from the market in 1971.

In some cases, a drug may be removed from the market temporarily to protect consumers. In 1982, there were several deaths eventually linked to Tylenol brand acetaminophen capsules. The capsules were laced with potassium cyanide. Several copycat crimes followed this incident. In 1987, Stella Nickell laced Excedrin capsules with cyanide, killing both her husband and a woman who purchased the tampered product. Crimes such as these led to the passage of the Federal Anti-Tampering Act of 1983.

Pesticides Research and Chemical Warfare: A Surprising Alliance

Naturally derived pesticides have been used to protect crops for thousands of years. Some 4500

years ago, the Sumerians dusted their crops with elemental sulfur. Around 3200 years ago, the Chinese used mercury and arsenic compounds to control body lice. Synthetic pesticide development and use is a product of the twentieth century.

Germany was responsible for much of the large-scale production of pesticides and warfare gases used in the early to mid-1900s. Fritz Haber, a German scientist, with contributions from Carl Bosch, sought a way to capture nitrogen in the air for use in large-scale fertilizer production. The Haber-Bosch process was instrumental in the manufacture of nitrogen-based explosives for the German Army during World War I. Bosch also researched the weaponization of toxic substances such as chlorine, phosgene, and mustard gas, leading to the largest deployment of chemical weapons in modern history. During World War I, the Germans launched a chemical attack using chlorine gas in Ypres, Belgium in 1915. Phosgene employment accounted for nearly 85% of all gas-related fatalities during that war. The human toxicity of tabun, a new organophosphate insecticide synthesized in 1937 by Gerhard Schrader, results from inhibition of acetylcholinesterase in the peripheral and central nervous systems.

DDT was recognized as an insecticide by Paul Hermann Müller in 1939. DDT was extremely effective in preventing the spread of malaria in developing countries. It was the chemical of choice for controlling insect populations in the United States as well. Not long after its introduction, DDT was discovered to cause eggshell thinning. Many birds didn't reproduce effectively, and their populations diminished over time. The work and research of Rachel Carson brought this to everyone's attention by publishing her book *Silent Spring* in 1962. The grassroots effort of environmental advocacy movements were instrumental in influencing the government to create the Environmental Protection Agency (EPA) in 1970.

Another critically important book is Theo Colburn's *Our Stolen Future* published in 1996, which brought the concept of endocrine disruption to the public and scientific forefront. The book reported that endocrine-active (or estrogen mimicking) compounds may be eliciting effects at doses considerably lower than toxicities caused by other mechanisms, and that reproductive and developmental risks can be significant.

High Profile Poisonings

Poisonings have continued unabated from ancient times forward. In 1978, Jim Jones, founder of the Peoples Temple, led over 900 of his followers, one-third of them children, to their deaths, by ordering them to drink a cyanide-laced punch drink in Jonestown, Guyana. An umbrella outfitted with a firing mechanism was used to administer the poison ricin into the leg of Georgi Markov, a Bulgarian dissident and writer. He died several days later. Nazi leaders such as Hitler, Himmler, and Goering committed suicide with cyanide. In 1995, five plastic bags of liquid sarin were punctured with metal-tipped umbrellas in Tokyo subway cars during rush hour releasing the deadly nerve gas. In 2004, Viktor Yuschchenko was poisoned with dioxin, resulting in severe facial disfigurement due to chloracne. In 2006, Alexander Litvinenko, a former officer of the Russian state security organization FSB died from radioactive polonium-210 poisoning. In February 2017, Kim Jong Nam, the half-brother of North Korean Leader Kim Jong Un, was assassinated at a Malaysian airport when two women rubbed his face with the lethal nerve agent VX. It is clear that poisons continue to be a weapon of choice in politics and in society.

Mass Environmental Exposures, the U.S. EPA, and

Environmental Legislation

Years prior to the advent of a full-fledged grassroots environmental movement, various events made clear the fragility of our environment. The Donora Smog was a historic air inversion in Pennsylvania that killed 20 people and sickened 7000 more in 1948. In 1952, during the so-called Great Smog of London, over five days, more than normal coal emissions mixed with fog in a temperature inversion resulted in thousands of deaths and tens of thousands of hospitalizations. It was the Great Smog that led to passage of the 1956 Clean Air Act in the United Kingdom. In Cleveland, the Cuyahoga River is remembered as the body of water polluted from decades of industrial waste, which caught fire in 1969 (and, in fact, on earlier occasions as well).

More direct cause–effect incidents involving chemicals began surfacing. Most companies created landfills for dumping chemical byproducts that accumulated from the manufacturing process. The increase in the manufacture of chemicals translated to both an increase in direct human exposure via ingestion of products kept in the home, and an increase in indirect human exposure via leaching of dumped chemicals into the ground water, air, and food supply.

Love Canal in Niagara Falls, New York, was used as a dump site by the Hooker Chemical Company for over a decade. In the 1970s, long after it was capped and an entire community built on top of it, weather patterns forced chemical waste into the groundwater and at surface. The entire area was found to be contaminated with a variety of toxic chemicals, which led to a cluster of illnesses among the residents living in the area. The activism around the contamination and subsequent cleanup led to legislation that would ensure that other chemically contaminated sites would receive government funding for cleanup. The Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA), commonly known as Superfund, was enacted on December 11, 1980. It authorizes the cleanup of uncontrolled or abandoned hazardous-waste sites as well as accidents, spills, and other emergency releases of pollutants and contaminants into the environment.

Superfund was amended owing to the release of methyl isocyanate from a Union Carbide insecticide plant in Bhopal, India, in 1984. With an immediate death toll of some 4000, a final death toll of many thousands more, and even more victims who suffered and are still suffering lingering effects, the Bhopal disaster remains probably the worst industrial accident in history. An important law authorized by Title III of the 1986 Superfund Amendments and Reauthorization Act (SARA) is the Emergency Planning and Community Right-to-Know Act (EPCRA). It requires public records of chemicals managed at facilities and provides the EPA with the authority to work with states and localities to prevent accidents and develop emergency plans in case of dangerous releases of chemicals. The EPA's Toxics Release Inventory (TRI), a publicly accessible online database, is an outgrowth of EPCRA.

For decades in the early part of the twentieth century, one of Japan's Chisso Corporation plants began releasing methylmercury in industrial wastewater to Minamata Bay. It bioaccumulated in the aquatic life in the Bay and was eaten by the local populace, as well as animals. With the situation not discovered until 1956, it took a severe toll on the population. Over 2000 victims suffered from severe nervous system symptoms, and many of those died. Itai-itai, another disease outbreak in Japan, was caused by cadmium poisoning from the release of large quantities of this chemical into the Jinz River from mining operations. Weak and brittle bones are among the main effects. In Seveso, Italy, an industrial accident resulted in the exposure of thousands of people to dioxin. Chloracne was among the main sequelae, and there was an

excess risk of lymphatic and hematopoietic tissue neoplasms in the most exposed zones. On August 15, 1984, Lake Monoun in West Province, Cameroon, exploded in a limnic eruption, in which dissolved carbon dioxide suddenly erupted from deep lake waters, forming a gas cloud with suffocating potential. The gas killed 37 people. On August 21, 1986, an even more deadly eruption occurred at Lake Nyos killing approximately 1746 people and more than 3000 livestock. Lake Monoun, Lake Nyos, and Lake Kivu are the only known volcanic lakes in the world to have high concentrations of gas dissolved deep below the surface.

The Toxic Substances Control Act (TSCA), the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), the Clean Air Act, the Clean Water Act, the Safe Drinking Water Act, and the Resource Conservation and Recovery Act (RCRA) give the EPA the authority to control hazardous waste from “cradle to grave.” All have been strengthened in various ways with amendments since their initial implementation. The Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) is a European Union regulation that requires all companies manufacturing or importing chemical substances into the European Union in quantities of one ton or more per year to register these substances with the European Chemicals Agency (ECHA) in Helsinki, Finland.

INTERNATIONAL ENVIRONMENTAL CONVENTIONS AND OTHER GLOBAL EFFORTS

Given that toxic agents do not respect national borders, some Multilateral Environmental Agreements (MEAs) are designed to manage potentially hazardous chemicals. Three MEAs are the Basel Convention on the Control of Transboundary Movements of Hazardous Wastes and their Disposal (adopted 1989; entered into force in 1992), the Rotterdam Convention on the Prior Informed Consent Procedure for Certain Hazardous Chemicals and Pesticides in International Trade (adopted 1998; entered into force in 2004), and the Stockholm Convention on Persistent Organic Pollutants (adopted 2001; entered into force in 2004).

Though most research focuses on single chemicals, we are exposed to many chemicals at a time and over time. Learning how they interact with each other in causing their effects upon organisms is a critical question. Related to this is the issue of the effects of chemicals or combinations thereof in common household products including furniture, cars, electronics, and baby products.

The upward trajectory of toxicology continues unabated. Its scientific foundation is becoming more assured, precise, and relevant. Challenges will remain and include intermittent funding and political constraints. Toxicology will continue to build upon its history and build a trail of new history. A better understanding of toxicant exposures, individual and combined, and their effects upon living organisms will lead to an era when the global environment will be significantly safer and the world’s populace healthier.

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QUESTIONS

Choose the one best answer.

1. Which one of the following statements regarding toxicology is true?
   - **a.** Modern toxicology is concerned with the study of the adverse effects of chemicals on ancient forms of life.
   - **b.** Modern toxicology studies embrace principles from such disciplines as biochemistry, botany, chemistry, physiology, and physics.
   - **c.** Modern toxicology has its roots in the knowledge of plant and animal poisons, which predates recorded history and has been used to promote peace.
   - **d.** Modern toxicology studies the mechanisms by which inorganic chemicals produce advantageous as well as deleterious effects.
   - **e.** Modern toxicology is concerned with the study of chemicals in mammalian species.

2. Knowledge of the toxicology of poisonous agents was published earliest in the:
   - **a.** Ebers papyrus.
   - **b.** *De Historia Plantarum.*
   - **c.** *De Maateria Medica.*
   - **d.** *Lex Cornelia.*
   - **e.** *Poisons and their Antidotes.*

3. Paracelsus, a physician-alchemist, formulated many revolutionary views that remain integral to the structure of toxicology, pharmacology, and therapeutics today. He focused on the primary toxic agent as a chemical entity and articulated the dose–response relation. Which one of the following statements is NOT attributable to Paracelsus?
   - **a.** Natural poisons are quick in their onset of actions.
   - **b.** Experimentation is essential in the examination of responses to chemicals.
   - **c.** One should make a distinction between the therapeutic and toxic properties of chemicals.

d. These properties are sometimes but not always indistinguishable except by dose.
e. One can ascertain a degree of specificity of chemicals and their therapeutic or toxic effects.

4. The art of toxicology requires years of experience to acquire, even though the knowledge base of facts may be learned more quickly. Which modern toxicologist is credited with saying that “you can be a toxicologist in two easy lesions, each of 10 years?”
   a. Claude Bernard.
   b. Rachel Carson.
   c. Upton Sinclair.
   d. Arnold Lehman.
   e. Oswald Schmiedeberg.

5. Which of the following statements is correct?
   a. Claude Bernard was a prolific scientist who trained over 120 students and published numerous contributions to the scientific literature.
   b. Ginger Jake paralysis was caused by tri-ortho-cresyl phosphate.
   c. *An Introduction to the Study of Experimental Medicine* was written by the Spanish physician Orfila.
   d. Magendie used autopsy material and chemical analysis systematically as legal proof of poisoning.
   e. Percival Potts was instrumental in demonstrating the chemical complexity of snake venoms.

CHAPTER 2

Principles of Toxicology

Lauren M. Aleksunes and David L. Eaton

KEY POINTS

- A *poison* is any agent capable of producing a deleterious response in a biological system.
- A *mechanistic toxicologist* identifies the cellular, biochemical, and molecular mechanisms by which chemicals exert toxic effects on living organisms.
- *Toxicogenomics* permits identification and protection of genetically susceptible individuals from harmful environmental exposures, and customizes drug therapies based on their individual genetic makeup.
- A *descriptive toxicologist* is concerned directly with toxicity testing, which provides information for safety evaluation and regulatory requirements.
- A *regulatory toxicologist* both determines from available data whether a chemical poses a sufficiently low risk to be marketed for a stated purpose and establishes standards for the amount of chemicals permitted in ambient air, industrial atmospheres, and drinking water.
- *Selective toxicity* means that a chemical produces injury to one kind of living matter without harming another form of life even though the two may exist in intimate contact.
- The individual or “graded” dose–response relationship describes the response of an *individual* organism to varying doses of a chemical.
- A quantal dose–response relationship characterizes the distribution of responses to different doses in a *population* of individual organisms.
- Hormesis, a “U-shaped” dose–response curve, results with some xenobiotics that impart beneficial or stimulatory effects at low doses but adverse effects at higher doses.
- Descriptive animal toxicity testing assumes that the effects produced by a compound in

laboratory animals, when properly qualified, are applicable to humans, and that exposure of experimental animals to toxic agents in high doses is a necessary and valid method of discovering possible hazards in humans.

INTRODUCTION

Toxicology is the study of the adverse effects of chemical, biological, or physical agents on living organisms and the environment. These toxic substances include naturally occurring harmful chemicals, or *toxins,* as well as foreign substances called *xenobiotics.* Toxins are poisons that originate from plants and microbial organisms and include venoms released by animals in order to injure predators. By comparison, xenobiotics include a variety of synthetic chemicals with different intended purposes. Generally, such toxic chemicals are referred to as toxicants, rather than toxins, because they are not produced by biological systems.

Toxic chemicals may also be classified in terms of physical state (gas, dust, liquid, size); chemical stability or reactivity (explosive, flammable, corrosive); general chemical structure (aromatic amine, halogenated hydrocarbon, etc.); or ability to cause significant toxicity (extremely toxic, very toxic, slightly toxic, etc.). Classification of toxic chemicals based on their biochemical mechanisms of action (e.g., alkylating agent, cholinesterase inhibitor, and endocrine disruptor) is usually more informative than classification by general terms such as irritants and oxidizers. However, more descriptive categories such as air pollutants, occupation-related exposures, and acute and chronic poisons may be useful to associate toxic chemicals that result in similar adverse events or are encountered under particular conditions.

Virtually every known chemical has the potential to produce injury or death if it is present in a sufficient quantity. Table 2–1 shows the dose of chemicals needed to produce death in 50% of treated animals (lethal dose 50 [LD_{50}]). It should be noted that measures of acute lethality such as LD_{50} do not accurately reflect the full spectrum of toxic responses, or hazards, associated with exposure to a chemical. For example, some chemicals may have carcinogenic, teratogenic, or neurobehavioral effects at doses that produce no evidence of acute or immediate injury. In addition, there is a growing recognition that various factors such as age, genetics, diet, underlying diseases, and concomitant exposures can account for an individual's susceptibility to a range of responses. For a particular chemical, multiple different effects can occur in a given organism, each with its own dose–response relationship.

TABLE 2–1 Approximate Acute LD_{50} Values of Some Representative Chemicals

Chemical	LD_{50} (mg/kg)*
Ethyl alcohol	10,000
Glyphosate	5,600
Sodium chloride	4,000
Ferrous sulfate	1,500
Morphine sulfate	900
Phenobarbital sodium	150
Chlorpyrifos	18
Picrotoxin	5
Strychnine sulfate	2
Nicotine	1
VX nerve gas	1
D-Tubocurarine	0.5
Hemicholinium-3	0.2
Tetrodotoxin	0.10
Dioxin (TCDD)	0.001
Botulinum toxin	0.00001

*LD_{50} is the dose (mg/kg body weight) causing death in 50% of exposed animals.

A *toxicologist* is an individual trained to examine and communicate the nature of a toxicant's properties and identify approaches to prevent or mitigate harm done to human, animal, and environmental health. Toxicological research identifies the cellular, biochemical, and molecular mechanisms of action of toxic chemicals and determines the extent to which these actions cause functional perturbations in critical organ systems. Using these data, a toxicologist then assesses the relationship between toxicant exposure (or dose) to the response (or outcome) and in turn the probability of an adverse event to occur. This determination requires an assessment of *risk* which is the quantitative estimate of the potential effects of a chemical on human and environmental health at particular exposure levels. Toxicology is a broad applied science that draws upon multiple disciplines including chemistry, biology, physiology, pathology, pharmacology, molecular biology, physics, statistics, and more.

SUBDISCIPLINES OF TOXICOLOGY

The professional activities of toxicologists fall into three main categories: mechanistic, hazard

assessment, and regulatory. Although all three categories have distinctive characteristics, each contributes to the others, and all are vitally important to chemical risk assessment (see Chapter 4).

A *mechanistic toxicologist* identifies the cellular, biochemical, and molecular mechanisms by which chemicals exert toxic effects on living organisms (see Chapter 3). The results of mechanistic studies have implications in many areas of toxicology. In risk assessment, mechanistic data may be useful in determining whether an adverse outcome (e.g., cancer and birth defects) observed in laboratory animals may occur in humans or that may not be relevant to humans. Mechanistic data are also useful in the design and production of safer alternative chemicals and in therapies for chemical poisoning and treatment of disease.

A *hazard assessment toxicologist* conducts toxicity testing that provides comprehensive information for the evaluation of a chemical's safety and to meet important regulatory requirements. A *hazard* is a chemical or action that causes harm, whereas the *risk* is the likelihood for a hazard to result in harm. Risk is determined by the extent of the exposure to the hazard. Toxicologists must be concerned with the risk posed by chemicals (drugs, food additives, insecticides, herbicides, solvents, etc.) to humans, fish, birds, and plants, as well as other factors that might disturb the balance of the ecosystem.

A *regulatory toxicologist* has the responsibility for deciding, on the basis of data provided by descriptive and mechanistic toxicologists, whether a drug or other chemical poses a sufficiently low risk (or, in the case of drugs, a favorable risk/benefit profile) to be marketed for a stated purpose. *Computational toxicologists* are needed to develop and implement computer-based models to predict adverse health effects resulting from the interaction of chemicals with biological organisms. *Occupational toxicologists* are responsible for conducting research and making recommendations for the prevention of work-related injury and illness. Regulatory toxicologists are also involved in the establishment of standards for the amount of chemicals permitted in ambient air, industrial atmospheres, and drinking water, often integrating scientific information from basic descriptive and mechanistic toxicology studies with the principles and approaches used for risk assessment.

Other specialized areas of toxicology are forensic, clinical, and environmental toxicology. *Forensic toxicology* is the application of analytical chemistry to toxicology. This field covers the medicolegal aspects of the deleterious effects of chemicals on animals and humans. The expertise of forensic toxicologists is used to aid in establishing the cause of death and determining its circumstances in a postmortem investigation (see Chapter 32). *Clinical toxicology* is the realm of medical science concerned with disease caused by or uniquely associated with toxic substances (see Chapter 33). Generally, clinical toxicologists are physicians who receive specialized training in emergency medicine and poison management. Efforts are directed at treating patients poisoned with drugs or other chemicals and at the development of new techniques to treat intoxications. *Environmental toxicology* focuses on the impact of chemical pollutants in the environment on biological organisms. *Ecotoxicology* is a specialized area within environmental toxicology that focuses on the impacts of toxic substances on population dynamics in an ecosystem. The transport, fate, and interactions of chemicals in the environment constitute a critical component of both environmental toxicology and ecotoxicology.

Information from the toxicological sciences, gained by experience or research, has a growing influence on our personal lives as well as on human and environmental health across the globe. Complementary fields such as *exposure science* advance toxicology by studying the magnitude

and duration of contact between toxic chemicals and biological organisms. Knowledge about the toxicological effects of a compound and the extent of exposure has important implications for drugs, consumer products, waste cleanup, manufacturing processes, regulatory action, civil disputes, and broad policy decisions. The growing influence of toxicology on societal issues carries with it substantial ethical, legal, and societal implications for toxicological research and testing. Recognizing the many critical roles that toxicologists play throughout society, the Society of Toxicology, a professional organization for toxicologists, has developed the Code of Ethics that frames the expected behaviors and attitudes of its members (Table 2–2). Adherence to these professional behaviors and ideals is critical for ensuring the public's trust of the data and assessments conducted by toxicologists.

TABLE 2–2 Toxicology Code of Ethics

- Conduct their work with objectivity and themselves with integrity. Being honest and truthful in reporting and communicating their research.
- Hold as inviolate that credible science is fundamental to all toxicological research and is the basis for communicating results.
- Recognize a duty to communicate information concerning health, safety, and toxicity in a timely and responsible manner, with due regard for the significance and credibility of the available data.
- Give due consideration to the ethical, legal, social, and policy implications of their research and communications.
- Be a thoughtful advocate for human, animal, and environmental health.
- Abstain from professional judgments influenced by undisclosed conflict of interest, disclose any material conflicts of interest, and avoid situations that imply a conflict of interest.
- Observe the spirit, as well as the letter of laws, regulations, and ethical standards with regard to the conduct of human and animal research.
- Practice high standards of environmental and occupational health, and safety for the benefit of themselves, their co-workers, their families, their communities, and society as a whole.
- Provide equal opportunity and equal consideration to all members without regard to sex, gender identity or expression, race, color, national or ethnic origin, religion or religious belief, age, marital status, sexual orientation, disabilities, or veteran status.

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SPECTRUM OF UNDESIRED EFFECTS

The spectrum of undesired effects elicited by chemicals can be broad. Some effects are harmful, whereas others are not. Prescription drugs produce a number of effects, but typically only one of these actions is intended to be therapeutic; all of the other responses are referred to as *side effects*. However, some of these side effects may be preferred for another therapeutic indication.

Drug effects that are never desirable and are always harmful to the well-being of animals and humans are referred to as the *adverse, deleterious,* or *toxic* effects of the drug.

Allergic Reactions

A chemical allergy is an adverse reaction of the immune system to a chemical in response to a previous exposure to that chemical or to a structurally similar one. The term *hypersensitivity* is most often used to define this allergic response, but *allergic reaction* and *sensitization reaction* may also describe the situation when a prior exposure (sensitization) to the chemical is required to produce its subsequent toxic effect. Once sensitization has occurred, allergic reactions may result from exposure to relatively very low doses of chemicals. For a given individual, allergic reactions can be dose-related. For example, it is well known that the allergic response to poison ivy or poison sumac in sensitized individuals is related to the extent of the skin's exposure to urushiol oils found in the leaves. Some sensitization reactions, such as allergic reactions to nuts, shellfish, and other foods as well as certain antibiotics and xenobiotics, may be severe and occasionally fatal. Hypersensitivity reactions are discussed in more detail in Chapter 12.

Idiosyncratic Reactions

Chemical idiosyncrasy refers to the abnormal reactivity of an individual to a chemical based on its genetics or other individual sensitivity factors. Idiosyncratic drug responses involve a combination of individual differences in the ability to (1) form a reactive intermediate (usually through oxidation to an electrophilic intermediate), (2) detoxify the reactive intermediate (usually through hydrolysis or conjugation), (3) mount an immune response through human leukocyte antigens (HLAs) as well as T cells, and/or (4) cause cell death. Patient-specific factors that extend beyond genetics include inflammatory stress, infection, mitochondrial dysfunction, and environmental factors. Typically, there is a delay between the time drug therapy is initiated and the clinical presentation of symptoms. Idiosyncratic reactions can occur in any organ system; however, the skin, liver, hematopoietic, and immune systems are the most often affected. Because of the life-threatening nature of idiosyncratic drug reactions, it is critical that researchers continue to identify modifying factors that initiate and/or heighten these responses.

Immediate versus Delayed Toxicity

The toxic effects of a chemical can develop rapidly after a single exposure or may be delayed for some time. *Immediate toxicity* can be observed for most chemicals, whereas *delayed toxicities* of chemicals may take months or years to be recognized. Because of the long latency period, decades may pass after exposure to a carcinogen before tumors are observed in humans.

Reversible versus Irreversible Toxic Effects

Some toxic effects of chemicals are *reversible*, whereas others are *irreversible*. The likelihood of a toxic response to be reversed largely depends on the ability of an injured tissue to adapt, repair, and regenerate. For tissues such as the liver and gastrointestinal tract that have a high ability to regenerate, many injuries are reversible. By comparison, the CNS has a much more limited

ability to divide and replace damaged neurons making damage largely irreversible. Cancers and birth defects caused by chemical exposures, once they occur, are often also considered irreversible toxic effects. Therefore, it is important to understand the regenerative and reparative capacity of a target organ in order to counteract a chemical's toxic effects.

Local versus Systemic Toxicity

Toxic responses are also characterized according to the proximity between the site of chemical exposure and the site(s) of molecular action. *Local effects* are those that occur where contact is first made by the toxicant and the biological system. Such effects are produced by the ingestion of toxic substances or the inhalation of irritant materials. By comparison, *systemic effects* require the absorption and distribution of a toxicant from its entry point to a distant site where the deleterious effects are produced. Most chemicals usually elicit their major toxicity in only one or two organs, the *target organs* of toxicity. While it may be true in some cases, the target organ of toxicity is not always the site of the highest concentration of the chemical.

Interactions of Chemicals

Throughout the day, an individual may contact many chemicals at any given time (in the workplace, cosmetics, medications, diet, hobbies, etc.). As a result, it is necessary to consider how various chemicals may interact with each other. Interactions may impact absorption, protein binding, receptor signaling, and the biotransformation and excretion of one or both of the interacting toxicants.

Additive—An *additive* effect occurs when the combined responses of two chemicals are equal to the sum of the responses to each chemical given alone (e.g., 2 + 3 = 5).

Synergistic—A *synergistic* effect is observed when the combined responses of two chemicals are much greater than the sum of the response to each chemical when given alone (e.g., 2 + 2 = 20).

Potentiation—*Potentiation* occurs when one substance does not produce any toxicity on a particular tissue or system but when added to another chemical makes that chemical much more toxic (e.g., 0 + 2 = 10).

Antagonism—*Antagonism* occurs when two chemicals administered together interfere with each other's actions or one interferes with the action of the other (e.g., 4 + 6 = 8; 4 + (−4) = 0; 4 + 0 = 1). There are four major types of antagonism: receptor, chemical, dispositional, and functional.

Receptor Antagonism—Receptor antagonism occurs when two chemicals that bind to the same receptor produce less of an effect when given together relative to the addition of their separate effects (e.g., 4 + 6 = 8) or when one chemical antagonizes the effect of the second chemical (e.g., 0 + 4 = 1). Receptor antagonists are often termed *blockers.*

Chemical Antagonism—Chemical antagonism or inactivation is simply a direct chemical reaction between two compounds that produces a less toxic product. For example, chelators bind to metal ions, such as arsenic, mercury, and lead, decreasing their toxicity.

Dispositional Antagonism—Dispositional antagonism occurs when the absorption, distribution, biotransformation, or excretion of a chemical is altered so that the concentration and/or duration of the chemical at the target organ is reduced.

Functional Antagonism—Functional antagonism occurs when two chemicals counterbalance each other by producing opposing effects on the same physiological function, often through different signaling pathways. For example, the marked fall in blood pressure during severe intoxication with a barbiturate can be effectively antagonized by the intravenous administration of a vasopressor such as norepinephrine. In this case, the barbiturate works through $GABA_A$ receptors and norepinephrine activates α-adrenergic receptors to produce opposing effects on vascular tone.

Tolerance

Repeated exposure to a chemical can reduce its pharmacologic and/or toxicologic actions, a process called *tolerance*. *Cross-tolerance* occurs when structurally related chemicals cause diminished responses. Typically, days or weeks of repeated exposure are required for tolerance to occur.

Dispositional tolerance occurs when the amount of chemical reaching the site of action decreases over time, leading to the reduced responsiveness of the tissue to stimulation.

Chemical or cellular tolerance may result from a lower availability of receptors and/or mediators (e.g., neurotransmitters).

CHARACTERISTICS OF EXPOSURE

Toxicity to a biological system requires that sufficient *concentrations* of the "active" form of a chemical accumulate at the site of action for a requisite period of *time*. Whether a toxic response occurs is dependent on multiple factors: chemical and physical properties of the chemical, the exposure scenario, how the chemical is metabolized by the system, the concentration of the active form at the particular target site(s), and the overall susceptibility of the biological system to injury. To characterize fully the potential hazard of a specific chemical, one needs to know not only the type of effect it produces, and the dose required to produce that effect, but also the information about the chemical, route of exposure, and disposition.

Route and Site of Exposure

Toxic chemicals enter the body via the gastrointestinal tract (ingestion), the lungs (inhalation), and the skin (topical, percutaneous, or dermal). Chemicals generally produce the greatest effect and the most rapid response when given directly into the bloodstream (the intravenous route). Chemicals can also enter the body to varying degrees through other routes. An approximate descending order of effectiveness for the other routes would be inhalation, intraperitoneal, subcutaneous, intramuscular, intradermal, oral, and dermal. The vehicle, or the inert material in which the toxicant is dissolved, and other formulation ingredients can markedly alter chemical absorption after ingestion, inhalation, or topical exposure. In addition, the route of administration can influence the toxicity of chemicals. For example, a chemical that acts on the CNS, but is efficiently detoxified in the liver, would be expected to be less toxic when given orally than when given via inhalation, because the oral route requires that nearly all the doses pass through the liver before reaching the systemic circulation and then the CNS. Typically, different routes of

toxicant entry into the body have been associated with certain types of exposures. Occupational exposure to chemicals most frequently results from breathing contaminated air (inhalation) and/or direct and prolonged contact of the skin with the substance (dermal exposure), whereas accidental and suicidal poisonings occur most frequently by oral ingestion.

Duration and Frequency of Exposure

The duration and frequency for exposure of experimental animals to chemicals is classified according to four categories: acute, subacute, subchronic, and chronic. *Acute exposure* refers to exposure to a chemical for less than 24 hours. While acute exposure usually refers to a single administration, repeated exposures may be given within a 24-hour period for some slightly toxic or practically nontoxic chemicals. Acute exposure by inhalation refers to continuous exposure for less than 24 hours, most frequently for 4 hours. Repeated exposure is divided into three categories: subacute, subchronic, and chronic. *Subacute exposure* refers to repeated exposure to a chemical for 1 month or less, *subchronic* for 1 to 3 months, and *chronic* for more than 3 months, although usually this refers to studies with at least 1 year of repeated dosing. These three categories of repeated exposure can be by any route, but most often they occur by the oral route.

In situations of human exposure to chemicals, the frequency and duration of exposure are not well-defined compared to controlled animal studies; nonetheless, many of the same terms are used to describe general exposure situations. Thus, workplace or environmental exposures may be described as *acute* (occurring from a single incident or episode), *subchronic* (occurring repeatedly over several weeks or months), or *chronic* (occurring repeatedly for many months or years).

For many chemicals, the toxic effects that follow a single exposure are quite different from those produced by repeated exposure. For example, the primary, acute toxic manifestation of benzene is CNS depression, but repeated exposures can result in bone marrow toxicity and an increased risk for leukemia. Chronic exposure to a toxic chemical may produce some immediate (acute) effects after each administration in addition to the long-term, low-level, or chronic effects of the toxic substance.

Toxicokinetic studies are performed by sampling the blood or tissue at various times after exposure to determine the concentration of a chemical and better understand the influence of exposures on toxicity endpoints. Concentration-time profiles for a given chemical are influenced by the frequency of exposures. The relationship between elimination rate and frequency of chemical exposure is shown in Fig. 2–1. A chemical that produces severe effects with a single dose may have no effect if the same total dose is given in several intervals. For the chemical depicted by line B in Fig. 2–1, in which the half-life for elimination (time necessary for 50% of the chemical to be removed from the bloodstream) is approximately equal to the dosing frequency, a theoretical toxic concentration (shown conceptually as two concentration units in Fig. 2–1) is not reached until the fourth dose, whereas that concentration is reached with only two doses for chemical A, which has an elimination rate much slower than the dosing interval (time between each repeated dose). Conversely, for chemical C, where the elimination rate is much shorter than the dosing interval, a toxic concentration at the site of toxic effect will never be reached regardless of how many doses are administered. Of course, it is possible that residual cell or tissue damage occurs with each dose even though the chemical itself is not accumulating. The important consideration, then, is whether the interval between doses is sufficient to allow for complete repair of tissue damage. It is evident that with any type of repeated exposure, the

production of a toxic effect not only is influenced by the frequency of exposure but also may be entirely dependent on the frequency rather than the duration of exposure. Chronic toxic effects may occur, therefore, if the chemical accumulates in the biological system (rate of absorption exceeds the rate of biotransformation and/or excretion), if it produces irreversible toxic effects, or if there is insufficient time for the system to recover from the toxic damage within the exposure frequency interval. For additional discussion of these relationships, see Chapters 5 and 7.

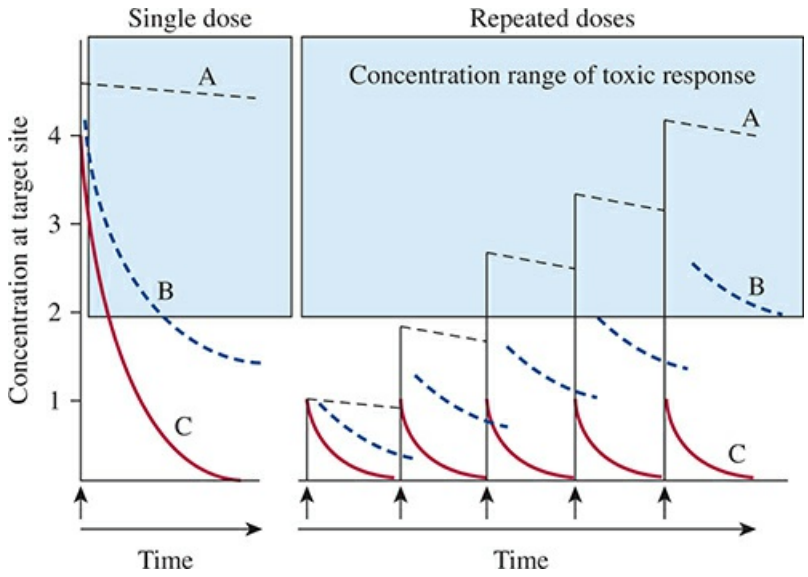

FIGURE 2–1 *Diagrammatic view of the relationship between dose and concentration at the target site under different conditions of dose frequency and elimination rate.* (Line A) A chemical with very slow elimination (e.g., half-life of 1 year). (Line B) A chemical with a half-life approximately equal to frequency of dosing (e.g., 1 day). (Line C) Rate of elimination faster than the dosing frequency (e.g., 5 hours). Blue-shaded area is representative of the concentration of chemical at the target site necessary to elicit a toxic response.

DOSE–RESPONSE RELATIONSHIPS

Dose–response relationships are defined as the association between the amount of a toxicant administered and the extent to which changes are observed in a biological system. When describing chemical exposures, it is important to consider both the dose of the chemical administered or measured in the environment (*external dose*) and the amount of chemical absorbed and found at the site of biological activity (*internal dose*).

The dose of a chemical or toxic agent can be expressed as a mass or concentration. The units of the concentration will depend upon whether the toxic chemical is found in the solid, liquid, or gaseous state. When considering the administration of chemicals to humans and animals, doses are conventionally expressed as milligrams per kilogram.

Dose–response relationships are routinely divided into two types: (1) the individual dose–response relationship, which describes the response of an *individual* organism to increasing doses of a chemical, often referred to as a "graded" response because the measured effect is

continuous over a range of doses, and (2) a quantal dose–response relationship, which characterizes the distribution of individual responses to different doses in a *population* of organisms.

Individual, or Graded, Dose–Response Relationships

Individual dose–response relationships are characterized by a continuous scale of doses that lead to an increase in the magnitude of a specific response. The graded dose–response relationship requires careful selection of a range of doses for evaluation and identification of a specific biochemical process. For example, Fig. 2–2 shows the dose–response relationship between different dietary doses of the organophosphorous insecticide chlorpyrifos and the extent of inhibition of two different enzymes acetylcholinesterase and carboxylesterase in the rat brain and liver, respectively. In the brain, the degree of inhibition of both enzymes is clearly dose-related, although the degree of inhibition per unit dose is different for the two enzymes. From the shapes of these two dose–response curves, it is evident that, in the brain, cholinesterase will be inhibited at a chlorpyrifos dose of 3 mg/kg that does not alter the activity of carboxylesterase. At higher doses of chlorpyrifos (5 mg/kg and higher), both enzymes will be inhibited, but the extent of inhibition will be greater for cholinesterase than carboxylesterase. The primary toxicological response that results from chlorpyrifos exposure is directly related to the degree of cholinesterase enzyme inhibition in the brain. Thus, clinical signs and symptoms for chlorpyrifos would follow a dose–response relationship like that for the brain cholinesterase enzyme. However, the observed response to varying doses of a chemical in the whole organism is often complicated by the fact that most chemicals have multiple sites or mechanisms of toxicity, each with their own “dose–response” relationship and subsequent adverse effect.

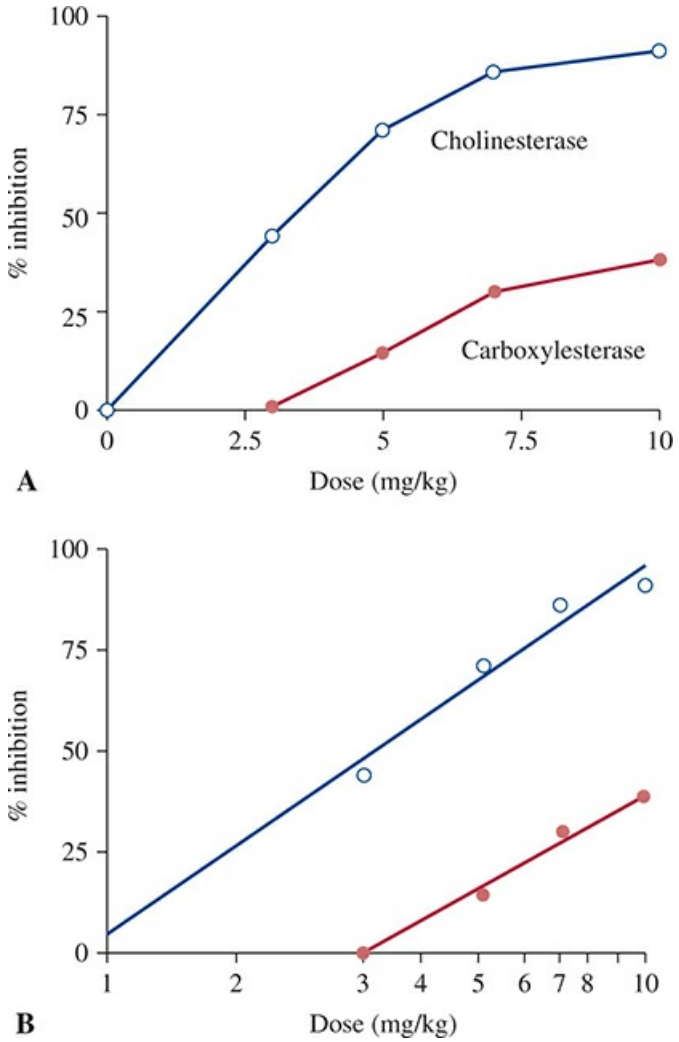

FIGURE 2–2 *Dose–response relationship between different doses of the organophosphate insecticide chlorpyrifos and esterase enzyme inhibition in the brain.* Open circles and blue lines represent acetylcholinesterase activity and closed circles represent carboxylesterase activity in the brains of pregnant female Long–Evans rats given five daily doses of chlorpyrifos. (**A**) Dose–response curve plotted on an arithmetic scale. (**B**) Same data plotted on a semi-log scale. (Data from Lassiter TL et al: Gestational exposure to chlorpyrifos: dose response profiles for cholinesterase and carboxylesterase activity, Toxicol Sci 1999: 52:92-100.)

Quantal Dose–Response Relationships

In contrast to the "graded" or continuous-scale dose–response relationship that occurs in individuals, the dose–response relationships in a *population* are defined as quantal—or "all or none"—in nature, that is, at any given dose, an individual in the population is classified as either a "responder" or a "nonresponder." Although these distinctions of "quantal population" and "graded individual" dose–response relationships are useful, the two types of responses are conceptually identical. The ordinate in both cases is simply labeled the *response,* which may be the degree of response in an individual or system or the fraction of a population responding, and

the abscissa is the range of administered *doses*.

A widely used approach for estimating the response of a population to a toxic exposure is the "effective dose" (ED). Generally, the midpoint, or 50%, response level for the population is used, giving rise to the "ED_{50}" value. However, any response level, such as an ED_1, ED_{10}, or ED_{30}, could be chosen. Where death is the measured endpoint, the ED_{50} would be referred to as the "lethal dose" LD_{50}.

The top panel of Fig. 2–3 shows that quantal dose–responses typically exhibit a normal or Gaussian distribution. The frequency histogram in this panel also shows the relationship between dose and effect. The bars represent the percentage of animals that responded at each dose minus the percentage that responded at the immediately lower dose. One can clearly see that only a few animals responded to the lowest dose and the highest dose. Larger numbers of animals responded to doses intermediate between these two extremes, and the maximum frequency of response occurred in the middle portion of the dose range. Thus, we have a bell-shaped curve known as a *normal frequency distribution.* The reason for this normal distribution is that there are differences in the susceptibility of individuals to chemical toxicity; this is known as biological variation. Animals responding at the left end of the curve are referred to as *hypersusceptible*, and those at the right end of the curve are termed *resistant.* If the number of individuals responding at each consecutive dose are added together, a cumulative, quantal dose–response relationship is obtained. When a sufficiently large number of doses are used with a large number of animals per dose, a sigmoid dose–response curve is observed, as depicted in the middle panel of Fig. 2–3. With the lowest dose (6 mg/kg), 1% of the animals responded. A normally distributed sigmoid curve such as this approaches a response of 0% as the dose is decreased and approaches 100% as the dose is increased, but—theoretically—it never passes through 0% and 100%. However, the minimal ED of any chemical that evokes a stated all-or-none response is referred to as the *threshold dose*.

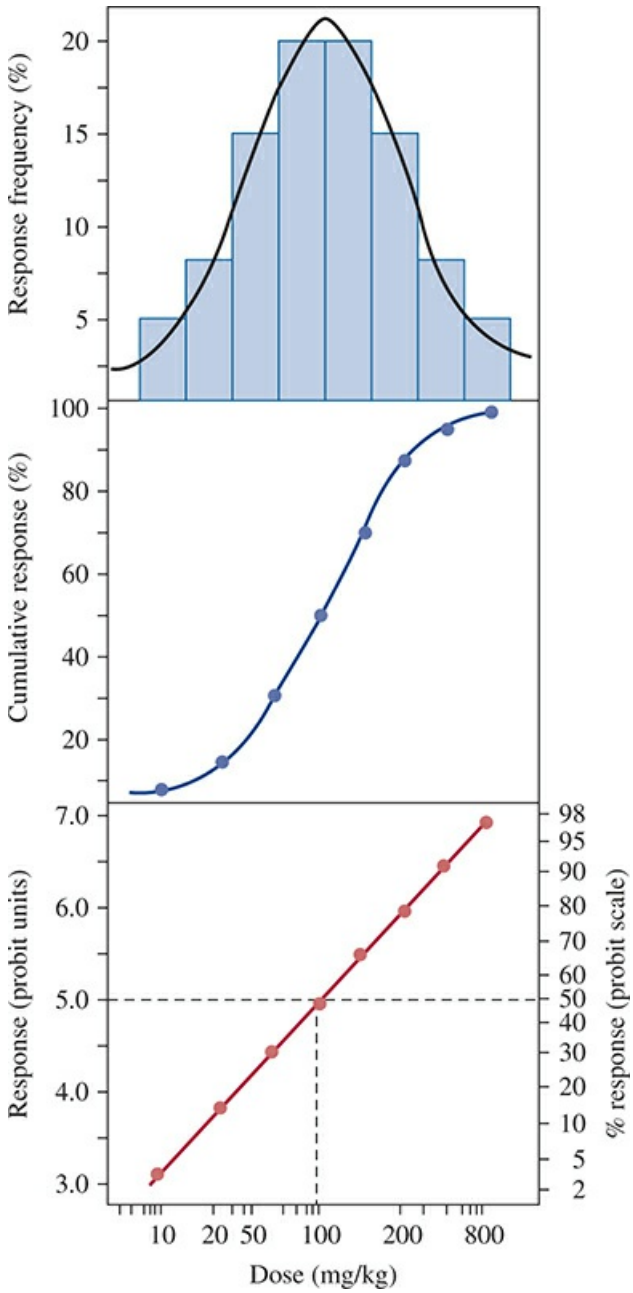

FIGURE 2–3 *Diagram of quantal dose–response relationship.* The abscissa is the log dose of the chemical. In the top panel the ordinate is response frequency, in the middle panel the ordinate is percent response, and in the bottom panel the response is in probit units (see text).

The sigmoid curve has a relatively linear portion between 16% and 84%. These values represent the limits of one standard deviation (SD) of the mean (and the median) in a population with truly normal or Gaussian distribution. In a normally distributed population, the mean ±1 SD represents 68.3% of the population, the mean ±2 SD represents 95.5% of the population, and the mean ±3 SD equals 99.7% of the population. Because quantal dose–response data usually exhibit a Gaussian distribution, one can convert the percent response to units of deviation from the mean or normal equivalent deviations (NEDs). Thus, the NED for a 50% response is 0; the NED of ±1

is equated with an 84.1% response. Units of NED are converted by the addition of 5 to the value to avoid negative numbers; these converted units are called *probit units*. The probit (from the contraction of *prob*ability un*it*), then, is a NED plus 5. In this transformation, a 50% response becomes a probit of 5, a ±1 deviation becomes a probit of 6, and a – 1 deviation is a probit of 4.

The data given in the top two panels of Fig. 2–3 are replotted in the bottom panel, with the response plotted in probit units. The data in the middle panel (which was in the form of a sigmoid curve) and the top panel (a bell-shaped curve) form a straight line when transformed into probit units (bottom panel). A probit transformation adjusts quantal data from an assumed normal population distribution to a straight line. The ED_{50} is obtained by drawing a horizontal line from probit unit 5, which is the 50% response point, to the dose–effect line. At the point of intersection, a vertical line is drawn, and this line intersects the abscissa at the ED_{50} point. Information with respect to the ED for 90% or for 10% of the population also may be derived by a similar procedure. Mathematically, it can be demonstrated that the range of values encompassed by the confidence limits is narrowest at the midpoint of the line (ED_{50}) and widest at both extremes (ED_{10} and ED_{90}) of the dose–response curve (dotted lines in Fig. 2–4). In addition to the ED_{50}, the slope of the dose–response curve can also be obtained. Figure 2–4 demonstrates the dose–response curves for the response of two compounds. Compound A exhibits a "flat" dose–response curve, showing that a large change in dose is required before a significant change in response will be observed. However, compound B exhibits a "steep" dose–response curve, where a relatively small change in dose will cause a large change in response. It is evident that the ED_{50} for both compounds is the same (8 mg/kg). However, the slopes of the dose–response curves are quite different. At one-half of ED_{50} of the compounds (4 mg/kg), less than 1% of the animals exposed to compound B would respond but 20% of the animals given compound A would respond.

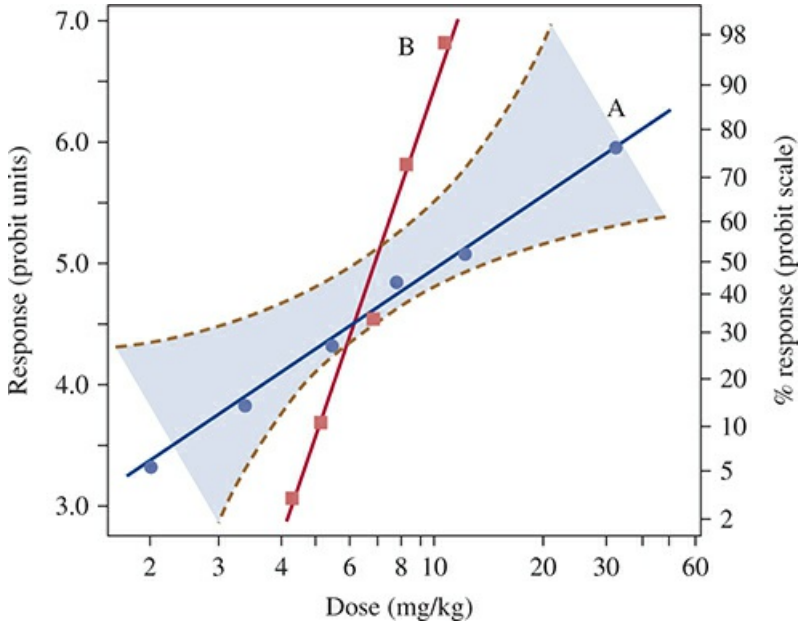

FIGURE 2–4 *Comparison of dose–response relationship for two different chemicals, plotted on a log dose-probit scale.* Note that the slope of the dose–response is steeper for chemical B than for chemical A. Dotted lines represent the confidence limits for chemical A.

In Figs. 2–3 and 2–4, the doses (mg/kg) have been specified on a logarithmic scale. For normally distributed quantal data, this transformation provides greater linearity in the dose–response relationship. However, this is not always the case. Some radiation effects give a better probit fit when the dose is expressed arithmetically rather than logarithmically. Additional models beyond probits including Hill, logit, and Weibull can be used to express dose–response relationships with rigorous mathematical analyses.

Dose Extrapolation Across Species

Researchers often work across multiple animal species, and there is a need to compare doses from one species to another. This is particularly important in drug development where preclinical data generated in laboratory animals are used to justify and design experiments for clinical testing in humans. While body weight is typically used to calculate a dose of a chemical for an individual organism, it is ineffective to generalize across species. Therefore, the extrapolation of doses across species often requires *allometry*, a field of study that examines relationships between body weight and other biological and physical parameters such as rate of basal metabolism (caloric consumption), heart rate, and blood flow. Allometric studies have revealed that the relationship between body weight and various other physiological parameters can be closely estimated by the following formula: $Y = aWb$, where Y is the biological parameter of interest (such as metabolic rate) and a and b are constants that relate Y to body weight. In general, organ sizes between species seem to scale best when b is equal to 1, whereas metabolically derived parameters scale better when b is 0.67 to 0.75.

One simple approach to estimate doses across species is to use body surface area (BSA) rather than body weight for comparison. The relationship between BSA and body weight across most mammalian species is described by the formula SA = 10.5 × (body weight [grams])$^{0.67}$. Empirical comparisons of toxicity data across species confirm that this relationship is appropriate for toxicological scaling. The exponent of body weight that gives the best correlation with toxicity was 0.73, with 95% confidence bounds of 0.69 to 0.77.

The FDA recommends calculating the *maximum recommended starting dose* for first-in-human clinical trials of an investigational drug using animal data, body surface allometry, and incorporation of a safety factor. This requires knowledge of the *no observable adverse effect level* (NOAEL) from animal studies. The NOAEL is the highest dose of chemical tested in an animal species that does not result in significant toxicities compared to control animals. Using the NOAEL dose in animals, conversion to a human equivalent dose (HED) can be made by the equation: HED = animal dose in mg/kg × (animal weight in kg/human weight in kg)$^{0.33}$. These conversions are largely used for safety testing.

Computational modeling, particularly physiologically based pharmacokinetic modeling (PBPK), is increasingly being used to scale doses across species. PBPK modeling uses key physiologic parameters and mechanistic data to mathematically describe and predict the disposition of chemicals (see Chapter 7). Toxicokinetic–toxicodynamic models can be developed that quantitatively define relationships between exposure and biological responses. This approach has been applied to various quantal and graded dose–response relationships including those assessing growth, development, and reproduction.

Shapes of Dose–Response Curves

Toxicology studies are designed to investigate responses across a wide range of doses. Characterization of adverse responses at low doses often depends upon having sensitive measures that detect meaningful, biologic changes. It is often assumed that toxicants will elicit *monotonic* responses where increasing doses will cause a steady unidirectional change in response (e.g., increase or decrease). Monotonic dose–response curves assume shapes that are "uphill" (Fig. 2–5A) or "downhill" (Fig. 2–5B) in nature. Along monotonic dose–response curves, there are dose-dependent transitions that are important in the mechanism(s) of toxicity. Typically, different slopes are observed along the dose-dependent transitions when the degree of involvement of various processes changes.

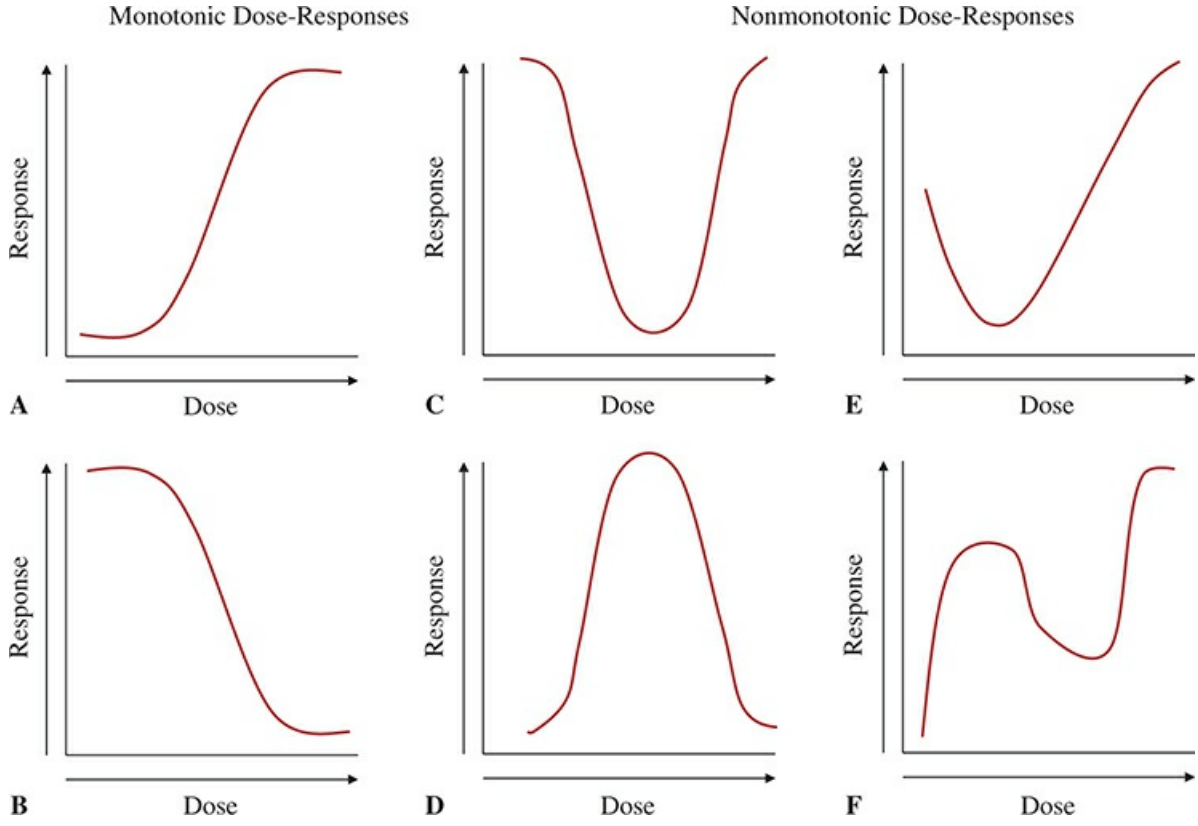

FIGURE 2–5 *Hypothetical curves for monotonic dose–responses and nonmonotonic dose–responses.* Traditional monotonic dose responses can exhibit increasing (**A**) and decreasing (**B**) changes in response with higher doses. Nonmonotonic dose–responses exhibit a number of shapes including a U-shape (**C**), inverted U-shape (**D**), J-shape (**E**), and variable slope (**F**).

Dose–response relationships also exist that are *nonmonotonic*, where multiple inflection points exist along the curve that change the shape. The shapes of nonmonotonic dose–response curves are variable and can resemble a biphasic "valley" (U-shaped [Fig. 2–5C] or J-shaped [Fig. 5E]) or an "entire hill" (an inverted U-shaped [Fig. 2–5D]). As discussed below, essential nutrients can exhibit U-shaped and inverted U-shaped curves. Similarly, some chemicals are thought to confer benefit to the organism at low exposures (called "hormetic effects" or "hormesis") and be harmful at higher doses. Still other dose–response curves resemble a "roller coaster" with multiple up and down inflection points (Fig. 2–5F).

Threshold and Linear, Nonthreshold Models— Extrapolation of the shape of monotonic dose responses at low doses can be performed by assessing *thresholds* and/or using *linear, nonthreshold* extrapolation. A *threshold* is a dose at which toxicity is first observed; at doses below this level, the probability of an individual responding is zero. By comparison, *linear, nonthreshold* models assume there is a direct and proportional relationship between dose and response, even at very low doses. The identification of a threshold in monotonic dose responses depends on the particular response being quantified, the sensitivity of the measurement, and the number of subjects studied.

The concept of thresholds has been applied to noncarcinogenic chemicals by employing a qualitative risk characterization approach known as *threshold of toxicological concern* (TTC). TTC suggests that there are levels of exposure for chemicals below which the risk to human health is not appreciable. The TTC applies in food safety, cosmetic ingredients, and impurities found in pharmaceutical preparations. TTC integrates available data including exposure, chemical structure, disposition, biotransformation, and toxicity and can be used to prioritize chemicals for further testing.

Nonmonotonic Dose–Response Curves

Essential Nutrients—For natural or endogenous chemicals required for normal physiological function and survival (e.g., vitamins and essential trace elements such as chromium, cobalt, zinc, manganese, and selenium), the "graded" dose–response relationship in an individual over the entire dose range can be U-shaped (Fig. 2–5C; Fig. 2–6). As essential elements for life, nutrient and vitamin concentrations need to be maintained within a specific range that allows for homeostasis. Doses or concentrations outside of this range can lead to *deficiency* (observed at very low doses when concentrations are below daily minimum requirements) or *toxicity* (observed at high, excessive doses). Often, the biological adverse responses that occur during periods of deficiency or toxicity will differ.

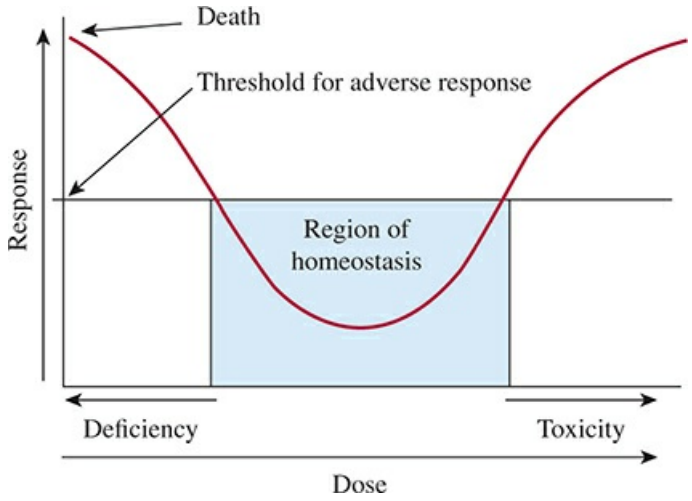

FIGURE 2–6 *Individual dose–response relationship for an essential substance such as a vitamin or trace element.* It is generally recognized that, for most types of toxic responses, a threshold exists such that at doses below the threshold, no toxicity is evident. For essential substances, doses below the minimum daily requirement, as well as those above the threshold

for safety, may be associated with toxic effects. The blue-shaded region represents the "region of homeostasis"—the dose range that results in neither deficiency nor toxicity.

Hormesis—Some non-nutritional toxic substances may also impart beneficial or stimulatory effects at low doses but that, at higher doses, produce adverse effects. This concept of "hormesis" has been described for radiation effects and chemicals. For example, chronic alcohol consumption is well recognized to increase the risk of esophageal cancer, liver cancer, and cirrhosis of the liver at relatively high doses, and this response is dose-related. However, there is also substantial clinical and epidemiological evidence that low to moderate consumption of alcohol reduces the incidence of coronary heart disease and stroke.

Endocrine Active Chemicals—Endocrine disrupting chemicals that have been shown to interfere with endocrine pathways in vivo and/or in vitro have heterogeneous sources and include industrial byproducts, plasticizers, plastics, pesticides, fungicides, and pharmaceuticals, as well as naturally occurring phytoestrogens. Nonmonotonic dose–response curves, where the slope of the dose–response curve changes sign (positive or negative) at least one time in the tested range of endocrine active chemicals, have been reported for many chemicals. Mechanisms proposed to explain the shape of the nonmonotonic dose–response curves for endocrine active chemicals include dose-dependent cytotoxicity, cell- and tissue-specific receptors and cofactors, receptor selectivity and down-regulation, receptor competition, and endocrine negative feedback loops.

Assumptions in Deriving the Dose–Response Relationship

Three assumptions need to be considered before traditional dose–response relationships can be used appropriately. First, the observed effect or response is indeed a causal one. For some data, it is not always apparent that the response is a result of chemical exposure. In its most strict usage, the dose–response relationship is based on the knowledge that the effect is a result of a known toxic agent or agents.

A second assumption seems simple and obvious: the magnitude of the response is in fact related to the dose. This assumption relies upon identifying a molecular target with which the chemical interacts to initiate the response and determining that the production of a response and the degree of response are related to the concentration of the chemical at the target site. In turn, the concentration at the site would be related to the dose administered.

The third assumption of the dose–response relationship is that there exist both an appropriate method of quantifying and a precise means of expressing the toxicity. The ideal endpoints are those closely associated with the molecular events resulting from exposure to the toxicant. For a given chemical, there may be multiple dose–response relationships, one for each toxicity endpoint. A hypothetical chemical that produces cancer through genotoxic effects, liver damage through inhibition of a specific enzyme, and CNS effects by reducing neurotransmitter release would likely have three distinct dose–response relationships.

Evaluating the Dose–Response Relationship

Therapeutic Index—Figure 2–7 illustrates a hypothetical quantal dose–response curve for a desirable effect of a chemical (effective dose, ED) such as anesthesia, a toxic effect (toxic dose, TD) such as liver injury, and the lethal dose (LD). Each of these responses spans a range of doses

and increases at varying rates (or estimated slopes). These curves highlight the importance of the (1) selection of relevant pharmacological/toxicological endpoints and (2) comparisons made between endpoints. The concept of the "therapeutic index" (TI) can be used to illustrate this relationship. Although the TI is directed toward a comparison of the therapeutic ED to the TD of a chemical, it is similarly applicable to considerations of comparative toxicities between chemicals. In its broadest sense, the TI is defined as the ratio of the dose required to cause a toxic effect to the dose needed to produce the desired therapeutic response. Similarly, an index of comparative toxicity is obtained by the ratio of doses of two distinct materials to produce an identical response or the ratio of doses of the same material necessary to yield different toxic effects.

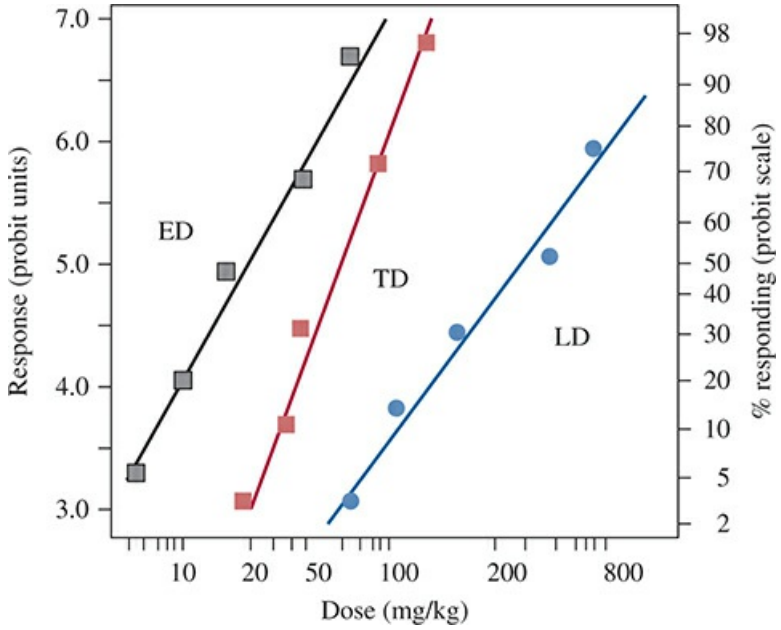

FIGURE 2–7 *Comparison of effective dose (ED), toxic dose (TD), and lethal dose (LD).* The plot is of log dose versus percentage of population responding in probit units.

The most commonly used index is the median dose that elicits an effective or toxic response in 50% of a population (ED_{50} or TD_{50}). The TI of a drug is an approximate statement about the relative safety of a drug expressed as the ratio of the adverse endpoint or TD (historically, the LD) to the effective therapeutic dose:

$$TI = \frac{TD_{50}}{ED_{50}}.$$

From Fig. 2–7, one can approximate a TI by using median doses. The larger the ratio, the greater relative safety is assumed. The ED_{50} is approximately 20, and the TD_{50} is about 60; thus, the TI is 3, a number indicating that reasonable care in exposure to the drug is necessary to avoid toxicity. However, the use of the median effective and median toxic doses is not without disadvantages, because median doses do not reflect the slopes of the dose–response curves for therapeutic and toxic effects.

Margins of Safety and Exposure—One way to overcome the limitation of comparing variable

slopes between the effective and toxic dose–response curves is to calculate the ED_{99} for the desired effect (efficacy in 99% of the population) and the TD_1 for the undesired effect (toxicity in 1% of the population), typically after a single administration of a chemical. These parameters are used in the calculation of the *margin of safety* (MOS):

$$\text{MOS} = \frac{\text{TD}_1}{\text{ED}_{99}}.$$

For nondrug chemicals, the term *MOS* is used in risk assessment as an indicator of the magnitude of the difference between an estimated "exposed dose" to a human population and NOAEL or other benchmark dose determined in experimental animals.

A measure of the degree of accumulation of a chemical and/or its toxic effects can also be estimated from quantal toxicity data. The *chronicity index* of a chemical is a unitless value obtained by dividing its one-dose TD_{50} by its 90-dose (90-day) TD_{50}, with both expressed in milligrams per kilogram per day. If no cumulative effect occurs over the doses, the chronicity index will be 1. If a compound caused cumulative effects, the chronicity index would be 90.

Potency versus Efficacy—*Efficacy* is an assessment of the extent to which a chemical can elicit a response and is often interpreted using the ordinate axis (or *y*-axis). By comparison, the *potency* is determined by the range of doses (on the *x*-axis) over which a chemical produces increasing responses. Figure 2–8 depicts the dose–response curves for four different chemicals. Chemical A is said to be more potent than chemical B because of their relative positions along the dose axis. Likewise, chemical C is more potent than chemical D. Maximal *efficacy* reflects the magnitude achieved on the response axis. Chemicals A and B have equal maximal efficacy, whereas the maximal efficacy of C is less than that of chemical D.

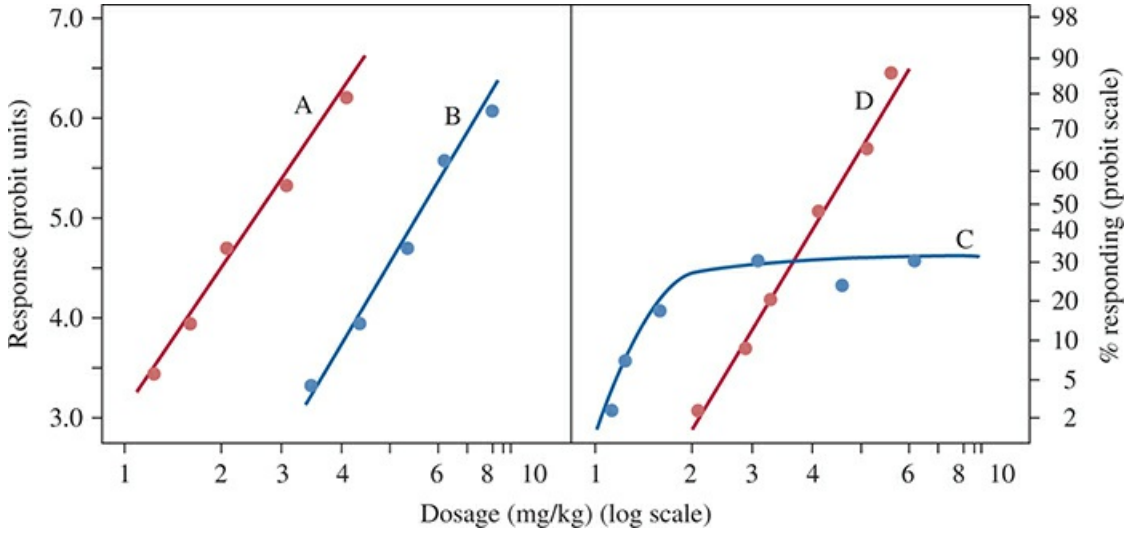

FIGURE 2–8 *Schematic representation of the difference in the dose–response curves for four chemicals (**A to D**), illustrating the difference between potency and efficacy (see text).*

ASSESSING TOXICOLOGICAL RESPONSES

Causation in Toxicology

The ability of an intervention to limit exposure to the chemical and in turn reduce the likelihood or extent of disease supports a "cause-and-effect" relationship. Likewise, the ability to draw analogies between related chemicals, due to physicochemical properties or molecular structures (or core features), can lessen the depth of experimental validation needed to assign causation when comparing to a previously characterized toxicant. This approach, often termed *read-across*, has been increasingly implemented, and this read-across computational approach allows scientists to utilize existing and extrapolated data to assess the likelihood that structurally related chemicals share a propensity for toxicity.

Evidence-based toxicology is composed of three methodological steps. First, the toxicologist collects and evaluates the relevant data regarding the chemical and outcome including source, exposure, dose, and diagnosis. Second, the relevant knowledge is collected in order to frame the question as well as to collate and evaluate the relevant literature. Third, the data and knowledge are integrated in order to formulate a conclusion. This includes evaluation of general causation, dose–response relationships, timing, alternative causes or confounders, rigorous statistical analyses of the results of individual studies and meta-analyses (combined studies), and coherence of the findings.

Experimentation and hazard identification are inherent to identifying cause-and-effect relationships. However, the risk or probability that toxicity will occur depends upon specific exposure scenarios. As a result, dose–response relationships and evaluation of human exposure paradigms are needed to characterize the risk and perform an assessment of the toxicological risk.

The intended purpose of the precautionary principle is the protection of the public's health and the environment. As such, the precautionary principle advocates regulatory restrictions that limit human exposure before sound, empirical data regarding the safety of a chemical are in place. Following this approach, the burden of proof is placed upon the manufacturer to establish the lowest level of risk before commercial usage. Challenges to the precautionary principle suggest there is a lack of clarity in its application and question how much certainty is needed to guarantee there is little to no risk of harm.

Mechanisms and Modes of Action

Mechanisms of action refer to the detailed molecular and biochemical changes that are required for a molecule to cause damage. This is a precise determination of "how" and the "extent" to which molecular events cause toxicity. Some possible mechanisms of action for carcinogenesis include the ability of the chemical to act as an electrophile, be genotoxic, alter DNA repair, cause epigenetic changes, modulate receptor signaling, and enhance proliferation. Various steps including the delivery of the chemical to the site of action, interaction with key signaling targets, cellular dysfunction, and disrepair will collectively determine the extent and reversibility of toxicity (Fig. 2–9). Factors such as pharmacokinetics, cell signaling, and maintenance, as well as molecular, cellular, and tissue responses will modulate each of these steps.

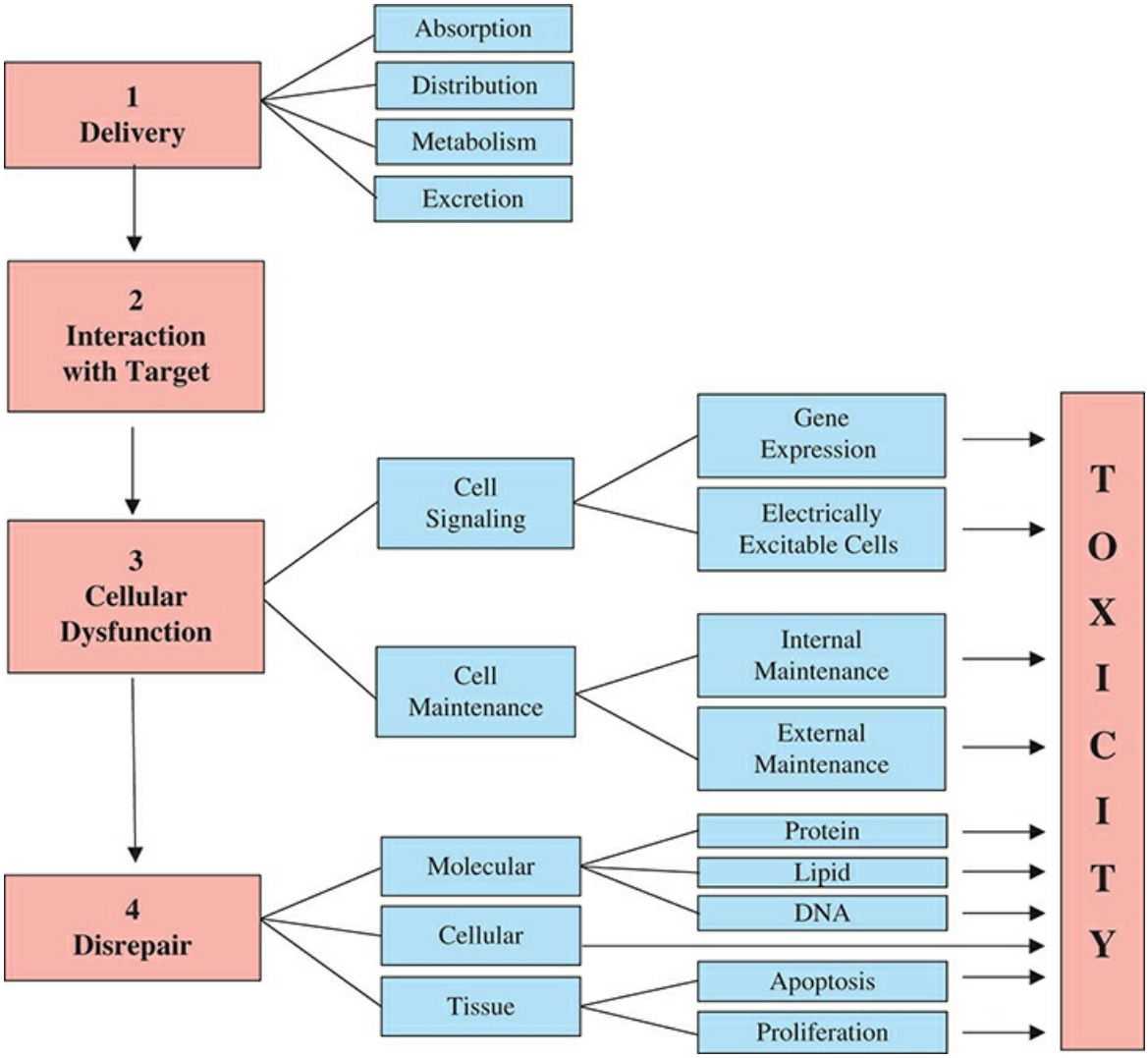

FIGURE 2–9 *Steps that result in chemical toxicity.*

A number of considerations are made regarding (1) key events, (2) associative events, and (3) modulating factors. *Key events* are causal precursors that are necessary for the adverse outcome. An example key event might be the formation of a specific DNA adduct. *Associative events* are processes that are not required for the mode of action but can serve as indicators or surrogate markers for the key events. An example of an associative event could be cellular proliferation in a nontarget tissue. *Modulating factors* can alter the dose–response relationship or likelihood of inducing a key event(s) leading to an adverse outcome. Modulating factors include generation of reactive oxygen species, altered cellular communication, activation of certain transcriptional pathways, immune responsiveness, life stage, genetic variation, and others.

Adverse Outcome Pathways

Toxicological evaluation has adopted the use of adverse outcome pathways (AOPs) to provide a conceptual framework and practical tool to portray and associate sequential biological events at the molecular, cellular, and organ levels following exposure to a chemical that ultimately results

in toxicity to an organism. AOP modeling utilizes data from computational (quantitative structure activity relationships), in vitro, and in vivo studies and integrates both mode and mechanism of action to describe toxicological processes.

In each AOP, a chemical's structural and physical properties are first evaluated using in silico approaches that may or may not include metabolism of the chemical to other forms. Then, the chemical is assessed for critical molecular initiating events, which include receptor/ligand interactions, macromolecule interactions, protein modifications and alkylation, nuclear receptor activation, and changes in membrane fluidity. With advancing biological complexity, a logical sequence of cellular, organ, and organism responses will be identified—each representing linked key events in the AOP that are required for progression of the toxicity. Responses that include the molecular initiating events and the cellular response are typically classified as the *toxicity pathway*. While the relationships between key events are most often linear, there is the potential for multiple steps to converge or diverge into various key events. The organism responses consist of target organ toxicities, malformations, cancer development, and/or lethality revealed from in vivo testing. Collectively, the molecular initiating events and key events comprise the *mode of action pathway*. While AOPs are similar to modes of action for a chemical, the AOP is anchored to both the molecular initiating event and the *adverse outcome*, a specialized key event of regulatory significance, which strengthens the certainty of the overall model. Lastly, the AOP pathway will advance to understanding how toxicant responses will impact an entire population.

VARIATION IN TOXIC RESPONSES

Selective Toxicity

Selective toxicity means that a chemical produces injury to one kind of living matter (such as a cell or organism) without harming another form of life even though the two may exist in intimate contact. By taking advantage of the biological diversity between species, it is possible to develop chemicals that are lethal for an undesired species and harmless for other species. Selective toxicity results because the chemical (1) is equally toxic to both organisms but accumulates preferentially in the target or (2) alters a unique cellular or a biochemical feature that is absent or irrelevant in the unaffected species. Differences in the absorption, biotransformation, or excretion of the toxicant, or intracellular metabolism or transport may dictate accumulation of the ultimate toxic compound in affected tissues.

Species Differences

Experimental animals are routinely used in toxicology as surrogates for humans; however, it is important to recognize the quantitative and qualitative differences in response to toxic substances among various species. Even among phylogenetically similar species (e.g., rats, mice, guinea pigs, and hamsters), large differences in response may be observed.

Modifying Factors

Not all humans respond to toxicants in the same manner and to the same degree as each other.

Multiple factors that modify one's susceptibility to adverse outcomes include genetic variation among a population, age and life stages, sex and hormonal status, microbiome, and circadian rhythm.

Genetics—Hereditary differences in a single gene that occur in more than 1% of the population are referred to as *genetic polymorphisms*. Increasingly, genetic variants are being identified that impact a variety of target organ toxicities.

Clinically, pharmacogenetic variants can have a critical influence on a patient's susceptibility to a drug toxicity. These variants may be in the coding or regulatory regions and influence pharmacokinetic/pharmacodynamic responses. The Pharmacogenetics Knowledge Database (PharmGKB) is an online resource that curates the literature on drug-related gene variants for use by scientists and researchers. The Clinical Pharmacogenetics Implementation Consortium (CPIC) routinely publishes peer-reviewed, evidence-based clinical guidelines that inform clinicians of particular variant alleles for which pharmacogenetic testing should be ordered while initiating therapy.

New approaches to identifying associations between diseases or adverse outcomes and common genetic variants (polymorphisms) include "genome-wide association studies" (GWAS) and next-generation sequencing of DNA. GWAS are based on a scan of hundreds of thousands of specific genetic variants (markers called "tag SNP") across the genome of persons affected by a particular disorder or adverse-response phenotype and persons who are not affected, with robust statistical tests to identify associations between a specific genetic marker and the phenotype (e.g., disease state or adverse drug response). Next-generation sequencing allows millions or billions of DNA strands to be sequenced in parallel, often revealing unique and rare variants.

Age—Life stage is an important factor that can alter susceptibility to toxicity. Metabolic processes that aid in xenobiotic clearance are often altered at juvenile and advancing ages. For example, newborns have relatively low gastric emptying, gastrointestinal motility, and expression of the metabolic enzymes including CYP2D6, CYP2E1, and CYP3A4. Likewise, as humans age to greater than 70 years old, reductions in liver, gastrointestinal, and kidney functions as well as lower receptor activity (e.g., beta-adrenergic receptor) along with more prevalent underlying pathologies can heighten the risk of adverse events.

Sex—Gender can be a determinant of xenobiotic disposition and toxicity. A notable sex-related example in humans is the effect of alcohol. Alcohol is absorbed and metabolized differently in females and males. This is in part due to the lower extent of body water in women compared to men of a similar weight. Toxicities such as liver disease and brain damage due to alcohol consumption appear to be more frequent and/or occur earlier in females compared to males. Critical transcription factors including the signal transducer and activator of transcription 5b (STAT5b) regulate liver gene expression in a sexually dimorphic manner. In addition to growth hormone–related signaling, sex hormones including estradiol and testosterone influence the expression and function of drug metabolizing enzymes and transporters, which can alter xenobiotic disposition and toxicity.

Circadian Rhythm—Circadian rhythm is a 24-hour cycle that regulates numerous molecular and physiological processes. Within the 24-hour cycle, there are diurnal (light cycle), nocturnal (dark cycle), and crepuscular (transition) periods. The circadian clock consists of a cellular clock

with specific genes that oscillate in expression. Timing in the circadian system is affected by such factors as light, activity, food consumption, and social cues. Though most changes in physiological processes during the 24-hour period are not readily apparent, they can impact susceptibility to toxicity.

Microbiome—Within the body, bacteria outnumber human cells by a ratio of 10:1. Typically, anaerobic and facultative aerobic bacteria comprise the resident microflora of the intestinal tract. The influence of commensal microbes on human health, including toxicologic responses, is garnering greater attention with the advent of highly sensitive methods in metagenomics. The ability to sequence the 16S rRNA subunit allows for comprehensive profiling of bacterial species.

TOXICITY TESTING

Two principal concepts underlie descriptive animal toxicity testing. The first is that the effects produced by a compound in laboratory animals, when properly qualified, are applicable to humans. Most, if not all, known chemical carcinogens in humans are carcinogenic in some species, but not necessarily in all species. For regulatory and risk assessment purposes, positive carcinogenicity tests in animals are usually interpreted as indicative of potential human carcinogenicity.

The second concept is that the exposure of experimental animals to high doses of chemicals is a necessary and valid method of discovering possible hazards in humans. This principle is based on the quantal dose–response concept that the incidence of an effect in a population is greater as the dose or exposure increases. Because the number of animals used in toxicology experiments is small compared with the size of human populations at risk, obtaining statistically valid results requires the use of relatively large doses so that the effect will occur frequently enough to be detected. However, the use of high doses can create problems in interpretation if the response(s) obtained at high doses does not occur at low doses.

Toxicity tests do not demonstrate that a chemical is safe but, rather, they serve to identify and characterize the toxic effects a chemical can produce. Depending on the eventual use of the chemical, the toxic effects produced by structural analogs of the chemical, as well as the toxic effects produced by the chemical itself, contribute to the determination of the toxicology tests that should be performed.

Although various countries often have different testing requirements for toxicity testing/product safety evaluation, efforts to “harmonize” such testing protocols have resulted in more standardized approaches. The International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use includes regulatory authorities from Europe, Japan, and the United States, as well as experts from the pharmaceutical industry in the three regions, who worked together to develop internationally recognized scientific and technical approaches to pharmaceutical product registration.

Typically, a tiered approach, with testing dependent upon the results of initial studies, is outlined in Fig. 2–10. Early studies assess purity, stability, solubility, and other physicochemical factors that could impact the ability of the test compound to be delivered effectively to animals. Then, the chemical structure of the test compound is compared with similar chemicals for which toxicological information is already available. Structure–activity relationships (SAR) derived

from a review of existing toxicological literature can provide additional guidance on the design of acute and repeated-dose experiments, including which specialized tests need to be completed. Once such basic information has been compiled and evaluated, the test compound is then administered to animals in acute and repeated-dose studies.

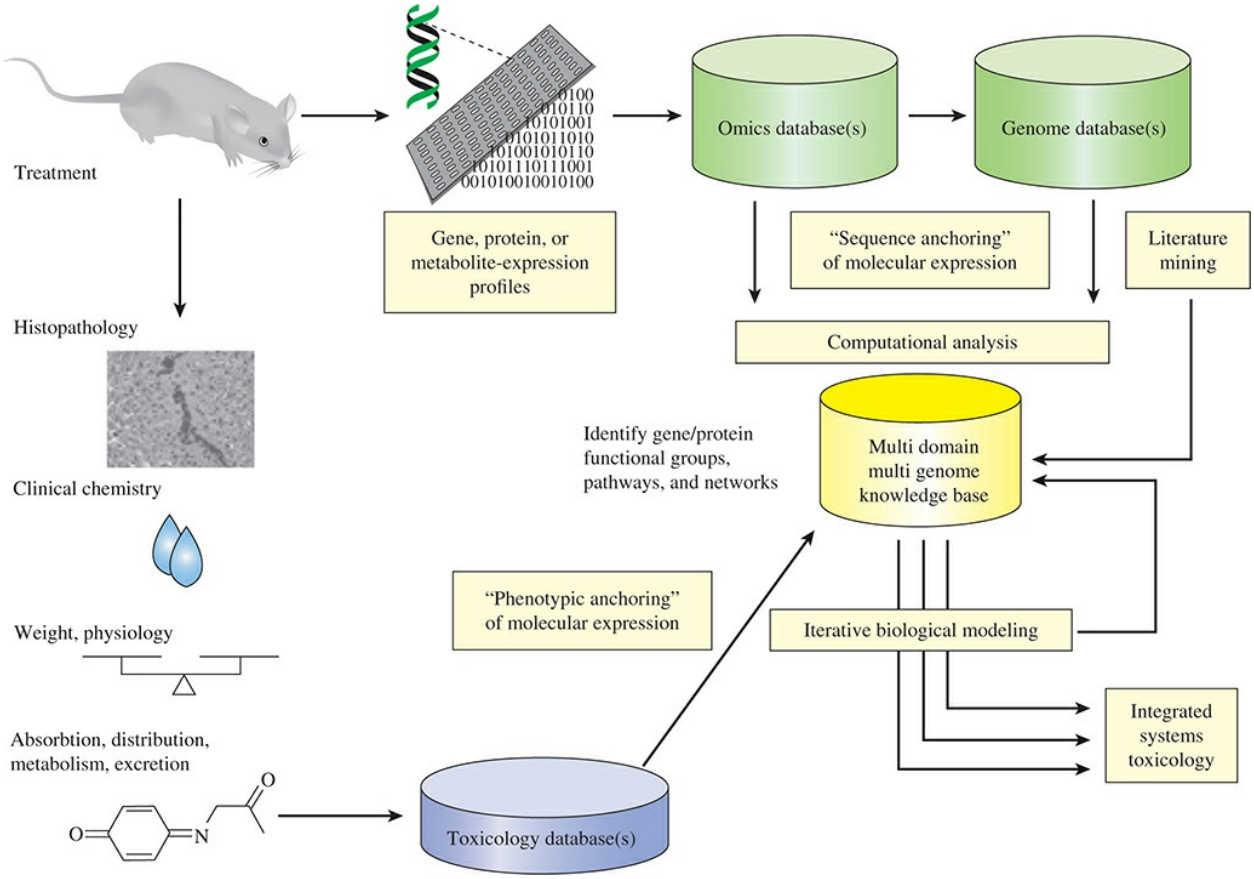

FIGURE 2–10 *Conceptual approach for incorporating "omics" technologies and resulting large databases into toxicological evaluation.* Data from experiments that evaluate the effects of a chemical on global patterns of gene expression (transcriptomics), protein content (proteomics), and small molecules/metabolites (metabonomics/metabolomics), combined with genomic information from both the test species (e.g., rats and mice) and the target species of interest (e.g., humans), are analyzed by computational tools (bioinformatics) for unique or potentially predictive patterns of toxicity. Essential to the use of omics data for predictive toxicology/safety assessment is the ability to reliably tie observed omics patterns to traditional measures of toxicity, such as histopathology and clinical chemistry (phenotypic anchoring). (Reprinted from Waters MD, Fostel JM: Toxicogenomics and systems toxicology: aims and prospects. *Nat Rev Genet* 2004; 5:969-948.)

Because of increased societal pressure to reduce or eliminate the use of animals in toxicity testing, while also ensuring that new chemicals do not pose unreasonable risks to human health or the environment, the EU promulgated the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) regulation, which is stimulating innovation in sampling and analysis, toxicology testing, exposure modeling, alternative toxicity testing, and risk assessment practices. Alternative in vitro approaches to toxicity assessment are transforming product safety

evaluation.

New “high-throughput” approaches to toxicity testing allow at least a basic hazard characterization for the thousands of untested chemicals currently in the marketplace, as well as the many new chemicals introduced each year. An extensive battery of in vitro tests is used to evaluate AOPs for chemicals. New technologies in genomics, transcriptomics, proteomics, metabolomics, and bioinformatics are combined with automated high-throughput technologies to create a tiered structure for toxicity testing. The approach to using biochemical and molecular pathway-based analyses, rather than apical endpoints (e.g., target organ damage, mutagenesis, carcinogenesis, reproductive and developmental effects), to identify potentially problematic chemicals early in their development is particularly attractive from a time frame and economic perspective. With validation, in vitro screening tests can reduce the number of animals needed for safety testing and potentially replace some traditional approaches. Established in vivo studies continue to serve an important role in hazard evaluation, especially for high-priority chemicals.

Acute Toxicity Testing

Safety testing is initiated with acute studies that identify target organs and provide an estimate of the intrinsic toxicity for a substance. Typically performed in rodents (rats and mice), these studies aim to approximate the LD (e.g., LD_{50}) of a chemical within 14 days after a single exposure. These data influence the design and dose selection for subchronic and chronic studies.

Several variables are considered (and often controlled) in the design of acute toxicity tests. There will typically be one control group and four to five treatment groups that each contain three to five animals per group. Both sexes in two species will often undergo this testing protocol. Typically, the route of administration will be the same that is anticipated or intended for human exposure. Approaches to determine the doses and dosing include: (1) fixed doses (5 to 2000 mg/kg OECD Guideline 420), (2) stepwise method (one group is tested at a time and observations determine the next step (OECD Guideline 423), and (3) up-and-down procedure, where an initial starting dose is estimated from in silico and in vitro testing and then successive doses are selected using half-log increases (OECD Guideline 425). Only a *single* dose of the vehicle and test chemical is administered. Inhalation exposures occur once for a 4-hour period. Assessments include twice-daily examination of animal weight, behavioral modifications, morbidity, and food consumption. After 14 days, a complete necropsy is performed.

The gross and microscopic conditions of the organs and tissues (up to 40) and the weight of the major organs are recorded and evaluated. Hematology measurements usually include hemoglobin concentration, hematocrit, erythrocyte counts, total and differential leukocyte counts, platelet count, clotting time, and prothrombin time. Clinical chemistry determinations commonly made include glucose, calcium, potassium, urea nitrogen, serum alanine and aspartate aminotransaminases, gamma-glutamyl transpeptidase, lactic dehydrogenase, alkaline phosphatase, creatinine, bilirubin, triglycerides, cholesterol, albumin, globulin, and total protein. Urinalysis is usually performed in the middle of and at the termination of the testing period and often includes determination of specific gravity or osmolarity, pH, proteins, glucose, ketones, bilirubin, and urobilinogen as well as microscopic examination of formed elements. If humans are likely to have significant exposure to the chemical by dermal contact or inhalation, subchronic dermal and/or inhalation experiments may also be required.

The LD_{50} is not a biological constant. Many factors influence toxicity and thus may alter the

estimation of the LD_{50}. Factors such as animal strain, age, and weight, type of feed, caging, pretrial fasting time, method of administration, volume and type of suspension medium, and duration of observation have all been shown to influence adverse responses to toxic substances. Because of this inherent variability in LD_{50} estimates, it is only necessary to characterize the LD_{50} within an order of magnitude range such as 5 to 50 mg/kg, 50 to 500 mg/kg, and so on.

Because of a growing interest to develop in silico and in vitro methods to reduce the number of animals needed for acute systemic toxicity tests, the *Registry of Cytotoxicity* (RC) was developed using linear regression analysis of the cytotoxic concentration values determined in mammalian cells in culture and the LD_{50} values reported in the literature from various laboratory species. Such an in vitro approach does provide a rapid first approximation of acute toxicity without the use of experimental animals and suggest a starting dose range for in vivo studies.

Subacute (Repeated-Dose) Toxicity Testing

Subacute toxicity tests are performed to obtain information on the toxicity of a chemical after repeated administration and as an aid to establish doses for subchronic studies. A typical protocol is to give three to four different doses of the chemicals to the animals by mixing them in their feed. For rats, 10 animals per sex per dose are often used; for dogs, three doses and three to four animals per sex are used. Clinical chemistry and histopathology are performed after either 14 or 28 days of exposure.

Subchronic Toxicity Testing

Subchronic exposure can last for different periods of time, but 90 days (13 weeks) is the most common test duration. The principal goals of the subchronic study are to establish a NOAEL, a *lowest observed adverse effect level* (LOAEL), and to further identify and characterize the specific organ or organs affected by the test compound after repeated administration. An alternative to the NOAEL approach uses all the experimental data to fit one or more dose–response curves and estimate a benchmark dose that is defined as “the statistical lower bound on a dose corresponding to a specified level of risk.” Advantages of determining a benchmark dose rather than a NOAEL include a lesser dependency upon the experimental doses selected, consideration of the shape of the dose–response curve, and an ability to account for variability and uncertainty within the data.

A subchronic study is usually conducted in two species (often rat and dog for FDA, and rat or mouse for EPA) by the route of intended exposure (usually oral). At least three doses are employed (a high dose that produces toxicity but does not cause more than 10% fatalities, a low dose that produces no apparent toxic effects, and an intermediate dose) with 10 to 20 rodents and 4 to 6 dogs of each sex per dose. Prior to the initiation of a subchronic (and chronic) safety study, a period of 1 to 2 weeks will be used for pretesting where animals in the control and (anticipated) treated groups are compared for various baseline values. When the test compound is administered in the diet over a prolonged period of time (subchronic and chronic studies), the concentration in the diet should be adjusted periodically (weekly for the first 12 to 14 weeks) to maintain a constant intake of material based on food consumption and rate of change in body weight. Animals should be observed once or twice daily for signs of toxicity, including changes in body weight, diet consumption, changes in fur color or texture, respiratory or cardiovascular

distress, motor and behavioral abnormalities, and palpable masses. All premature deaths should be recorded and necropsied. Severely moribund animals should be terminated immediately to preserve tissues and reduce unnecessary suffering. At the end of the 90-day study, all remaining animals are terminated, and blood and tissues are collected for further analysis. Subchronic toxicity studies not only characterize the dose–response relationship of a test substance after repeated administration but also provide data for a more reasonable prediction of appropriate doses for chronic exposure studies.

Chronic Toxicity Testing

Long-term or chronic exposure studies are performed similarly to subchronic studies except that the period of exposure is longer than three months. In rodents, chronic exposures are usually for six months to two years. Chronic studies in nonrodent species are usually for one year but may be longer. The length of exposure is somewhat dependent on the intended period of exposure in humans.

Dose selection is critical in these studies to ensure that premature mortality from chronic toxicity does not limit the number of animals that survive to a normal life expectancy. Most regulatory guidelines require that the highest dose administered be the estimated *maximum tolerable dose* (MTD, also commonly referred to as the “minimally toxic dose”). MTD may be defined as the dose that suppresses body weight gain slightly (i.e., 10%) in a 90-day subchronic study. However, the use of parameters other than weight gain, such as physiological and pharmacokinetic considerations and urinary metabolite profiles, may be used as indicators of an appropriate MTD. Generally, one or two additional doses, usually fractions of the MTD (e.g., one-half and one-quarter MTD), and a control group are tested.

Developmental and Reproductive Toxicity

Developmental toxicology is the study of adverse effects on the developing organism occurring anytime during the life span of the organism that may result from exposure to chemical or physical agents before conception (either parent), during prenatal development, or postnatally until the time of puberty. *Teratology* is the study of defects induced during development between conception and birth (see Chapter 10). *Reproductive toxicology* is the study of the occurrence of adverse effects on the male or female reproductive system that may result from exposure to chemical or physical agents (see Chapter 21).

Mutagenicity

Mutagenesis is the ability of chemicals to cause changes to DNA that are transmitted during cell division. *Germline mutations* damage DNA in sperm and ova, which undergo meiotic division and therefore have the potential to transmit mutations to future generations. *Somatic mutations* refer to nonheritable mutations in all other cell types. Somatic mutations may lead to cell death or transmission of the genetic defect through mitotic division. Mutagenic tests are often used to screen for potential carcinogens. Numerous in vivo and in vitro procedures have been devised to test chemicals for their ability to cause mutations (see Chapter 9).

Carcinogenicity

Carcinogenicity studies are performed when there is reason to suspect that a chemical may be carcinogenic, or when there may be widespread, long-term exposures to humans (e.g., food additives, drinking water contaminants, or pharmaceuticals administered repeatedly for long time periods). Chemicals that test positive in several mutagenicity assays are likely to be carcinogenic, and thus are frequent candidates for oncogenicity bioassay assessment (see Chapter 8). Studies to evaluate the carcinogenic (oncogenic) potential of chemicals are usually performed in rats and mice and extend over the average lifetime of the species (18 months to 2 years for mice, 2 to 2.5 years for rats). Both gross and microscopic pathological examinations are made not only on animals that survive the chronic exposure but also on those that die prematurely.

Neurotoxicity Assessment

Neurotoxicity is defined as the adverse changes in the chemistry, structure, or function of the nervous system following exposure to a chemical or physical agent (see Chapter 16). Neurotoxic effects can occur in central or peripheral nervous systems and disrupt sensory, motor, autonomic, or cognitive functions. The developing nervous system is particularly sensitive to chemical exposures (see Chapter 10) necessitating testing on adult and perinatal/juvenile animals.

Immunotoxicity Assessment

Under normal conditions, the immune system is responsible for host defense against pathogenic infections and certain cancers. However, xenobiotic exposures can alter immune system development and/or function and lead to immunostimulation (sensitization or autoimmunity) or immunosuppression (see Chapter 12).

Sensitization

Skin sensitization, also known as allergic contact dermatitis, is the immune-mediated reaction of the skin to a chemical. The appearance of skin sensitization can range in humans from erythema, edema, and vesicles to bullae, whereas in rodents only erythema and edema may be observed (see Chapter 19).

Eye and Skin Irritation and Corrosion

Irritant chemicals cause reversible injury to the eyes (see Chapter 17) or skin (see Chapter 19), whereas *corrosive* damage is irreversible. Several new validated methods are available to test a chemical's ability to cause irritation and/or corrosion. Typical ocular endpoints include evaluation of the cornea (opacity), iris (congestion and reaction to light), and the conjunctivae (redness and chemosis). The skin is evaluated for erythema/eschar and edema and reversibility of these findings during a 14-day period.

Other Toxicity Tests

Most of the tests described above will be included in a “standard” toxicity testing protocol because they are required by the various regulatory agencies. Additional tests may be required or included in the protocol to provide information relating a special route of exposure, such as inhalation. Inhalation toxicity tests in animals are usually carried out in a dynamic (flowing) chamber to avoid particulate settling and exhaled gas complications (see Chapter 15).

SYSTEMS TOXICOLOGY

The integration of classical toxicology testing with the quantitative analysis of molecular and functional changes occurring across multiple levels of biological organization has resulted in the field of *systems toxicology*. The molecular functions include *transcriptomics* (complete set of RNA transcripts found in the genome), *epigenomics* (global modifications to genetic material), *proteomics* (profile of proteins present within cells or tissues), *metabonomics/metabolomics* (endogenous and exogenous by-products of cellular metabolism), and *lipidomics* (a subset of the metabolome that is enriched by lipids). Each of these molecular processes is vulnerable to changes by exposure to chemicals. Systems toxicology also integrates the *exposome*, or the cumulative environmental exposures (including chemicals, diet, lifestyle, etc.) over a lifetime that impact the development and progression of diseases, into the testing paradigm. The goal of predictive toxicity testing is to prioritize the testing of myriad chemicals and in turn reduce the number of animals needed for research and regulatory studies.

Transcriptome

The transcriptome (all of the mature mRNA species present in a cell at a given point in time) is dynamic and represents the steady state between the rate of synthesis (transcription) and degradation of mRNAs in a cell. The reverse transcription–polymerase chain reaction (RT-PCR) allows one to quantitatively determine the relative number of mRNA species in a sample for specific genes. Using microarray technologies, where tens of thousands of unique oligonucleotides (or cDNAs) are anchored on a solid matrix, toxicologists can quantitatively assess the expression of thousands of unique mRNAs in a single sample, thus capturing an “expression profile” of the entire transcriptome in a single analysis.

Next-generation sequencing (NGS) allows use of DNA or RNA samples for whole-genome sequencing, RNA sequencing, targeted sequencing (such as exome sequencing or 16S sequencing), and other approaches. Whole-transcriptome sequencing identifies and quantifies the abundance of all RNA transcripts including coding mRNAs and noncoding RNAs (such as microRNAs). Compared to microarrays, RNA-sequencing has higher resolution and reproducibility and can be used to study alternative splicing events and novel transcripts.

Epigenome

The epigenome includes global changes in the DNA genome that affect gene expression. Epigenetics may be defined as a mitotically or meiotically heritable change in gene expression that occurs independently of an alteration in DNA sequence. Gene expression may be silenced or suppressed, or in some instances activated, by DNA methylation or histone deacetylation—

changes that do not alter the nucleotide sequence of the silenced genes. Subtle epigenetic changes that are not associated with cytotoxicity or mutations can also result from environmental exposures and thus may have important toxicological implications. Epigenetic changes have been causally implicated in cancer, neurodevelopment disorders, autoimmune diseases, diabetes and metabolic disorders, asthma, behavioral disorders, and endocrine disorders.

Proteome

The *proteome* represents the complete set of proteins expressed within a cell, organ, or organism. Increases and decreases in proteins may result from changes in transcription or from posttranslational modifications that enhance translation or turnover. Enrichment of different proteins as a result of toxicant exposure can be determined. Mass spectrometry is the primary technology used for proteomics because of its high sensitivity and accuracy in identifying proteins, down to the subfemtomolar range. Mass spectrometry can be used to identify and quantify protein enrichment and to assess posttranslational modifications such as phosphorylation and ubiquitylation. These alterations to proteins can impact their localization, activity, and/or interaction with other macromolecules.

Metabonomics/Metabolomics

These two terms describe the analysis of small molecules that serve as substrates, products, and cofactors in the multitude of enzymatic reactions and other metabolic processes that define living cells. Metabonomics is the profiling of metabolite levels and their systematic and temporal changes resulting from diet, lifestyle, environment, genetics, and xenobiotic exposures in whole organisms, whereas metabolomics principally refers to studies in plants and in vitro or single-cell systems. Nuclear magnetic resonance provides structural identification of metabolites; however, mass spectrometry is more sensitive.

Exposome

The *exposome* represents the cumulative environmental exposures (including diet, lifestyle, exercise, inherent metabolic activity, infections, and xenobiotics) across an entire life span that affect human health. Defining the exposome depends upon highly sensitive analytical methods that can detect changes in biomarkers of exposure, effect, and/or susceptibility that represent the interaction between an exposure and a biological system. Biomarkers include exogenous chemicals (or their metabolites), biochemical, physiological, or other alterations in an organism, and intrinsic and acquired factors that will influence susceptibility to toxicity. The discovery and validation of appropriate biomarkers has enabled the conductance of environment-wide association studies (EWAS) in which candidate biomarkers are used to evaluate the causality, prevention, detection, prognosis, and treatment of diseases with regards to the total environment.

High-Content Screening

High-content screening or cellomics is an automated microscopy analysis that often uses fluorescence to visualize cellular or subcellular changes in cells, organs, or small organisms

(such as zebrafish) in a high-throughput format. Quantitative measurements include morphology, apoptosis, autophagy, cell proliferation, DNA damage, mitochondria functioning, mitosis, oxidative stress, and cytotoxicity to name a few. In fact, multiple phenotypes can be profiled simultaneously.

Computational Toxicology

Computational toxicology integrates biology, chemistry, and computer science to evaluate chemicals for potential adverse liabilities and prioritize them for further testing. This discipline uses models and algorithms based on existing data sets for defined endpoints to predict the likelihood that a new chemical entity will possess the same toxic liabilities.

Innovative Testing Models

Novel in vitro approaches for predictive safety testing include organs- and body-on-a-chip and stem cells. Organs-on-a-chip are microfluidic flow-based systems that are engineered with multiple cell types that spatially recapitulate three-dimensional organs. Microfabricated devices allow for cell–cell communication and dynamic mechanical forces such as breathing (lungs), shear (circulation), peristalsis (intestines), and blinking (eyes) that are important for organ functioning. Other in vitro models include induced pluripotent, embryonic, and adult stem cells.

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QUESTIONS

1. Five identical experimental animals are treated with 1 mg of one of the following toxins. The animal treated with which toxin is most likely to die?
   - **a.** ethyl alcohol (LD_{50} = 10,000 mg/kg).
   - **b.** botulinum toxin (LD_{50} = 0.00001 mg/kg).
   - **c.** nicotine (LD_{50} = 1 mg/kg).
   - **d.** ferrous sulfate (LD_{50} = 1500 mg/kg).
   - **e.** picrotoxin (LD_{50} = 5 mg/kg).

2. Place the following mechanisms of toxin delivery in order from most effective to least effective—1: intravenous; 2: subcutaneous; 3: oral; 4: inhalation; 5: dermal.
   a. 1, 5, 2, 4, 3.
   b. 4, 1, 2, 3, 5.
   c. 1, 4, 2, 3, 5.
   d. 4, 2, 1, 5, 3.
   e. 1, 4, 3, 2, 5.

3. A toxin with a half-life of 12 h is administered every 12 h. Which of the following is true?
   a. The chemical is eliminated from the body before the next dose is administered.
   b. The concentration of the chemical in the body will slowly increase until the toxic concentration is attained.
   c. A toxic level will not be reached, regardless of how many doses are administered.
   d. Acute exposure to the chemical will produce immediate toxic effects.
   e. The elimination rate of the toxin is much shorter than the dosing interval.

4. Urushiol is the toxin found in poison ivy. It must first react and combine with proteins in the skin in order for the immune system to recognize and mount a response against it. Urushiol is an example of which of the following?
   a. antigen.
   b. auto-antibody.
   c. superantigen.
   d. hapten.
   e. cytokine.

5. Toxic chemicals are most likely to be biotransformed in which of the following organs?
   a. central nervous system.
   b. heart.
   c. lung.
   d. pancreas.
   e. liver.

6. When chemicals A and B are administered simultaneously, their combined effects are far greater than the sum of their effects when given alone. The chemical interaction between chemicals A and B can be described as which of the following?
   a. potentiative.
   b. additive.
   c. antagonistic.
   d. functionally antagonistic.
   e. synergistic.

7. With respect to dose–response relationships, which of the following is true?
   a. Graded dose–response relationships are often referred to as “all or nothing” responses.
   b. Quantal dose–response relationships allow for the analysis of a population’s response to

varying dosage.

c. Quantal relationships characterize the response of an individual to varying dosages.

d. A quantal dose–response describes the response of an individual organism to varying doses of a chemical.

e. The dose–response always increases as the dosage is increased.

8. When considering the dose–response relationship for an essential substance:

a. there are rarely negative effects of ingesting too much.

b. the curve is the same for all people.

c. adverse responses increase in severity with increasing or decreasing dosages outside of the homeostatic range.

d. the relationship is linear.

e. deficiency will never cause more harm than over-ingestion.

9. The therapeutic index of a drug:

a. is the amount of a drug needed to cure an illness.

b. is lower in drugs that are relatively safer.

c. describes the potency of a chemical in eliciting a desired response.

d. describes the ratio of the toxic dose to the therapeutic dose of a drug.

e. explains the change in response to a drug as the dose is increased.

10. Penicillin interferes with the formation of peptidoglycan cross-links in bacterial cell walls, thus weakening the cell wall and eventually causing osmotic death of the bacterium. Which of the following is true?

a. Treatment with penicillin is a good example of selective toxicity.

b. Penicillin interferes with human plasma membrane structure.

c. Penicillin is a good example of a drug with a low therapeutic index.

d. Penicillin is also effective in treating viral infections.

e. Penicillin is completely harmless to humans.

CHAPTER 3

Mechanisms of Toxicity

Lois D. Lehman-McKeeman

KEY POINTS

- Toxicity involves toxicant delivery to its target or targets and interactions with endogenous target molecules that may trigger perturbations in cell function and/or structure or that may initiate repair mechanisms at the molecular, cellular, and/or tissue levels.
- Biotransformation to harmful products is called *toxication* or *metabolic activation*.
- Biotransformations that eliminate the ultimate toxicant or prevent its formation are called *detoxications*.
- Apoptosis, or programmed cell death, is a tightly controlled, organized process whereby individual cells break into small fragments that are phagocytosed by adjacent cells or macrophages without producing an inflammatory response.
- Sustained elevation of intracellular Ca^{2+} is harmful because it can result in (1) depletion

of energy reserves by inhibiting the ATPase used in oxidative phosphorylation, (2) dysfunction of microfilaments, (3) activation of hydrolytic enzymes, and (4) generation of reactive oxygen and nitrogen species (ROS and RNS).

- Cell injury progresses toward cell necrosis (death) if molecular repair mechanisms are inefficient or the molecular damage is not readily reversible.
- Chemical carcinogenesis involves insufficient function of various repair mechanisms, including (1) failure of DNA repair, (2) failure of apoptosis (programmed cell death), and (3) failure to terminate cell proliferation.

INTRODUCTION

Mechanisms of toxicity describe how an adverse effect occurs. Such events involve many molecular, biochemical, and cellular processes that may act in isolation or in a complex combination to produce a given response. The focus of this chapter is to establish the fundamental concepts for how toxicity occurs, to illustrate how to apply these concepts to understanding mechanisms of toxicity, and to give examples of mechanisms of toxicity. Mechanistic toxicology data are also useful for developing more predictive biomarkers of toxicity, developing approaches to antagonize or prevent toxicity, and gaining insight into fundamental physiologic, biochemical, and molecular processes that underlie normal and abnormal organ function. Knowledge of mechanisms of toxicity is essential for developing risk assessments for chemical exposure, as such data are relevant to determining the likelihood that chemical exposure may cause harmful effects.

Mechanisms of toxicity can be simplified visually to a four-step process depicted in Fig. 3–1, encompassing (1) delivery of the toxicant to its target; (2) interactions between the toxicant and its target or the microenvironment; (3) progression to cellular dysfunction; and (4) inappropriate repair or adaptation. Target delivery perturbs cell function and/or structure to initiate repair mechanisms at the molecular, cellular, and/or tissue levels, and adaptive mechanisms may develop to diminish delivery, boost repair capacity, and/or compensate for dysfunction. Efforts to repair injury can be overwhelmed, with serious, irreversible consequences including cell death or chronic changes like fibrosis or cancer.

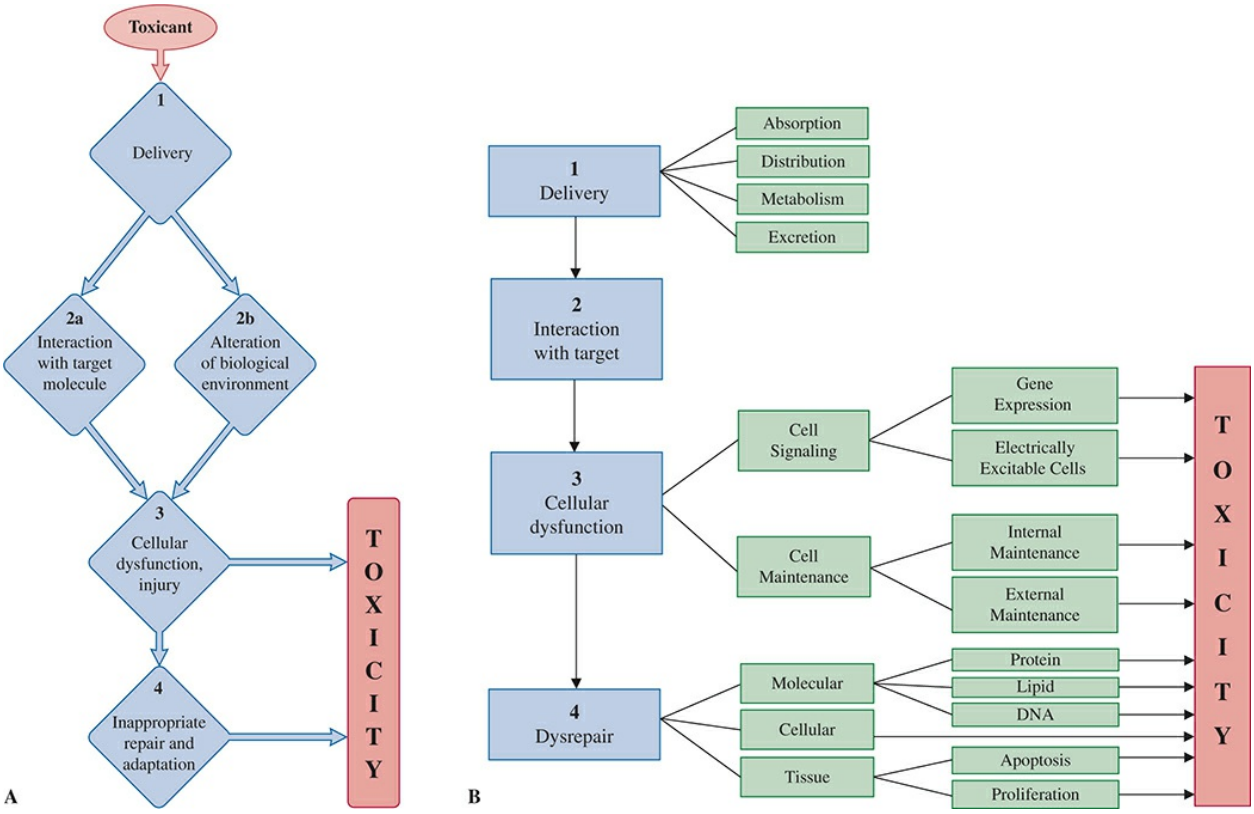

FIGURE 3–1 *Overview of critical events that contribute to the development of toxicity after chemical exposure.* These fundamental processes serve as the basis for the organization of this chapter. Panel A shows more details of critical events underlying the four major processes outlined in this chapter. Panel B shows more details of the mechanistic events involved in toxic outcomes.

STEP 1: DELIVERY TO THE TARGET

Exposure at the target site is an absolute requirement for any mechanism of toxicity. Xenobiotic disposition, defined as the absorption, distribution, biotransformation, and elimination of a toxicant, is a critical determinant of target organ toxicity along with sensitivity of target sites to toxicity. Moreover, the kinetics that describe these processes contributes to the severity of a toxic response. The fundamental principles of disposition, biotransformation, and kinetics are discussed in detail in Chapters 5, 6, and 7, respectively, and the overview in Fig. 3–2 illustrates how these processes can increase or decrease toxicity.

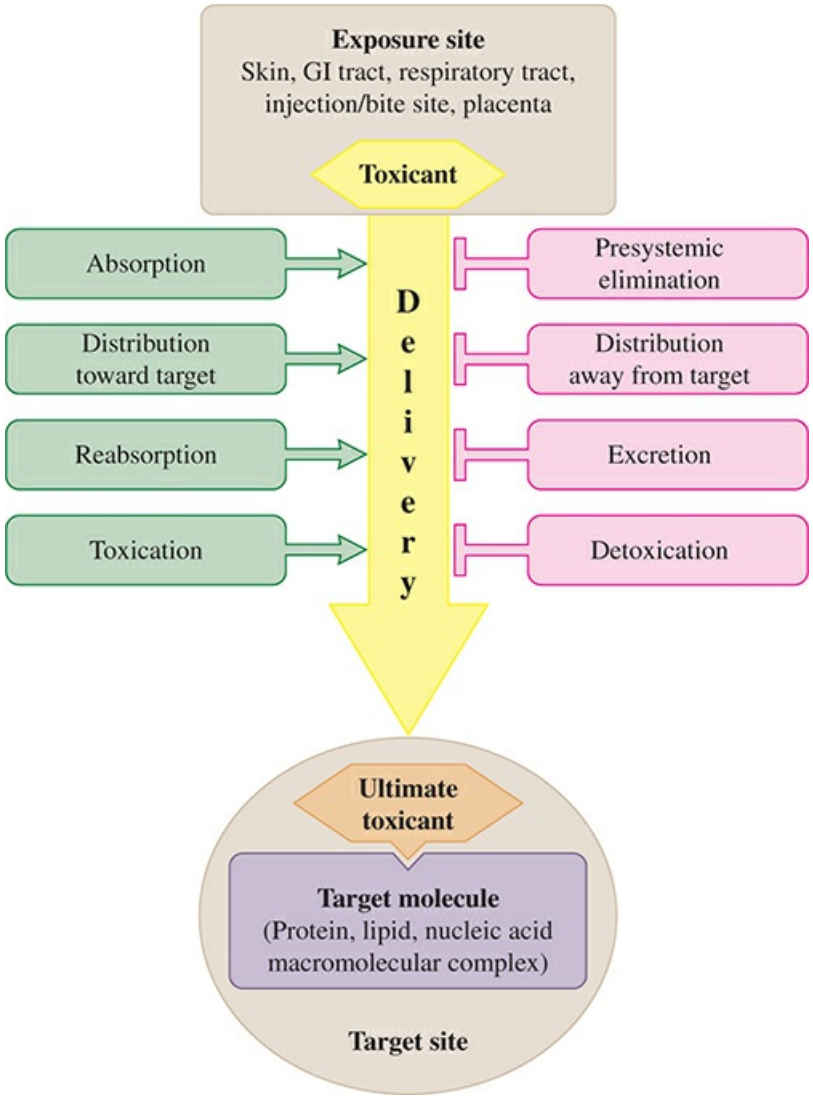

FIGURE 3–2 *The process of toxicant delivery to the target is the first step in the development of toxicity.* Factors illustrated on the left increase delivery to the target, whereas those on the right denote reduced delivery to the target.

Absorption and Presystemic Elimination

Absorption is defined as the process by which toxicants cross body membranes to enter the systemic circulation. It can occur from many sites, including the gastrointestinal tract, skin, or lungs, reflecting oral, dermal, or inhalation exposure, respectively. Absorption occurs along the entire length of the gastrointestinal tract and is usually determined by the extent to which the compound exists in its nonionized, lipid-soluble form. In the skin, the stratum corneum layer of the epidermis is a major barrier to absorption, but once through this barrier, compounds are typically absorbed by diffusion into the venous or lymphatic capillaries to enter the systemic circulation. In the lungs, gases and vapors diffuse quickly through the alveolar space and into the bloodstream. In contrast, aerosols and particles are absorbed throughout the lung based on their size and water solubility. Larger particles will be deposited in the upper regions of the

tracheobronchiolar regions, and only the smallest particles can penetrate all the way into the alveolar sacs. In all organs, the rate of absorption is determined by the concentration at the site of exposure, rate of dissolution of the chemical, the total area that is exposed, the vascular composition of the region, and physicochemical properties of the toxicant.

Presystemic elimination is a process by which a toxicant is eliminated prior to reaching the systemic circulation. The gastrointestinal tract epithelium may eliminate or modify a compound directly. Additionally, many toxicants absorbed from the gastrointestinal tract pass via the portal circulation directly into the liver where they are modified by biotransformation enzymes and excreted into bile. This is also referred to as first-pass metabolism, a process that reduces systemic exposure to a toxicant. At the same time, if a toxicant targets the liver, a significant first-pass effect will increase toxicity. There are also examples of presystemic elimination with respect to the skin and lungs.

Distribution of Toxicants Occurs by Passive or Active Processes

Tissue distribution is the process by which a toxicant reaches its target site. There are four properties that facilitate distribution to a target. These include the (1) porosity of the capillary endothelium, (2) presence of specialized transport processes, (3) potential for accumulation within cellular organelles, and (4) binding to proteins or other macromolecules.

The porosity of the capillary epithelium enables passive diffusion of toxicants. In particular, the capillaries of the liver (sinusoids) and kidney (peritubular capillaries) have relatively large fenestrae (50 to 150 nm) that enable ready passage of even protein-bound compounds. In contrast, the capillaries that form the blood–brain barrier are joined tightly and lack fenestrae to prevent significant distribution of hydrophilic compounds into the brain. Similarly, the seminiferous tubules of the testis are protected by a blood–testis barrier.

There are many specialized ion channels and transporters that regulate uptake and efflux of many xenobiotics and endogenous substrates. At least 52 families of transporters identified in the human genome represent nearly 400 unique proteins which function in energy-dependent processes to transport organic cations, organic anions, amino acids, nucleosides, and bile acids as substrates. The role of these proteins in toxicant disposition is discussed extensively in Chapter 5.

Physicochemical properties of toxicants often contribute to their accumulation in cells or cellular organelles. For example, amphipathic molecules, defined as those containing hydrophilic and hydrophobic portions, can accumulate in lysosomes or mitochondria to produce toxicity. Accumulation in lysosomes usually occurs by a pH-dependent trapping mechanism leading to lysosomal changes such as phospholipidosis.

Some chemicals accumulate in tissue storage sites that are not the major organ of toxicity and thereby prevent toxicity. For example, lead is deposited in bone where it substitutes for calcium in hydroxyapatite without adversely affecting bone, and highly lipophilic compounds such as chlorinated hydrocarbon insecticides will concentrate in fat (adipocytes), where they are generally nontoxic. Although such storage limits toxicity, any condition that causes these stored toxicants to be released or mobilized from the storage site will increase the likelihood of toxicity.

Finally, binding to proteins can influence distribution and target organ toxicity. Keratins are highly abundant proteins in skin and hair, and with a high level of cysteine residues, keratins sequester thiol-reactive metal ions including arsenic and mercury. The metal-binding protein metallothionein binds numerous heavy metals with very high affinity and protects against the

toxicity of metals such as cadmium.

Elimination Processes Affect Delivery of Toxicants

The route and rate of excretion largely depend on the physicochemical properties of the toxicant. The major excretory organs for non-volatile compounds are the liver and kidney, which are most efficient at removing water-soluble, usually ionized compounds. Important considerations for excretion of hydrophilic, ionized compounds include (1) compounds (<60 kDa) must be dissolved in plasma water to be filtered through the renal glomerulus; (2) transporters in liver or kidney are highly specialized to secrete and enhance excretion of water-soluble organic anions or cations; and (3) only water-soluble compounds are readily excreted in urine or bile.

The excretion of non-volatile highly lipophilic compounds is much less efficient than that of water-soluble compounds. Lipophilic compounds are readily absorbed by transcellular diffusion, and many lipophilic compounds are eliminated slowly allowing for accumulation in the body with repeated exposure. Biotransformation may facilitate elimination of these compounds, but other processes for excretion of highly lipophilic compounds include excretion in the bile (in a lipid or phospholipid micelle), secretion directly into the intestinal contents, and excretion in milk lipids in mammary glands. In contrast, volatile lipophilic compounds diffuse through pulmonary capillaries to be excreted in expired air.

Whereas excretory pathways reduce toxicant exposure, some toxicants are reabsorbed after initial elimination steps and this reabsorption may increase total exposure to the toxicant. In the kidney, reabsorption mechanisms are highly pH-dependent, as the generally acidic milieu of urine favors reabsorption of weak acids. However, altering urine pH can also be used to enhance excretion and thereby reduce toxicity. Acidification of urine favors the excretion of weak bases, whereas urinary alkalization will favor excretion of weak acids.

Toxicants delivered to the gastrointestinal tract by biliary excretion, intestinal secretion, or secretion from salivary glands and the pancreas are typically absorbed by diffusion across the intestinal mucosa. Enterohepatic circulation is a cycle in which a compound is excreted into bile to enter the intestine where it is modified to facilitate intestinal reabsorption rather than fecal elimination. This initiates a cycle in which the compound is reabsorbed in the intestine, only to return to the liver where it is again excreted into bile, returns to the intestine, and is again reabsorbed. This cycle is frequently observed with glucuronide conjugates because conjugation favors elimination into bile, with subsequent hydrolysis of the glucuronide by β-glucuronidase activity of intestinal microorganisms, and the resulting aglycones are reabsorbed. Enterohepatic circulation is especially important in bile acid homeostasis as nearly 95% of the bile acids in the gut return to the liver.

The Balance between Metabolic Activation and Metabolic Detoxification Reactions Contributes to Toxicity

Although some xenobiotics are directly toxic, many others produce toxicity through metabolites formed following exposure. Biotransformation of xenobiotics that increases toxicity is referred to as metabolic activation or a toxification process. Metabolic activation is an important mechanism of toxicity because it generates intermediates that adversely affect the microenvironment, alter cellular macromolecules and organelles, and/or stress endogenous protective mechanisms.

Another major feature of metabolic activation is that it often explains target organ specificity of a toxic outcome or contributes to species differences in toxicity. As an example, the fungal-derived toxicant, 4-ipomeanol, is metabolized to a reactive intermediate that in rodents is catalyzed by a cytochrome P450 enzyme, CYP4B1, an enzyme present in high levels in the lung. As a result, the reactive metabolite is mainly formed in rodent lungs where marked bronchiolar toxicity is observed. In contrast, ipomeanol is hepatotoxic in humans because it is metabolically activated in the liver rather than the lung. In this example, the same reactive metabolite causes toxicity, but species differences in the expression of the enzymes involved in forming the toxic metabolite determine the target organ affected.

Formation of Electrophiles—An electrophile is a molecule containing an electron-deficient atom with a partial or full positive charge that allows it to react by accepting electron pairs from electron-rich atoms in nucleophiles. Such reactants are often produced by insertion of an oxygen atom, which withdraws electrons from the atom it is attached to, making that atom electrophilic. This is the case when aldehydes, ketones, epoxides, arene oxides, sulfoxides, nitroso compounds, phosphonates, and acyl halides are formed. Consequently, a major outcome of metabolic activation is that electrophiles are often formed, and these metabolites are commonly involved in mechanisms of toxicity. Formation of many of these metabolites is catalyzed by cytochrome P450s (see Chapter 6).

Cationic electrophiles may be produced as a result of heterolytic bond cleavage. For example, 7,12-dimethylbenzanthracene and 2-acetylaminofluorene are hydroxylated to form benzylic alcohols and *N*-hydroxy arylamines (amides), respectively. These metabolites are then esterified, typically by sulfotransferases. Heterolytic cleavage of the C–O or N–O bonds of these esters results in a hydrosulfate anion and the concomitant formation of a benzylic carbonium ion or arylnitrenium ion, respectively (see Chapter 6). The antiestrogen tamoxifen undergoes similar activation by hydroxylation and sulfation to form a carbocationic metabolite. Dimethylnitrosamine (DMN) is C-hydroxylated by CYP2E1, after which it undergoes spontaneous decomposition and heterolytic cleavage of the C–N bond to form a methyl carbonium cation ($^{+}CH_3$), and this reactive metabolite is the penultimate species involved in carcinogenic effects of DMN. In a similar process, the tob acco-specific nicotine-derived nitrosamine ketone [(methylnitrosamino)-1-(3-pyridyl)-1-butanone; referred to as NNK], the most potent carcinogen in tobacco, may generate either methyl carbonium or piridyloxobutyl carbonium cations, depending on the site of C-hydroxylation that initiates its decomposition.

Formation of Free Radicals—A free radical is a molecule or molecular fragment that contains one or more unpaired electrons in its outer orbital. Radicals are formed by three major actions, namely (1) accepting an electron, (2) losing an electron, or (3) homolytic fission of a covalent bond. These three reactions are summarized here.

1. ***Free radicals formed by accepting electrons***: Xenobiotics can accept an electron from cellular reductases (such as cytochrome P450 reductase) to generate a free radical. Once formed, free radicals typically transfer the extra electron to molecular oxygen, forming a superoxide anion radical $(O_2^{\bullet})$ and regenerating the parent xenobiotic, which is ready to gain a new electron. This cycle, referred to as “redox cycling,” ultimately amplifies the toxic response because one electron acceptor xenobiotic molecule can generate many $O_2^{\bullet}$ molecules.
2. ***Free radicals generated by losing electrons***: Nucleophiles generate free radicals when they

lose an electron.

3. ***Free radicals formed by homolytic bond fission***: Reductive fission is a cleavage reaction in which two electrons in a bond are divided equally between the products. The hydroxyl radical ($HO^{\bullet}$) is a free radical generated by homolytic fission. The Fenton reaction describes the reductive homolytic fission of hydrogen peroxide (HOOH) to the harmful $HO^{\bullet}$ and the harmless HO^- (hydroxide anion; Fig. 3–3). This reaction is a major toxicity mechanism for HOOH and its precursor $O_2^{\bullet-}$ that is catalyzed by transition metal ions, typically Fe(II), Cu(I), Cr(V), Ni(II), or Mn(II). HOOH is a direct or indirect by-product of several enzymatic reactions, including monoamine oxidase, acyl-coenzyme A oxidase, xanthine oxidase, aldehyde oxidase, and CYP2E1.

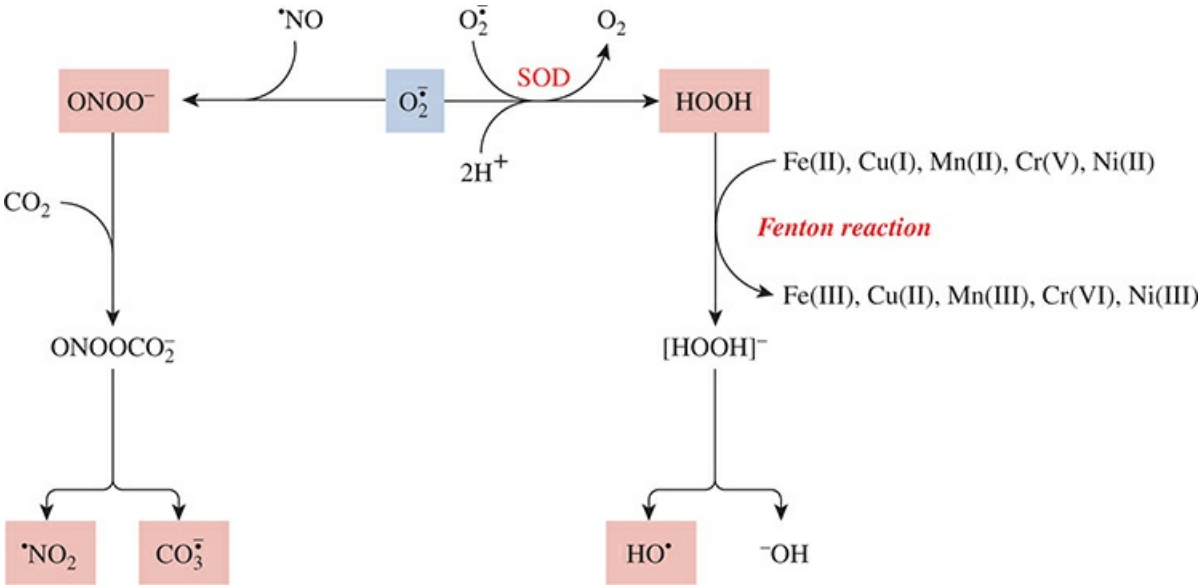

FIGURE 3–3 *Two pathways for toxicity of superoxide anion radical $O_2^{\bullet-}$ from conversion of $ONOO^-$ and HOOH (nonradical products) to $^{\bullet}NO2$, $CO_3^{\bullet-}$, and HO• (radicals).* Conversion of $O_2^{\bullet-}$ to HOOH is spontaneous or is catalyzed by SOD. Homolytic cleavage of HOOH to hydroxyl radical and hydroxyl ion (Fenton reaction) is catalyzed by the transition metal ions shown. Hydroxyl radical is the ultimate toxicant for xenobiotics that form $O_2^{\bullet-}$. In the other pathway, $O_2^{\bullet-}$ reacts avidly with nitric oxide ($^{\bullet}NO$), the product of $^{\bullet}NO$ synthase (NOS), forming peroxynitrite ($ONOO^-$). Spontaneous reaction of $ONOO^-$ with carbon dioxide (CO_2) yields nitrosoperoxy carbonate that with homolytic cleavage forms nitrogen dioxide ($^{\bullet}NO_2$) and carbonate anion radical $(CO_3^{\bullet-})$. All three radical products indicated in this figure are oxidants, whereas $^{\bullet}NO_2$ is also a nitrating agent.

Homolytic cleavage is also involved in free radical generation from peroxynitrite ($ONOO^-$; Fig. 3–3). The facile reaction of $ONOO^-$ with CO_2 yields nitroso-peroxycarbonate $(ONOOCO_2^-)$, which spontaneously homolyzes into two radicals, namely nitrogen dioxide ($^{\bullet}NO_2$) and the carbonate anion radical $(CO_3^{\bullet-})$. Formation of $ONOO^-$ and the latter radicals is involved in the toxicity of $O_2^{\bullet-}$ and $^{\bullet}NO$. As $^{\bullet}NO$ is the product of nitric oxide synthase (NOS), this mechanism is especially relevant in and around cells that express NOS constitutively (i.e., neurons and

endothelial cells) as well as in cells that express the inducible form of NOS.

Detoxification—Biotransformation that prevents the formation of a toxic metabolite or eliminates it once formed is an important mechanism that reduces the likelihood of toxicity. Any such process is called detoxification. Detoxification mechanisms are catalyzed mainly by biochemical mechanisms that include phase II biotransformation reactions such as glucuronidation, sulfation, and glutathione conjugation. These reactions, including the enzymes and cofactors required for activity, are discussed in detail in Chapter 6. An important concept in detoxification is that these processes, albeit protective, can be saturated. When protective mechanisms are overwhelmed and saturated, toxicity ensues.

Detoxification of Electrophiles—Specific mechanisms for the detoxification of electrophilic chemicals include epoxide hydrolase-catalyzed biotransformation of epoxides and arene oxides to less toxic diols and dihydrodiols, respectively, and carboxylesterase-catalyzed hydrolysis of organophosphate ester pesticides. Quinones are reduced to hydroquinones by NAD(P)H:quinone oxidoreductase (NQO1) and NRH:quinone oxidoreductase (NQO2). α,β-Unsaturated aldehydes (e.g., the lipid peroxidation product 4-oxonon-2-enal) are reduced to alcohols or to their saturated derivative by carbonyl reductase, but they can also be oxidized to acids by aldehyde dehydrogenases.

An extremely important mechanism for the detoxification of electrophilic toxicants is conjugation with glutathione. Glutathione (GSH) is a tripeptide composed of glycine, cysteine, and glutamic acid, with the glutamic acid linked to cysteine by a γ-carboxyl group (γ-glutamine-cysteinylglycine). It is a major non-protein sulfhydryl in most tissues, with constitutive concentrations of 5 to 10 mM in the liver. Conjugation with GSH occurs spontaneously or can be catalyzed by glutathione *S*-transferases. Some metal ions, including Ag^+, Cd^{2+}, Hg^{2+}, and CH_3Hg^+, also readily react with and are detoxified by GSH conjugation.

Beyond biotransformation, covalent binding of electrophiles to proteins is a detoxification reaction provided the bound protein has no critical function and does not become a neoantigen or otherwise harmful. Carboxylesterases, for example, inactivate organophosphates not only by hydrolysis but also by covalent binding.

Detoxification of Free Radicals—Detoxification of $O_2^{\bullet-}$ is an important mechanism to prevent toxicity because it can otherwise be converted into much more reactive compounds. Detoxification of $O_2^{\bullet-}$ is achieved by the coordinated action that starts with superoxide dismutases (SOD), followed by reactions catalyzed by catalase, glutathione peroxidase, or peroxiredoxin (Fig. 3–4). SODs are high-capacity enzymes located in the cytosol (Cu, Zn-SOD) and the mitochondria (Mn-SOD) that convert $O_2^{\bullet-}$ to HOOH. The HOOH is reduced to water by catalase in peroxisomes (or in the mitochondria in cardiac muscle), by glutathione peroxidases in the cytosol and mitochondria, and by peroxiredoxins in the cytosol, mitochondria, and endoplasmic reticulum. In contrast to $O_2^{\bullet-}$, no enzyme eliminates $HO^{\bullet}$. Furthermore, $HO^{\bullet}$ is extremely reactive, with such a short half-life (10^{-9} seconds) that affords little time for the $HO^{\bullet}$ to reach and react with antioxidants. Therefore, the only effective protection against $HO^{\bullet}$ is to prevent its formation by elimination of its precursor, HOOH, via conversion to water.

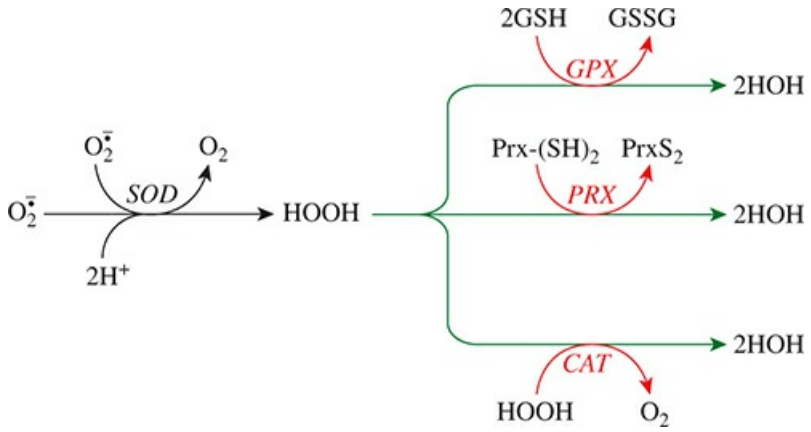

FIGURE 3–4 *Detoxification of superoxide anion radical* $(O_2^{\bullet-})$ *and HOOH.* Superoxide dismutase (SOD) converts $O_2^{\bullet-}$ to HOOH, which is further detoxified by glutathione peroxidase (GPX), peroxiredoxin (Prx-$(SH)_2$), and catalase (CAT). When GPX reduces HOOH, it forms glutathione disulfide (GSSG), which is reduced back to GSH by glutathione reductase (requires NADPH; see Fig. 3–6). When Prx-$(SH)_2$ reduces HOOH, its catalytic thiol group (–R–S–H) is oxidized to a sulfenic acid group (–R–S–OH), which in turn reacts with another SH group of Prx, forming HOH and Prx disulfide ($PrxS_2$). Finally, catalase converts HOOH into two moles of water. Catalase is a high-capacity system, whereas Prx and GPX can be saturated at high concentrations of HOOH.

Glutathione peroxidase and peroxiredoxins can detoxify $ONOO^-$ by reduction to nitrite (ONO^-) the same way they reduce HOOH to water (Fig. 3–5). In addition, $ONOO^-$ reacts with oxyhemoglobin, heme-containing peroxidases, and albumin, all of which could be important sinks for $ONOO^-$. Also, $ONOO^-$ toxicity can be reduced by scavenging $^{\bullet}NO$ with oxyhemoglobin (forming metHb and nitrate) and by SODs decreasing $O_2^{\bullet-}$ levels.

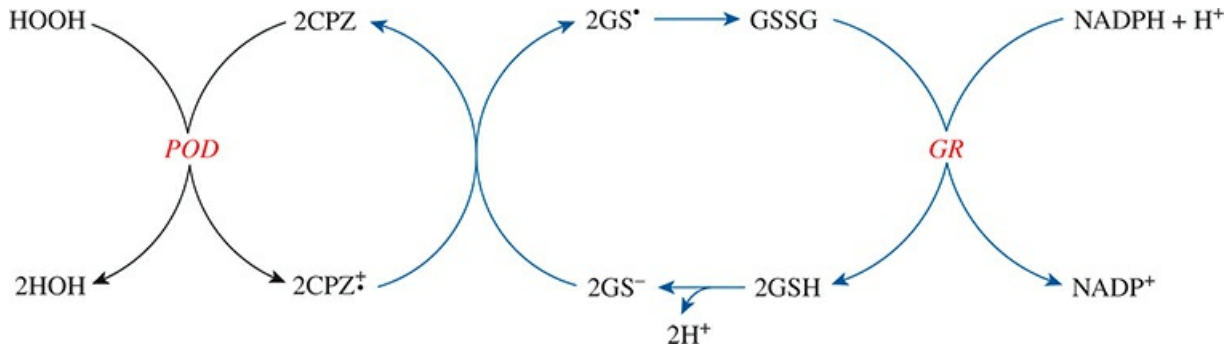

FIGURE 3–5 *Detoxification of peroxidase (POD)–generated free radicals such as chlorpromazine free radical* $(CPZ^{+\bullet})$ *by glutathione (GSH).* The by-products are glutathione thiyl radical ($GS^{\bullet}$) and glutathione disulfide (GSSG), from which GSH is regenerated by glutathione reductase (GR), a reaction that requires NADPH.

Peroxidase-generated free radicals are eliminated by electron transfer from glutathione. This results in the oxidation of glutathione, which is reversed by NADPH-dependent glutathione reductase (Fig. 3–5). This pathway illustrates that glutathione is important in detoxification of

both electrophiles and free radicals.

Detoxification of Nucleophiles—Nucleophiles are generally detoxified by conjugation at the nucleophilic functional group. Hydroxylated compounds are conjugated by glucuronidation, sulfation, or rarely methylation, whereas thiols are methylated or glucuronidated and amines and hydrazines are acetylated. These reactions prevent peroxidase-catalyzed conversion of the nucleophiles to free radicals and biotransformation of phenols, aminophenols, catechols, and hydroquinones to electrophilic quinones and quinoneimines. One alternative mechanism for the elimination of thiols, amines, and hydrazines is oxidation by flavin-containing monooxygenases (see Chapter 6). Finally, a specific nucleophile detoxification mechanism is the biotransformation of cyanide to thiocyanate by either rhodanese or mercaptopyruvate sulfurtransferase.

Detoxification of Toxicants with No Functional Groups—Oxidative reactions introduce functional groups (i.e., hydroxyl or carboxyl moiety), a reaction most often by cytochrome P450 enzymes, after which the oxidative metabolites are conjugated by glucuronidation or sulfation reactions. These processes are referred to as Phase I and Phase II metabolism, respectively. With some exceptions, the final products are inactive, highly hydrophilic organic acids that are readily excreted. Carbonyl reduction of ketones, aldehydes, and esters is catalyzed by at least five enzymes (e.g., carbonyl reductase, 3 members of aldo-keto reductase superfamily, and 11-β-hydroxysteroid dehydrogenase-1).

Detoxification of Protein Toxicants—It is generally thought that extracellular and intracellular proteases are involved in the inactivation of toxic polypeptides. Several toxicants found in venoms, such as α- and β-bungarotoxin, erabutoxin, and phospholipase, contain intramolecular disulfide bonds that are required for their activity. These proteins are inactivated by thioredoxin, an endogenous dithiol protein that reduces the essential disulfide bond.

When Detoxification Fails—Although detoxification reactions are generally protective, there are many mechanisms of toxicity that ensue when detoxification reactions are inadequate. The most important reason for such failure is that the exposure to the toxicant overwhelms vital detoxification processes. This will occur typically in a dose-dependent manner and results from saturation of detoxification enzymes, consumption of essential co-substrates, or depletion of cellular antioxidants such as GSH, ascorbic acid, and α-tocopherol. Thus, the dose-response relationship is an important quantitative aspect underlying most toxicity mechanisms.

Other notable examples of detoxification “failure” occur when a reactive toxicant inactivates a detoxicating enzyme. Some conjugation reactions that detoxify a reactive intermediate are reversed to initiate toxicity. For example, 2-naphthylamine is *N*-hydroxylated and glucuronidated in liver, with the glucuronide eliminated in urine. However, in the urinary bladder, the glucuronide is hydrolyzed, and the released arylhydroxylamine is converted to the reactive electrophilic arylnitrenium ion. Ultimately, 2-naphthylamine causes bladder cancer because of this local activation of a previously detoxified metabolite. In another example, isocyanates and isothiocyanates form labile GSH conjugates from which they can be released. Thus, methylisocyanate readily forms a GSH conjugate in the lung after inhalation, and once formed, this conjugate is distributed to other tissues, where the reactive electrophilic parent compound may be regenerated.

STEP 2: REACTION OF THE ULTIMATE TOXICANT WITH THE TARGET MOLECULE

Toxicity is initiated by a reaction between the ultimate toxicant and its target molecule (step 2a in Fig. 3–1), with subsequent events leading to dysfunction or injury. The determinants of interaction of the ultimate toxicant with the target molecule include (1) the attributes of target molecules, (2) the types of reactions between ultimate toxicants and target molecules, and (3) the effects of toxicants on the target molecules. Additionally, some toxicities result from alteration of the biological (micro)environment (step 2b in Fig. 3–1) in which critical endogenous molecules, cell organelles, cells, and organs function are perturbed.

Attributes of Target Molecules

Three attributes of a target molecule that determine whether it is associated with or responsible for toxicity are the ability to (1) react with the target and adversely affect its function, (2) reach an effective concentration at the target site, and (3) alter the target in a way that is mechanistically related to toxicity. But, not all targets for chemicals contribute to the harmful effects. Covalent binding to proteins, generally considered to be harmful, may be without adverse consequences or may be a form of detoxification if binding spares toxicologically relevant targets.

Types of Reactions

The major interactions between the toxicant and target molecules are noncovalent or covalent in nature, but other reactions including hydrogen abstraction, electron transfer, or enzymatic reaction can occur.

Noncovalent Binding—Non-covalent binding is attributed to apolar interactions or the formation of hydrogen and ionic bonds between toxicants and their targets including membrane or intracellular receptors, ion channels, and some enzymes. Such forces also are responsible for the intercalation of chemicals into the double helix of DNA. In most cases, noncovalent binding is reversible because of the comparatively low bonding energy.

Covalent Binding—Covalent binding is of great toxicological importance because binding is essentially irreversible and permanently alters endogenous molecules. Covalent adduct formation is common with electrophilic toxicants because they react with nucleophilic atoms in proteins and nucleic acids. In general, soft electrophiles prefer to react with soft nucleophiles (low charge-to-radius ratio in both), whereas hard electrophiles react more readily with hard nucleophiles (high charge-to-radius ratio in both).

The covalent reaction between HOOH (a soft electrophile) and protein thiol groups is of special biological significance because it produces protein sulfenic acid (Prot-S-OH) as illustrated here:

$$\text{Prot-SH} + \text{HOOH} \rightarrow \text{Prot-S-OH} + \text{HOH}$$

However, the S atom in a protein–sulfenic acid is electrophilic and will further react with another thiol group of the same protein, a different protein or glutathione (GSH), resulting in formation of an intramolecular disulfide, intermolecular disulfide, or a mixed disulfide of the protein and glutathione, respectively:

$$\text{Prot}_1\text{-S-OH} + \text{Prot}_2\text{-SH} \rightarrow \text{Prot}_1\text{-S-S-Prot}_2 + \text{HOH}$$
$$\text{Prot-S-OH} + \text{GSH} \rightarrow \text{Prot-S-SG} + \text{HOH}$$

Hydroperoxides, including lipid hydroperoxides (LOOH), may also produce protein–sulfenic acids, protein–disulfides, and glutathionylated proteins, which represent posttranslational modifications with a role in redox signaling.

Hydrogen Abstraction—Neutral free radicals, such as those generated in reactions depicted in Fig. 3–3, readily abstract H atoms from endogenous compounds, converting them into radicals. Abstraction of hydrogen from thiols (R-SH) creates thiyl radicals (R-S$^{\bullet}$), which on recombination with HO$^{\bullet}$ forms sulfenic acids (R-S-OH). Radicals also remove hydrogen from CH_2 groups of amino acids and convert them to carbonyls. These carbonyls can react covalently with amines, forming cross-links with other proteins or DNA. Abstraction of hydrogen from fatty acids produces lipid radicals and initiates lipid peroxidation.

Electron Transfer—Some chemicals can oxidize the Fe^{2+} in hemoglobin to Fe^{3+}, producing metHb, which is toxic by its reduced oxygen carrying capacity. Nitrite can oxidize ferrous iron in hemoglobin, whereas dapsone hydroxylamine, 5-hydroxy primaquine, and phenylhydrazine are co-oxidized with oxyhemoglobin, forming metHb and HOOH.

Enzymatic Reactions—Snake venoms contain enzymes that cause toxicity by generalized proteolytic degradation of many biomolecules. Plant toxins ricin and abrin are *N*-glycosidases, and they hydrolyze a specific glycosidic bond in ribosomal RNA to block protein synthesis. Botulinum toxicant is a Zn-protease that hydrolyzes the fusion proteins required for exocytosis of acetylcholine preventing its release and leading to paralysis. The lethal factor of anthrax toxicant is also a Zn-protease, which inactivates mitogen-activated protein kinase kinase (MAP2K), an integral kinase involved in many signal transduction pathways. Diphtheria toxicant blocks the function of elongation factor 2 (EF2) to inhibit protein synthesis.

Effects of Toxicants on Target Molecules

Dysfunction of Target Molecules—Some toxicants activate protein target molecules, mimicking endogenous ligands. This is the case for many drugs that are designed to be agonists of specific functions. Alternatively, chemicals may inhibit the function of target molecules.

Destruction of Target Molecules—In addition to adduct formation, toxicants alter the primary structure of endogenous molecules by means of cross-linking and fragmentation. Cross-linking imposes both structural and functional constraints on the linked molecules to alter function.

Free radicals such as $Cl_3COO^{\bullet}$ and HO$^{\bullet}$ initiate peroxidative degradation of lipids by hydrogen abstraction from fatty acids. Lipid peroxidation is an important mechanism of toxicity because it not only destroys lipids in cellular membranes but also generates additional

endogenous toxic free radicals (e.g., $LOO^{\bullet}$ and $LO^{\bullet}$) and electrophiles (e.g., 4-oxonon-2-enal and 4-hydroxynon-2-enal), which readily react with additional targets, such as membrane proteins, or diffuse intracellularly to more distant molecules including DNA. Single-strand breaks (SSB) are typically initiated by hydroxyl radicals that cause H abstraction from deoxyribose in DNA and ultimately cleave the phosphodiester bond. Double-strand breaks (DSB), which are typically more toxic or lethal to the affected cell, are caused by multiple hydroxyl radical attacks on a short length of DNA.

Neoantigen Formation—Haptens are small molecules that can elicit an immune response only when attached to a larger carrier, usually a protein. The protein carrier, often albumin, does not cause an immune response by itself. Accordingly, an immune-mediated mechanism of toxicity can occur after modification of proteins by toxicants. Haptenized proteins released from cells may evoke antibody-mediated (humoral) and/or T-cell-mediated (cellular) immune response (see Chapter 12).

Toxicity Not Initiated by Reaction with Target Molecules

Some xenobiotics not only interact with a specific endogenous target molecule to induce toxicity but also may alter the biological microenvironment (see step 2b in Fig. 3–1). There are three important examples of toxicity mediated through changes in the microenvironment. First, solvents and detergents directly alter the lipid phase of cell membranes and destroy transmembrane solute gradients that are essential to cell functions. Second, chemicals that alter H^+ ion concentrations in the aqueous biophase alter the microenvironment by disrupting essential acid–base balance. Finally, some xenobiotics cause harm merely by occupying a site or space.

Idiosyncratic Toxicity: When No Mechanism Explains the Outcome

Characterized by its low, frequently rare incidence and its poor predictability, idiosyncratic toxicity is most often described for adverse reactions to drugs, and the liver and skin tend to be the most frequently affected organs. Idiosyncratic reactions do not follow the general outline of toxic mechanisms illustrated in Fig. 3–1, nor do they follow the general rules regarding disposition and dose dependence. Rather, idiosyncratic reactions are distinguished from other adverse reactions because they do not show typical dose–response relationships and concentration dependence. Idiosyncratic drug reactions are referred to as type B reactions, whereas more common, concentration-dependent reactions are type A reactions.

By their very nature, the mechanism of idiosyncratic reactions is not well-defined. However, there is emerging evidence that nongenetic and genetic factors contribute to susceptibility to such toxicity. Nongenetic factors could include current disease states, including infection, inflammation, and pregnancy. Genetic factors include polymorphisms in drug metabolizing enzymes and drug transporters, enzymes that reduce ROS, and the MHC proteins. In fact, there is growing evidence that immune-mediated effects, particularly those involving the adaptive immune system, are highly likely to contribute to idiosyncratic responses.

Although the low frequency and multifactorial nature of idiosyncratic toxicity make it difficult to explain, there is a growing effort to predict this type of toxicity. Research efforts have

been directed toward developing in vitro and in vivo methods that predict intrinsic immunogenicity of drugs and chemicals, along with models that assess mitochondrial dysfunction, failure to adapt to modest injury, or inflammatory stress factors. Additionally, some mechanistic approaches apply quantitative systems pharmacology (QSP) to evaluate multiple factors and biological processes simultaneously. QSP is an in silico modeling technique that leverages and assimilates known physiology and the results of in vitro experiments in order to make predictions about how drugs affect biological processes.

STEP 3: CELLULAR DYSFUNCTION AND RESULTANT TOXICITIES

Toxicity-induced cellular dysfunction is determined by the function of the target molecule. If the target molecule is involved in cellular signaling and regulation, then altered gene expression and/or cellular function will occur. However, if the target molecule is involved predominantly in essential processes, the resultant dysfunction may compromise cell survival, and the nature of cell death varies with the mechanism of cell dysfunction (Fig. 3–6).

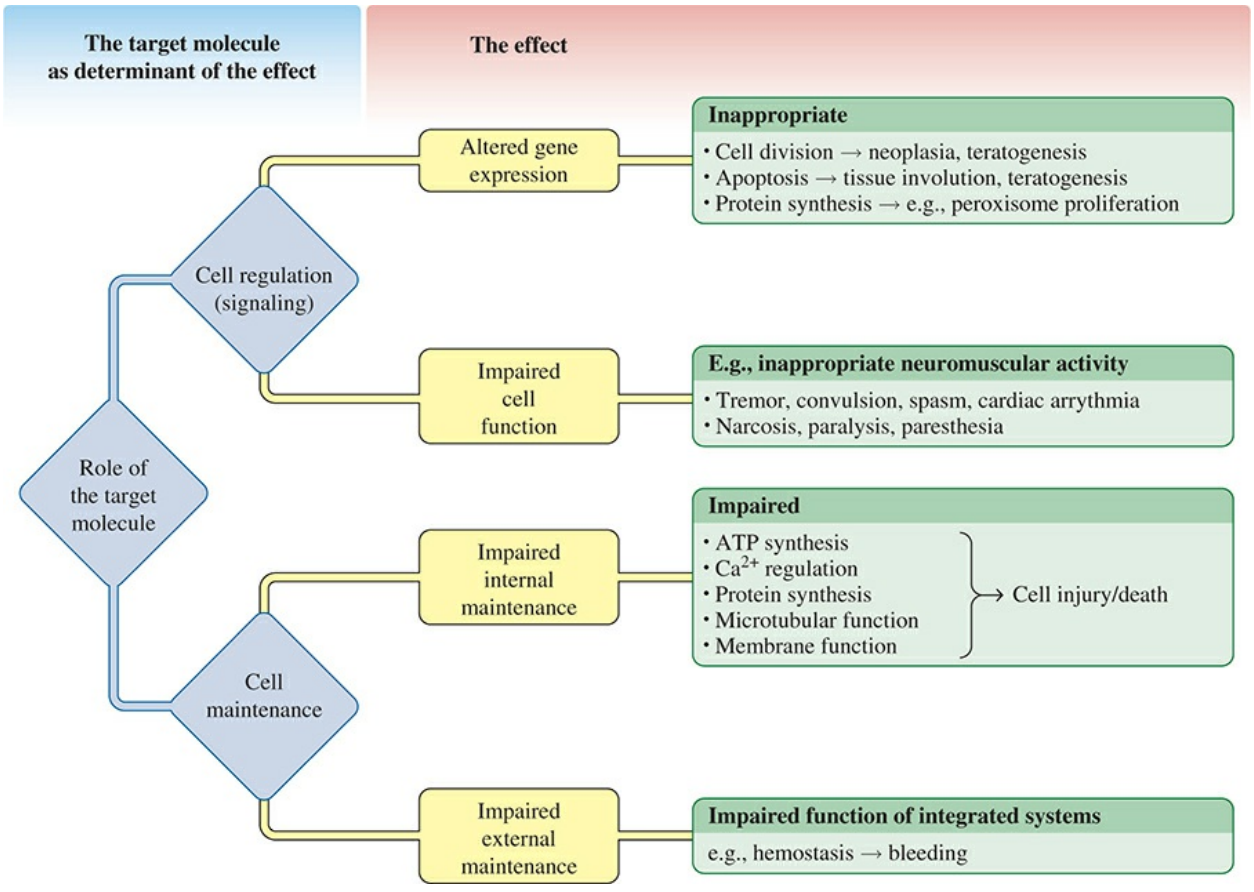

FIGURE 3–6 *The third step in the development of toxicity: alteration of cell functions.* The major effects are manifested as altered regulation of cell signaling or maintenance of cell function, with specific effects including altered gene expression, impaired cell function,

impaired intracellular homeostasis, and extracellular homeostasis. Examples of such changes are included on the right side of the figure.

Toxicant-Induced Cellular Dysregulation

Transcriptional processes as well as numerous pathways involved in signal transduction cascades contribute to toxicant-induced alterations in cell regulation and gene expression. There are many cellular receptors that function to transmit signals to regulatory regions of genes and/or to functional proteins. Receptor activation may alter gene expression to increase or decrease protein expression. Additionally, the phosphorylation state of proteins can be altered, which will activate or inhibit their function. Programs controlling the fate of cells primarily affect gene expression, whereas those regulating the ongoing activities primarily influence the activity of functional proteins. Often, however, one signal evokes changes in both gene expression and cell function responses because of the complex branching and interconnection of many signaling networks.

Altered Gene Expression—Gene expression is the process by which information from a gene is used in the synthesis of a functional gene product. The genetic information may be transcribed from DNA into messenger RNA (mRNA), which in turn is translated into proteins. There are other noncoding RNAs, including small interfering double-stranded RNA (siRNA) and microRNA (miRNA). miRNAs are short sequences, typically 20 to 25 base pairs in length, derived from regions of RNA transcripts that fold back on themselves to form short hairpins, whereas siRNAs are generated from longer regions of double-stranded RNA. Both siRNAs and miRNAs interfere with complementary nucleotide sequences to repress protein synthesis. Chemicals alter gene expression by affecting mRNAs, miRNAs, or both. Altered gene expression may also be affected directly at transcription, at signal transduction pathways, and at the synthesis, storage, or release of the extracellular signaling molecules.

Dysregulation of Transcription—Gene transcription is controlled largely by interplay between transcription factors (TFs) and the regulatory or promoter region of genes coding for mRNA and miRNA. By binding to distinctive nucleotide sequences in the promoter, ligand- and signal-activated TFs facilitate or impede the formation of the preinitiation complex to increase or repress transcription. Xenobiotics may interact with TFs (or other components of the preinitiation complex) and/or may alter the promoter region of the gene.

Several endogenous compounds, such as hormones (e.g., steroids and thyroid hormones) and vitamins (retinoids and vitamin D), influence gene expression by binding to and activating TFs or intracellular receptors. Many xenobiotics also mimic these natural ligands to alter cellular processes. Ligand-activated TFs control many fundamental cellular processes, and accordingly, adverse effects of compounds that act through TFs often result from perturbation of cell proliferation or by inducing cell death. In addition to altering the fate of specific cells, compounds that act on ligand-activated TFs can also evoke changes in the metabolism of endobiotics and xenobiotics by inducing overexpression of relevant metabolizing enzymes.

Transcriptional Dysregulation by Chemical Alteration of Regulatory Region of Genes—Xenobiotics may alter the regulatory gene regions through direct chemical interaction or by changing methylation patterns. Methylation of cytosine bases in CpG islands in the promoter regions of genes is a major epigenetic mechanism, which together with coupled histone

modifications influences transcriptional activity of the downstream gene. The operational definition of an epigenetic mechanism is one that affects heritable traits that are not explained by alterations in the DNA sequence. Thus, increased promoter methylation (hypermethylation) usually silences genes, whereas decreased methylation (hypomethylation) permits their activation. Importantly, when DNA replication occurs, the methylation pattern is copied from the parent strand to the daughter strand by DNA methyltransferase-1 (DNMT1), making the pattern heritable. Moreover, environmental conditions can alter promoter methylation to cause heritable changes in gene regulation.

DNA methylation is also an important process in genomic imprinting that takes place during formation of germ cells. Most genes in both sperm and ova similarly undergo or avoid epigenetic alterations. Offspring carries maternal and paternal alleles of most genes. However, some imprinted genes are epigenetically altered (generally methylated and silenced) only in the male or female germ cells, so that only the paternal or the maternal gene copy is expressed in the offspring. This is the case for the insulin-like growth factor-2 gene, which is expressed only from the paternal allele. Because imprinted genes have only a single active copy, they are especially susceptible for epigenetic dysregulation.

Role of miRNAs in Toxicant-Induced Alterations in Gene Expression— miRNAs repress translation or increase mRNA degradation to decrease gene and/or protein expression. Accordingly, any toxicant that increases miRNA activity will decrease gene expression, whereas a decrease in miRNA increases gene expression through the loss of repression. These small noncoding RNAs serve to regulate many fundamental cellular functions, resulting in a broad range of potential adverse effects on development, immune responses, metabolism, and diseases, as well as specific toxicological outcomes. There are at least 2500 mature miRNAs that have been identified in the human genome and may contribute to many mechanisms of toxicity.

Dysregulation of Signal Transduction—Signal transduction is the process by which a chemical or physical signal is transmitted through a cell in a stepwise series of events, often involving kinase or phosphatase activity, which modifies protein activity by phosphorylation status to invoke a response. Many extracellular signaling molecules, including growth factors, cytokines, hormones, neurotransmitters, and some secreted proteins, engage intracellular signal transducing networks and ultimately activate TFs.

Signaling in the mitogen-activated protein kinase (MAPK) pathway, which is also referred to as the extracellular signal–regulated kinase (ERK) is initiated by the binding of a signaling molecule to a cell surface receptor such as epidermal growth factor (EGF) binding to epidermal growth factor receptor (EGFR). Mitogen binding causes Ras (a GTPase) to switch to GTP to phosphorylate and activate the kinase activity Raf, which in turn phosphorylates MEK which phosphorylates ERK. Raf, MEK, and ERK are also referred to as MAP3K (i.e., MAP kinase kinase kinase), MAP2K, and MAPK, respectively. There are numerous receptors and ligands that can activate this critical pathway that regulates cell division including IGF, fibroblast growth factor, and transforming growth factor α (TGF-α).

Figure 3–7 is a complex illustration of numerous networks with emphasis on those involved in cell cycle regulation that identifies some signal-activated TFs that control transcriptional activity of genes that determine cell fate, with the MAPK pathway (4) shown in the middle of the figure. Pathways that are fundamental to understanding adverse effects are summarized here.

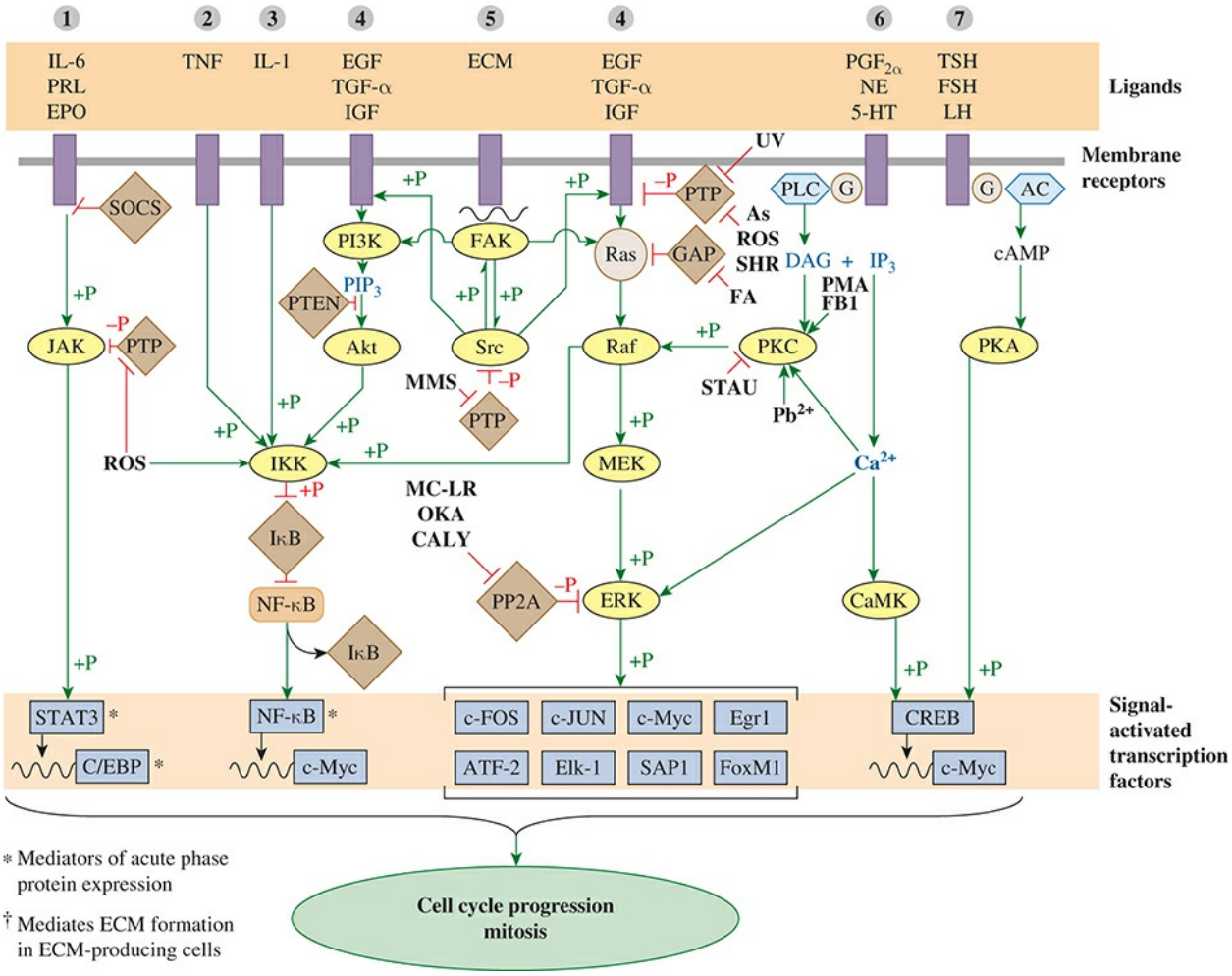

FIGURE 3–7 *Selected signal transduction pathways to illustrate phosphorylation cascades, potential interactions across pathways, and ultimately transcriptional regulation of the cell cycle.* The MAPK pathway is presented as activated by EGF in the middle of this figure (4) along with the numerous transcription factors it activates. The symbols of cell membrane receptors are numbered 1 to 7 and some of their activating ligands are indicated. G protein–coupled receptors are depicted in the numbers at the top of the figure and include the (1) JAK-STAT pathway; (2 and 3) NF-κB activation; (4) phosphatidilinositol-3-kinase (PI3K) and MAPK pathway; (5) integrin receptor–mediated activation of the focal adhesion kinase pathway that influences extracellular matrix (ECM) regulation; (6) phospholipase C activation of the protein kinase C (PKC) pathway; and (7) hormone-mediated activation of the cAMP and protein kinase A (PKA) pathway. The complexity of the figure illustrates the numerous interconnections between the pathways. For example, the G protein–coupled receptor (6) can relay signal into the MAPK pathway via PLC-catalyzed formation of second messengers that activate PKC, whereas signals from TNF and IL-1 receptors (2 and 3) are channeled into the PI3K–Akt–NF-κB pathway by phosphorylating IKK. The integrin receptor (5) can also engage the growth factor receptors and Ras as well as PI3K via FAK and the tyrosine kinase Src. For illustration purposes, the scheme is oversimplified. Virtually all components of the signaling network (e.g., G proteins, PKCs, and MAPKs) are present in multiple and functionally different forms whose distribution may be cell-specific. The pathways depicted are not equally

relevant for all cells. In addition, these pathways determine the fate of cells, and they also control certain aspects of the ongoing cellular activity. *Abbreviations and symbols*: Circles represent G proteins, oval symbols protein kinases, rectangles transcription factors, wavy lines genes, and diamond symbols inhibitory proteins, such as protein phosphatases (PTP, PP2A) and the lipid phosphatase PTEN, the GTPase-activating protein GAP, and inhibitory binding proteins such as IκB and suppressor of cytokine signaling (SOCS). Arrowheads indicate stimulation or formation of second messengers (e.g., DAG, IP_3, PIP_3, cAMP, and Ca^{2+}), whereas blunt arrows indicate inhibition. Phosphorylation and dephosphorylation are indicated by +P and −P, respectively. Abbreviations for interfering chemicals are printed in black (As, arsenite; CALY, calyculin A; FA, fatty acids; FB1, fumonisin B; MC-LR, microcystin-LR; OKA, okadaic acid; MMS, methylmethane sulfonate; PMA, phorbol miristate acetate; ROS, reactive oxygen species; SHR, SH-reactive chemicals, such as iodoacetamide; STAU, staurosporin).

The lower portion of Fig. 3–7 indicates important TFs activated by signaling pathways. For example, c-Fos and c-Jun, which dimerize (called AP-1) to bind to the tetradecanoylphorbol acetate (TPA) response element (TRE), play an important role in regulating cell division because they activate cyclin D to drive the G_1/S-phase transition of the cell cycle. There are other signal-activated TFs that also upregulate transcription of cyclin D and induce cell proliferation including c-Myc, NF-κB (nuclear factor kappa–light chain enhancer of activated B cells), CREB (cAMP response element binding protein), and STAT (signal transducer and activator of transcription).

Signaling through the transforming growth factor β (TGF-β) signaling pathway is also an important pathway that influences cell growth, differentiation, and wound repair, and there are two transmembrane receptors (type I and type II) that function as serine/threonine kinases required for its signal transduction. The Wnt and hedgehog (Hh) pathways as important regulators of cell division and differentiation have critical roles in embryonic development, tissue regeneration, and carcinogenesis. Activation of Wnt and Hh pathways regulate cellular levels of β-catenin and Gli (glioma-associated transcription factor), respectively, which in turn control transcription of numerous genes involved in cell cycle regulation, proteosomal activity, and cell differentiation. Chemicals that influence Wnt signaling have numerous adverse effects including abnormal development, cell proliferation, and carcinogenesis. Signal transduction may occur through successive protein–protein interactions and protein phosphorylation reactions at hydroxyl groups in specific serine, threonine, or tyrosine residues. Most cell surface growth factor receptors are kinases that phosphorylate their downstream targets. In addition to the MAPK pathway already described, the PI3K pathway (Fig. 3–7) leads to phosphorylation of phosphatidylinositol (4,5)-bisphosphate (PIP_2), a lipid commonly found in the plasma membrane, to generate phosphatidylinositol-3,4,5-triphosphate (PIP_3). Furthermore, Akt catalyzes the inactivating phosphorylation of glycogen synthase kinase 3 (GSK3), a ubiquitous multifunctional constitutionally active protein kinase. Inactivation of GSK3α and GSK2β causes numerous effects ranging from promotion of glycogen synthesis to suppression of apoptosis (see later). Additionally, Akt inactivates the FoxO family of TFs, which prevents protein degradation in the skeletal muscle and promotes proliferation, thereby countering muscle atrophy. Most intracellular signal transducer proteins exist in the phosphorylated and dephosphorylated state, with protein kinases and phosphatases catalyzing these reactions, respectively. Some growth factor receptors amplify their signals by inducing formation of HOOH.

Chemically Altered Signal Transduction and Proliferation— Xenobiotics that facilitate phosphorylation of signal transducers often promote mitosis and tumor formation. Examples include phorbol esters and fumonisin B that activate protein kinase C (PKC). The activated PKC promotes mitogenic activity through two major phosphorylation reactions. These are the phosphorylation of Raf, the first protein kinase in the MAPK pathway, and by phosphorylation of a protein phosphatase that dephosphorylates the TF c-Jun at specific sites (Thr 231, Ser 234, and Ser 249), thereby permitting its binding to DNA. Chemicals that modulate PKC activity often mimic diacylglycerol (DAG), an endogenous activator of PKC. The other PKC activator is Ca^{2+}, which can be mimicked by Pb^{2+}. Lead ions increase PKC activity at picomolar concentrations, when Pb^{2+} occupies only high-affinity binding sites on PKC, but Pb^{2+} inhibits PKC activity at high, micromolar concentrations, where the low-affinity sites are also occupied.

Hyperphosphorylation of proteins may result not only from increased phosphorylation by kinases but also from decreased dephosphorylation by phosphatases. Inhibition of phosphatases, including the lipid phosphatase PTEN, appears to be the underlying mechanism of the mitogenic effect of various chemicals, oxidative stress, and ultraviolet (UV) irradiation. PTP and dual-specificity phosphatases (i.e., enzymes that remove phosphate from phosphorylated tyrosine as well as serine and threonine residues) as well as PTEN contain a catalytically active cysteine that is susceptible to inactivation by HOOH. Xenobiotics such as the SH-reactive iodoacetamide, the organometal compound tributyltin, arsenite, as well as HOOH cause phosphorylation of the epidermal growth factor (EGF) receptor (Fig. 3–7) by interfering with the PTP that dephosphorylates and inactivates this receptor.

Protein phosphatase 2A (PP2A) is the major soluble Ser/Thr phosphatase in cells and is likely responsible, at least in part, for reversing the growth factor–induced stimulation of MAPK, thereby keeping the extent and duration of MAPK activation under control. Several natural toxicants are extremely potent inhibitors of PP2A, including the blue-green algae poison microcystin-LR and the dinoflagellate-derived okadaic acid. With chronic, low-dose exposure, both compounds are tumor promoters. However, acute high-dose exposure to microcystin induces severe liver injury, whereas acute exposure to okadaic acid causes gastrointestinal effects associated with shellfish poisoning. In these acute conditions, hyperphosphorylation of proteins other than those involved in proliferative signaling (e.g., hepatocellular microfilaments in microcystin poisoning) is likely responsible for the toxicity.

Apart from phosphatases, there are also inhibitory binding proteins that can keep signaling under control. Such is the case for IκB, which binds to NF-κB, preventing its transfer into the nucleus and its function as a TF (Fig. 3–7). When phosphorylated by its designated IκB kinase (IKK), IκB is degraded by the proteasome to release NF-κB. The released NF-κB is a critical regulator of inflammatory processes. Through its signaling, a broad, pro-inflammatory program is controlled, including the release of cytokines (e.g., TNF and IL-1β), chemokines (e.g., MCP-1), cell adhesion molecules (e.g., ICAM-1, E-selectin, and P-selectin), enzymes producing inflammatory lipid mediators (e.g., PLA2 and COX-2), and acute-phase proteins (e.g., C-reactive protein and α1-acid glycoprotein). However, IKK can also be phosphorylated (activated) by other protein kinases, such as Raf (MAPK cascade) and Akt. NF-κB activation via Akt-mediated IKK phosphorylation is involved in the proliferative (and possibly carcinogenic) effect of arsenite, nicotine, and NNK.

Aberrant mitogenic signals may originate in the GTP/GDP-binding protein Ras. Signaling through Ras is normally terminated via stimulation of its own GTPase activity by a GTPase-activating protein (GAP; Fig. 3–7) that returns Ras into its inactive GDP-bound state. Fatty

acids, which may accumulate in response to phospholipase A activation and exposure to peroxisome proliferators, inhibit GAP and can delay turning off Ras, leading to increased cell proliferation. Finally, genotoxic carcinogens may mutate Ras and lead to loss of its GTPase activity, thereby sustaining signaling of the MAPK pathway and increasing the likelihood of malignant transformation.

Chemically Altered Signal Transduction with Antiproliferative Effect— Cell proliferation is generally tightly controlled and is turned off when repair of injured cells is complete. However, an insult that decreases proliferative signaling after cell injury may compromise replacement of injured cells and can direct a cell to apoptotic death (discussed later in this chapter). Indeed, inhibitors of PKC (staurosporin), PI3K (wortmannin), and IκB degradation (gliotoxin) induce apoptosis. Similarly, TGF-β and glucocorticoids increase IκB, which in turn decreases NF-κB activation and c-Myc expression, and both mechanisms may contribute to the apoptotic effect of TGF-β and glucocorticoids, particularly in lymphoid cells.

Dysregulation of Extracellular Signal Production—Hormones of the anterior pituitary exert mitogenic effects on endocrine organs by acting on cell surface receptors. Pituitary hormone production is under negative feedback control by hormones of the peripheral organs, and perturbation of this circuit adversely affects pituitary hormone secretion and, in turn, endocrine organ function (see Chapter 20). Decreased secretion of pituitary hormone produces the opposite adverse effect, with apoptosis followed by involution of the target organ. For example, estrogens produce testicular atrophy in males by means of feedback inhibition of gonadotropin secretion.

Impaired Ongoing Cellular Activity— Ongoing control of specialized cells is exerted by signaling molecules acting on membrane receptors that transduce the signal by regulating Ca^{2+} entry into the cytoplasm or stimulating the enzymatic formation of intracellular second messengers. Ca^{2+} or other second messengers ultimately alter phosphorylation of functional proteins, changing their activity and, in turn, cellular functions almost instantly. Perturbation of ongoing cellular activity by chemicals may be due to an alteration in (1) the concentration of neurotransmitters, (2) receptor function, (3) intracellular signal transduction, or (4) the signal-terminating processes.

Dysregulation of Electrically Excitable Cells—Many xenobiotics influence cellular activity in excitable cells, such as neurons and skeletal, cardiac, and smooth muscle cells. Cellular functions such as the release of neurotransmitters and muscle contraction are controlled by transmitters and modulators synthesized and released by adjacent neurons. Mechanistic details concerning altered regulation of excitable cells are provided here as this information is not considered in other chapters of this book. These mechanisms are summarized in Fig. 3–8, and chemicals that interfere with these mechanisms are listed in Table 3–1.

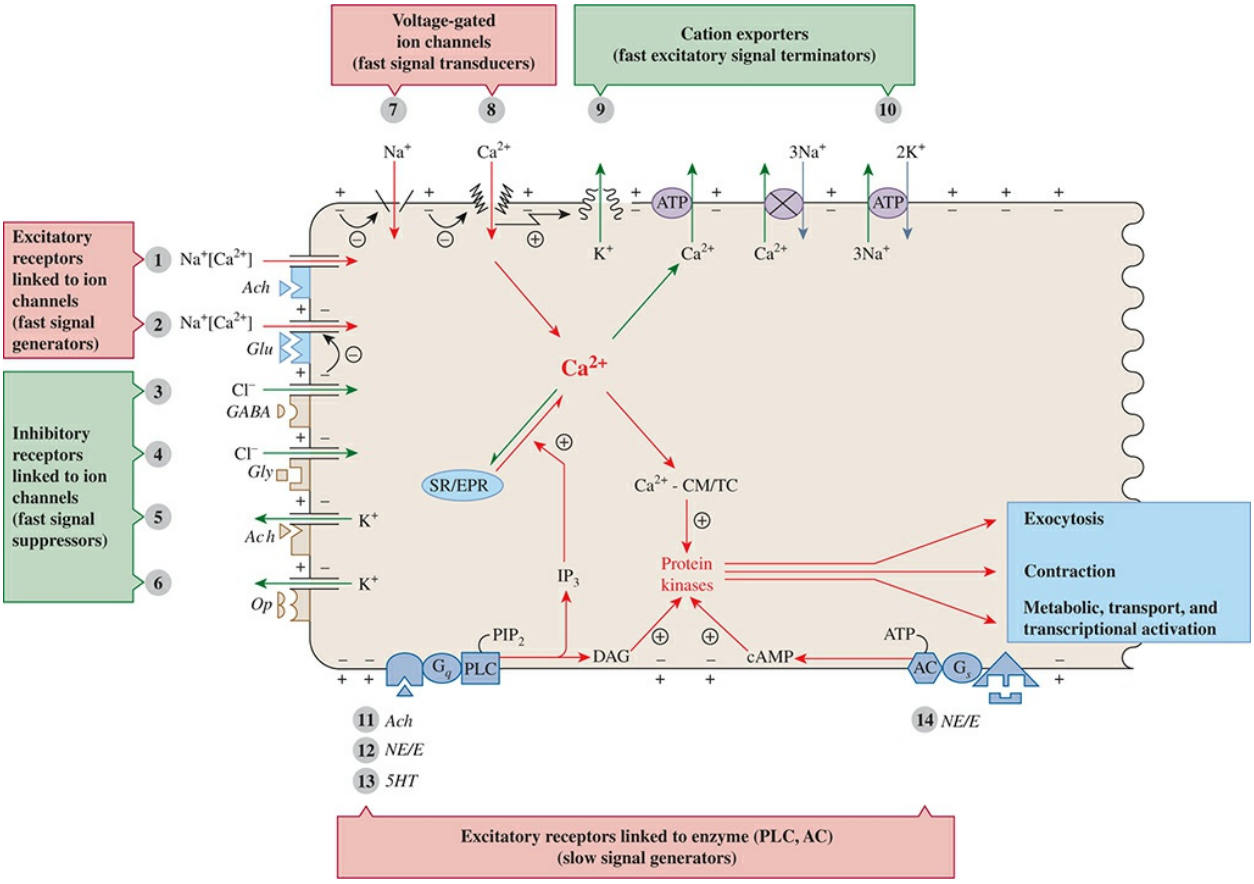

FIGURE 3–8 *Signaling mechanisms for neurotransmitters.* This simplified scheme depicts major cellular signaling mechanisms in neurons, muscle, and exocrine cells. Chemicals acting on the numbered elements are listed in Table 3–4. Fast signaling is initiated by the opening of ligand-gated Na^+/Ca^{2+} channels (1, 2). The resultant cation influx decreases the inside negative potential (i.e., evokes depolarization) and triggers the opening of the voltage-gated Na^+ and Ca^{2+} channels (7, 8). As a second messenger, Ca^{2+} activates intracellular Ca^{2+}-binding proteins such as calmodulin (CM) and troponin C (TC), which in turn enhance the phosphorylation of specific proteins. The signal is terminated by channels and transporters (9, 10) that remove cations from the cells to reestablish the inside negative resting potential (repolarization) and restore the resting Ca^{2+} level. Fast signaling can be suppressed by opening the ligand-activated Cl^- or K^+ channels (3–6), which increase intracellular hyperpolarization to counteract opening of the voltage-gated Na^+ and Ca^{2+} channels (7, 8). Signal transduction from other receptors (11–13) that are coupled to G*q* proteins involves generation of the second messenger inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) by phospholipase C (PLC), whereas signaling from receptor 14 that is coupled to Gs protein involves production of cyclic AMP (cAMP) by adenylyl cyclase (AC). These second messengers influence cellular activities by mobilizing Ca^{2+} from the sarcoplasmic or endoplasmic reticulum (SR and EPR), as is the case for IP_3, or by activating protein kinases, as is the case for cAMP and DAG activation of PKA and PKC, respectively. The figure does not depict that inhibitory receptors 5 and 6 are G*i* protein–coupled that function by opening K^+ channels and inhibit AC. *Abbreviations*: Ach, acetylcholine; Glu, glutamate; GABA, γ-aminobutyric acid; Gly, glycine;

Op, opioid peptides; NE, norepinephrine; E, epinephrine; 5HT, 5-hydroxytryptamine; G, G protein; PIP_2, phosphatidylinositol 4,5-bisphosphate. Positive and negative signs (circled) indicate activation and inhibition, respectively.

TABLE 3–1 Agents Acting on Signaling Systems for Neurotransmitters

Receptor/Channel/Pump		Agonist/Activator		Antagonist/Inhibitor	
Name*	**Location**	**Agent**	**Effect**	**Agent**	**Effect**
1. Acetylcholine nicotinic receptor	Skeletal muscle	Nicotine Anatoxin-a Cytisine *Ind*: ChE inhibitors	Muscle fibrillation and then paralysis	Tubocurarine, lophotoxin (from sea whip) α-Bungarotoxin (banded krait venom) α-Cobrotoxin (cobra venom) α-Conotoxin (marine cone snail) Erabutoxin b (banded krait venom) *Ind*: botulinum toxicant	Muscle paralysis
	Neurons	See above	Neuronal activation	Pb^{2+}, general anesthetics	Neuronal inhibition
2. Glutamate receptor	CNS neurons	*N*-Methyl-*d*-aspartate Kainate, domoate Quinolinate Quisqualate *Ind*: hypoxia, HCN → glutamate release	Neuronal activation → convulsion, neuronal injury ("excitotoxicity")	Phencyclidine Ketamine General anesthetics	Neuronal inhibition → anesthesia Protection against "excitotoxicity"
3. $GABA_A$ receptor	CNS neurons	Muscimol, avermectins, sedatives (barbiturates, benzodiazepines), general anesthetics (halothane), alcohols (ethanol)	Neuronal inhibition → sedation, general anesthesia, coma, depression of vital centers	Bicuculline Picrotoxin Pentylenetetrazole Cyclodiene insecticides Lindane, TCAD *Ind*: isoniazid	Neuronal activation → tremor, convulsion
4. Glycine receptor	CNS neurons, motor neurons	Avermectins (?), general anesthetics	Inhibition of motor neurons → paralysis	Strychnine *Ind*: tetanus toxicant	Disinhibition of motor neurons → tetanic convulsion
5. Acetylcholine M_2 muscarinic receptor	Cardiac muscle	*Ind*: ChE inhibitors	Decreased heart rate and contractility	Belladonna alkaloids (e.g., atropine), atropine-like drugs (e.g., TCAD)	Increased heart rate
6. Opioid receptor	CNS neurons, visceral neurons	Morphine and congeners (e.g., heroin, meperidine) *Ind*: clonidine	Neuronal inhibition → analgesia, central respiratory depression, constipation, urine retention	Naloxone	Antidotal effects in opiate intoxication
7. Voltage-gated Na^+ channel	Neurons, muscle cells, etc.	Aconitine, veratridine Grayanotoxin (rhododendrons) Batrachotoxin (poison dart frogs) Scorpion toxins Ciguatoxin (fish) DDT, pyrethroids	Neuronal activation → convulsion	Tetrodotoxin (pufferfish), saxitoxin (algae and cyanobacteria) μ-Conotoxin Local anesthetics Phenytoin Quinidine	Neuronal inhibition → paralysis, anesthesia Anticonvulsive action

8. Voltage-gated Ca^{2+} channel	Neurons, muscle cell, etc.	Maitotoxin (dinoflagellates) Atrotoxin (?) Latrotoxin [?] (black widow spider)	Neuronal/muscular activation, cell injury	ω-Conotoxin Pb^{2+}	Neuronal inhibition → paralysis
9. Voltage/Ca^{2+}-activated K^+ channel	Neurons, smooth and skeletal muscle, cardiac muscle	Pb^{2+}	Neuronal/muscular inhibition	Ba^{2+}, apamin (bee venom), dendrotoxin, 20-HETE, hERG inhibitors (e.g., cisapride, terfenadine)	Neuronal/muscular activation → convulsion/spasm vasoconstriction, PMV tachycardia (torsade de pointes)
10. Na^+, K^+-ATPase	Universal			Digitalis glycosides Oleandrin Chlordecone	Increased cardiac contractility, excitability Increased neuronal excitability → tremor
11. Acetylcholine M_3 muscarinic receptor	Smooth muscle, glands	*Ind*: ChE inhibitors	Smooth muscle spasm, salivation, lacrimation	Belladonna alkaloids (e.g., atropine), atropine-like drugs (e.g., TCAD)	Smooth muscle relaxation → intestinal paralysis, decreased salivation, decreased perspiration
Acetylcholine M_1 muscarinic receptor	CNS neurons	Oxotremorine *Ind*: ChE inhibitors	Neuronal activation → convulsion	See above	
12. Adrenergic α_1 receptor	Vascular smooth muscle	(Nor)epinephrine *Ind*: cocaine, tyramine, amphetamine, TCAD	Vasoconstriction → ischemia, hypertension	Prazosin	Antidotal effects in intoxication with α_1-receptor agonists
13. 5-HT_2 receptor	Smooth muscle	Ergot alkaloids (ergotamine, ergonovine)	Vasoconstriction → ischemia, hypertension	Ketanserine	Antidotal effects in ergot intoxication
14. Adrenergic β_1 receptor	Cardiac muscle	(Nor)epinephrine *Ind*: cocaine, tyramine, amphetamine, TCAD	Increased cardiac contractility and excitability	Atenolol, metoprolol	Antidotal effects in intoxication with β_1-receptor agonists

Abbreviations: ChE, cholinesterase; CNS, central nervous system; 20-HETE, 20-hydroxy-5,8,11, 14-eicosatetraenoic acid; Ind, indirectly acting (i.e., by altering neurotransmitter level); PMV, polymorphic ventricular; TCAD, tricyclic antidepressant.
*Numbering of the signaling elements in this table corresponds to those in Fig. 3–8. This table is simplified. Virtually all receptors and channels listed occur in multiple forms with different sensitivity to the agents. The reader should consult additional literature for more detailed information.

Altered regulation of neural and/or muscle activity is the basic mechanism of action of many drugs and is responsible for toxicities associated with drug overdosage, pesticides, and microbial, plant, and animal toxicants. As neurons are signal-transducing cells, the influence of toxic chemicals is seen not only on the neuron affected by the toxicant but also on downstream cells influenced by neuronal activity. Thus, the action of tetrodotoxin is to block voltage-gated Na^+ channels in motor neurons (item 7 in Fig. 3–8), but the observed effect is skeletal muscle paralysis. Similarly, tarantula venoms, including ceratotoxins (CccTx1-3) and phrixotoxin 3, also cause paralysis through inhibition of voltage-gated Na^+ channels. In contrast, cyclodiene insecticides, which block GABA receptors in the central nervous system (item 3 in Fig. 3–8), induce neuronal excitation and convulsions.

Alteration in Neurotransmitter Levels—Chemicals may alter synaptic levels of neurotransmitters by interfering with their synthesis, storage, release, or removal from the vicinity of the receptor. Toxicity is associated with an exaggeration of the normal physiologic activity of the neurotransmitter. In contrast, if synaptic levels of a neurotransmitter are decreased,

the outcome is usually the opposite of the normal activity (Fig. 3–8). Inhibition of acetylcholinesterase by organophosphate or carbamate insecticides or chemical warfare agents (e.g., soman) prevents the hydrolysis of acetylcholine, resulting in massive stimulation of cholinergic receptors (receptors 1, 5, and 11 in Fig. 3–8) and a cholinergic crisis. Inhibition of the neuronal reuptake of norepinephrine by cocaine or tricyclic antidepressants is responsible for overexcitation of α_1-adrenergic receptors on vascular smooth muscles, resulting in nasal mucosal ulceration and myocardial infarction in heavy cocaine abusers, whereas overstimulation of β_1-adrenergic receptors contributes to life-threatening arrhythmias. Similar cardiac complications may result from amphetamine abuse, because amphetamine enhances the release of norepinephrine from adrenergic neurons and competitively inhibits neuronal reuptake of this transmitter. A hypertensive crisis can occur with the combined use of tricyclic antidepressants and monoamine oxidase inhibitors, drugs that block different mechanisms of norepinephrine elimination. Concomitant use of drugs that increase the concentration of serotonin (5-HT) by enhancing its neuronal release and decreasing its neuronal reuptake (e.g., fluoxetine) or its biotransformation (e.g., monoamine oxidase inhibitors) induces serotonin syndrome with cognitive and behavioral changes, autonomic dysfunction, and neuromuscular abnormalities. Some cytotoxic antineoplastic drugs (e.g., cisplatin) and radiation cause nausea and emesis by inducing release of 5-HT from enterochromaffin cells of the intestinal mucosa, which stimulates the 5-HT_3 receptors (5-HT-gated cation channel, functionally similar to item 1 in Fig. 3–8) on the adjacent vagal afferent neurons, thereby evoking the vomiting reflex. Intestinal release of 5-HT and stimulation of vagal afferent neurons are also involved in the emetic effect of ipecac syrup. The α_2-adrenergic receptor agonist clonidine induces release in the brain of β-endorphin, an endogenous peptide that stimulates opioid receptors (item 6 in Fig. 3–8). This explains why clonidine intoxication mimics several symptoms of morphine poisoning, including depressed respiration and pinpoint pupils.

Toxicant–Neurotransmitter Receptor Interactions—Some chemicals interact directly with neurotransmitter receptors, including (1) agonists that associate with the ligand-binding site on the receptor and mimic the natural ligand, (2) antagonists that occupy the ligand-binding site but cannot activate the receptor, (3) activators, and (4) inhibitors that bind to a site on the receptor that is not directly involved in ligand binding. In the absence of other actions, agonists and activators mimic, whereas antagonists and inhibitors block, the physiologic responses characteristic of endogenous ligands. For example, muscimol, a mushroom poison, is an agonist at the inhibitory $GABA_A$ receptor (item 3 in Fig. 3–8), whereas barbiturates, benzodiazepines, general anesthetics, and alcohols are activators. Thus, all these chemicals inhibit central nervous system activity, resulting in sedation, general anesthesia, and coma, and may ultimately inhibit the medullary respiratory center, depending on the dose administered. There are also similarities in the responses evoked by agonist/activators on excitatory receptors and those elicited by antagonists/inhibitors on inhibitory sites. Glutamate receptor agonists and muscarinic receptor agonists cause neuronal hyperactivity in the brain and ultimately convulsions, as do inhibitors of $GABA_A$ receptor. Likewise, chemicals acting as agonists/activators on inhibitory receptors and those acting as antagonists/inhibitors on excitatory receptors may exert similar effects. Finally, the numerous subtypes of neurotransmitter receptors may be affected differentially by toxicants. For example, the neuronal nicotinic acetylcholine receptor is extremely sensitive to inhibition by lead ions, whereas the muscular nicotinic receptor subtype is not.

Some sensory neurons have membrane receptors that are stimulated by noxious chemicals and operate as ligand-gated cation channels. The transient receptor potential (TRP) channels open to enable Na^+ and Ca^{2+} influx. TRPV1 is stimulated by capsaicin, the pungent ingredient of hot peppers, and mediates the burning sensation of the tongue and reflex stimulation of the lacrimal gland associated with exposure to pepper spray. However, the TRPA1 receptor is activated by thiol-reactive and oxidant chemicals, such as the lacrimator compounds in tear gas, acrolein, methyl isocyanate, and corrosive gases to evoke considerable irritation and pain, along with lacrimation, bronchial secretion, sneezing, coughing, bronchospasm, and neurogenic inflammation.

Toxicant–Signal Transducer Interactions—Many chemicals alter neuronal and/or muscle activity by acting on signal transduction processes. Voltage-gated Na^+ channels (item 7 in Fig. 3–8) are activated by a number of toxicants derived from plants and animals (Table 3–1) as well as some synthetic chemicals such as DDT, resulting in overexcitation. In contrast, chemicals that block voltage-gated Na^+ channels (such as tetrodotoxin and saxitoxin) cause paralysis.

Toxicant–Signal Terminator Interactions—The cellular signal generated by cation influx is terminated by removal of the cations through channels or by transporters (Fig. 3–8). Inhibition of cation efflux by Ba^{2+} may prolong excitation by blockade of Ca^{2+}-activated K^+ channels (item 9 in Fig. 3–8) to cause potentially lethal neuroexcitatory and spasmogenic effects.

Glycosides from digitalis and other plants inhibit Na^+,K^+-ATPase (item 10 in Fig. 3–8) and thus increase the intracellular Na^+ concentration, which, in turn, decreases Ca^{2+} export by Ca^{2+}/Na^+ exchange. The resultant rise in the intracellular concentration of Ca^{2+} enhances the contractility and excitability of cardiac muscle. Failure of Na^+,K^+ pump function is also believed to contribute to neuronal damage resulting from hypoxia, hypoglycemia, and cyanide intoxication. Inasmuch as 70% of the ATP produced in neurons is used to drive the Na^+,K^+ pump; cessation of ATP synthesis causes a cell to become or remain depolarized.

Dysregulation of the Activity of Other Cells—Many exocrine secretory cells are controlled by muscarinic acetylcholine receptors (item 11 in Fig. 3–8). Salivation, lacrimation, and bronchial hypersecretion after organophosphate insecticide poisoning are due to stimulation of these receptors. In contrast, blockade of these receptors contributes to the hyperthermia characteristic of atropine poisoning.

Toxic Alteration of Cellular Maintenance

The major toxicity manifested by toxicant-induced alterations in cellular maintenance is cell death. This is usually an irreversible process that can disrupt the structural and functional integrity of the organ or organism, and it invokes numerous repair or adaptive processes to compensate for the loss of function. Understanding mechanisms of cell death provides for deeper recognition of the cellular processes that initiate this toxic outcome along with the compensatory changes that accompany these processes.

Mechanisms of Toxic Cell Death

There are three common, major events underlying cell death. These are ATP depletion, sustained rise in intracellular Ca^{2+}, and overproduction of ROS and RNS. These events and the chemicals that may cause them are individually characterized, after which the features of difference pathways leading to cell death are discussed. Classically, cell death was considered to occur via necrosis, a process deemed to be irreversible damage. However, additional pathways that lead to cell death involve more complex signaling mechanisms and a specific, regulated program. Mitochondrial changes are at the core of every cell death, regardless of the pathways or mediators involved.

Primary Metabolic Disorders Jeopardizing Cell Survival—Cell viability is maintained by ATP as it supports intermediary metabolism and is the major source of cellular energy. ATP is utilized in numerous biosynthetic reactions, activating endogenous compounds by phosphorylation and adenylation, and it is incorporated into cofactors as well as nucleic acids. It is required for muscle contraction and polymerization of the cytoskeleton, fueling cellular motility, cell division, vesicular transport, and the maintenance of cell morphology. ATP drives many different ion transporters in plasma and ER membranes, and in the membrane of lysosomes and neurotransmitter-containing vesicles. These pumps also support essential cell functions. For example, the Na^+ concentration gradient across the plasma membrane generated by the Na^+,K^+ pump drives Na^+-glucose and Na^+–amino acid cotransporters as well as the Na^+/Ca^{2+} antiporter, facilitating the entry of these nutrients and the removal of Ca^{2+}.

Chemical energy is released by hydrolysis of ATP to ADP and AMP. The ADP is rephosphorylated in the mitochondria by ATP synthase (Fig. 3–9). *Oxidative phosphorylation* is the process by which ATP is synthesized when coupled to the oxidation of hydrogen to form water. In addition to ATP synthase, oxidative phosphorylation requires the (1) delivery of hydrogen in the form of NADH to the initial electron transport complex; (2) delivery of oxygen to the terminal electron transport complex; (3) delivery of ADP and inorganic phosphate to ATP synthase; (4) flux of electrons along the electron transport chain to O_2, accompanied by ejection of protons from the matrix space across the inner membrane; and (5) return of protons across the inner membrane into the matrix space down an electrochemical gradient to drive ATP synthase.

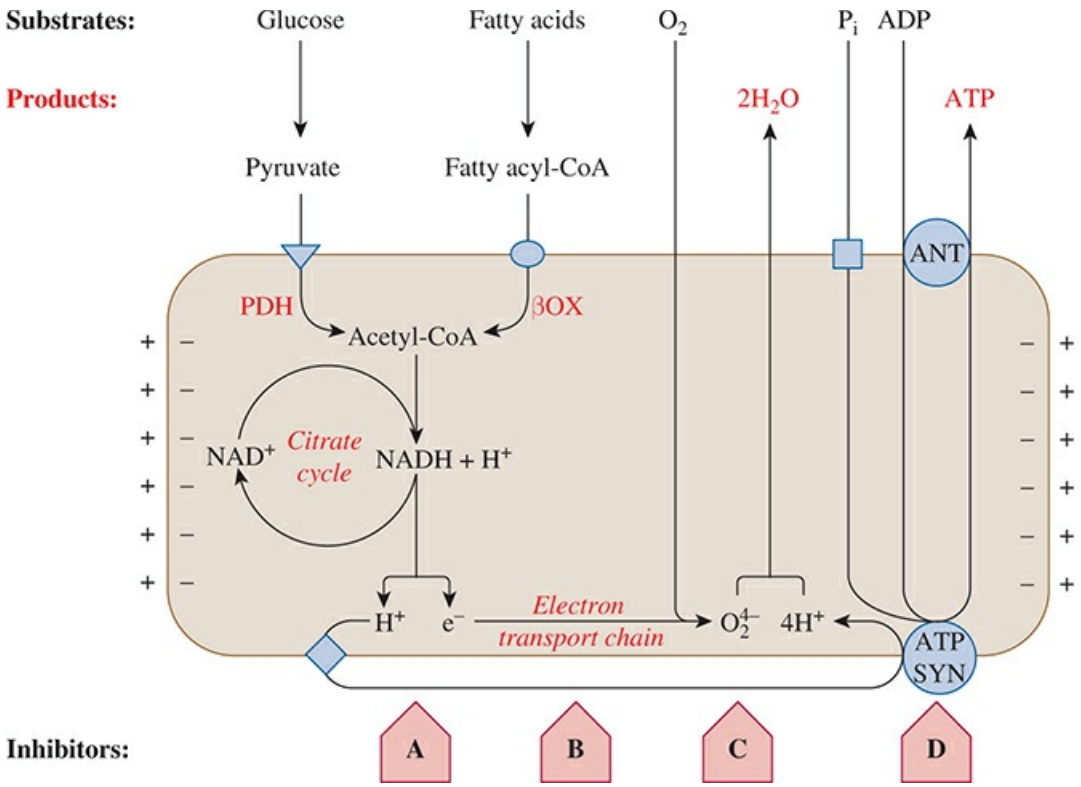

FIGURE 3–9 *ATP synthesis (oxidative phosphorylation) in mitochondria.* Arrows with letters A to D indicate the ultimate sites of action of four categories of agents that interfere with oxidative phosphorylation. Toxicants that induce DNA damage (Class E) are not illustrated. For simplicity, this scheme does not indicate the outer mitochondrial membrane and that protons are extruded from the matrix space along the electron transport chain at three sites. *Abbreviations*: βOX, beta-oxidation of fatty acids; e^-, electron; P_i, inorganic phosphate; ANT, adenine nucleotide translocator; ATP SYN, ATP synthase (F_oF_1-ATPase).

Several chemicals that impede the various steps in oxidative phosphorylation to disrupt mitochondrial ATP synthesis have been divided into five groups (Table 3–2). Substances in class A interfere with the delivery of hydrogen to the electron transport chain. Class B chemicals inhibit the transfer of electrons along the electron transport chain to oxygen. Class C agents interfere with oxygen delivery to the terminal electron transporter, cytochrome oxidase. Chemicals in class D inhibit the activity of ATP synthase, the key enzyme for oxidative phosphorylation. At this site, the synthesis of ATP may be inhibited in one of four ways: (1) direct inhibition of ATP synthase, (2) interference with ADP delivery, (3) interference with inorganic phosphate delivery, and (4) deprivation of ATP synthase from its driving force, the controlled influx of protons into the matrix space. Protonophoric chemicals (uncouplers) such as 2,4-dinitrophenol and pentachlorophenol import protons into the mitochondrial matrix, dissipating the proton gradient that drives the controlled influx of protons into the matrix, which in turn drives ATP synthase. Finally, chemicals causing mitochondrial DNA injury to impair synthesis of specific proteins encoded by the mitochondrial genome (e.g., subunits of complex I and ATP synthase) are listed in group E.

TABLE 3–2 Agents That Impair Mitochondrial ATP Synthesis*

Class A. Inhibitors of hydrogen delivery to the electron transport chain

1. Glycolysis (critical in neurons): hypoglycemia; iodoacetate, koningic acid, and NO^+
2. Gluconeogenesis (critical in renal tubular cells): coenzyme A depletors (see below)
3. Fatty acid oxidation (critical in cardiac muscle): hypoglycin, 4-pentenoic acid, 4-ene-valproic acid
4. Pyruvate dehydrogenase: arsenite, DCVC, *p*-benzoquinone
5. Citrate cycle
   (a) Aconitase: fluoroacetate, $ONOO^-$
   (b) Isocitrate dehydrogenase: DCVC
   (c) Succinate dehydrogenase: malonate, DCVC, PCBD-Cys, 2-bromohydroquinone, 3-nitropropionic acid, *cis*-crotonalide fungicides
6. Depletors of TPP (inhibit TPP-dependent PDH and α-KGDH): ethanol (when chronically consumed)
7. Compounds that deplete coenzyme A (CoA)
   (a) Thiol-reactive electrophiles: 4-(dimethylamino)phenol, *p*-benzoquinone
   (b) Drugs enzymatically conjugated with CoA: salicylic acid (the metabolite of aspirin), valproic acid
8. Compounds that deplete NADH
   (a) Alloxan, t-butylhydroperoxide
   (b) Activators of poly(ADP-ribose) polymerase (PARP): agents causing DNA damage (e.g., MNNG, hydrogen peroxide, $ONOO^-$)

Class B. Inhibitors of electron transport

1. Inhibitors of electron transport complexes
   (a) NADH–coenzyme Q reductase (complex I): rotenone, amytal, MPP^+, paraquat
   (b) Coenzyme Q–cytochrome *c* reductase (complex III): antimycin-A, myxothiazole
   (c) Cytochrome oxidase (complex IV): cyanide, hydrogen sulfide, azide, formate, $^{\cdot}NO$, phosphine (PH_3)
   (d) Multisite inhibitors: dinitroaniline and diphenylether herbicides, $ONOO^-$
2. Electron acceptors: CCl_4, doxorubicin, menadione, MPP^+

Class C. Inhibitors of oxygen delivery to the electron transport chain

1. Chemicals causing respiratory paralysis: CNS depressants (e.g., opioids), convulsants
2. Chemicals impairing pulmonary gas exchange: CO_2, NO_2, phosgene, perfluoroisobutene
3. Chemicals inhibiting oxygenation of Hb: carbon monoxide, methemoglobin-forming chemicals
4. Chemicals causing ischemia: ergot alkaloids, cocaine

Class D. Inhibitors of ADP phosphorylation

1. ATP synthase: oligomycin, cyhexatin, DDT, chlordecone
2. Adenine nucleotide translocator: atractyloside, DDT, free fatty acids, lysophospholipids
3. Phosphate transporter: *N*-ethylmaleimide, mersalyl, *p*-benzoquinone
4. Chemicals dissipating the mitochondrial membrane potential (uncouplers)
   (a) Cationophores: pentachlorophenol, dinitrophenol, benzonitrile, thiadiazole herbicides, salicylate, CCCP, cationic amphiphilic drugs (bupivacaine, perhexiline), valinomycin, gramicidin, calcimycin (A23187)
   (b) Chemicals permeabilizing the mitochondrial inner membrane: PCBD-Cys, chlordecone
5. Multisite inhibitor drugs: phenformin, propofol, salicylic acid (when overdosed)

Class E. Chemicals causing mitochondrial DNA damage

1. Antiviral drugs: zidovudine, zalcitabine, didanosine, fialuridine
2. Antibiotics: chloramphenicol (when overdosed), linezolid
3. Ethanol (when chronically consumed)

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCVC, dichlorovinyl-cysteine; α-KGDH, α-ketoglutarate dehydrogenase; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; MPP+, 1-methyl-4-phenylpyridinium; PCBD-Cys, pentachlorobutadienylcysteine; PDH, pyruvate dehydrogenase; TPP, thiamine pyrophosphate.
*The ultimate sites of action of these agents are indicated in *Fig. 3–9*.

ATP synthase is also present in the outer segment disk of the retinal rods as well as the plasma membrane of certain cells and the concentric membranes that form the myelin sheath around the axon of neurons. In plasma and myelin membranes, ATP is formed in the external membrane by ecto-ATP synthase. ATP supply to axons, through the ecto-ATP synthase in myelin, may explain why demyelinated axons die, that is, they have no ATP source. In contrast, myelinated axons are sensitive to hypoxia but can synthesize ATP, if required.

Impairment of oxidative phosphorylation is detrimental to cells because with failure of ADP rephosphorylation, ATP is depleted and there is accumulation of ADP and its breakdown products. A rise in cytosolic H^+ and Mg^{2+} results from hydrolysis of adenosine diphosphates and triphosphates (existing as Mg salts), which produces cellular acidosis from the release of phosphoric acid and Mg^{2+}. Increased conversion of pyruvate to lactate also may contribute to

acidosis. The lack of ATP compromises the operation of ATP-requiring ion pumps, leading to the loss of ionic and volume-regulatory controls. Furthermore, there is an increase in intracellular Na^+ that follows acidosis and hypermagnesemia. The increase in Na^+ likely results from failure of the ATP-requiring Na^+ pumps, and this effect leads to the formation of plasma membrane blebs, an early morphologic change in cell death. Cellular acidosis directly decreases the activity of phospholipases and inhibits mitochondrial permeability transition (MPT; discussed in the section "Mitochondrial Permeability Transition and Necrotic Cell Death"). Finally, in its terminal stages, the intracellular pH rises, and this increases phospholipase activity, which enhances degradation of membrane phospholipids to initiate irreversible membrane damage.

Sustained Rise of Intracellular Ca 2 +— Intracellular Ca^{2+} levels are highly regulated. There is a 10,000-fold difference between extracellular and cytosolic Ca^{2+} concentration that is maintained by the impermeability of the plasma membrane to Ca^{2+} and by transport mechanisms that remove Ca^{2+} from the cytoplasm. Ca^{2+} is actively pumped out of the cytosol across the plasma membrane and it is sequestered in the ER and mitochondria. Importantly, mitochondria express a low-affinity Ca^{2+} transporter that plays a significant role in Ca^{2+} sequestration when the cytoplasmic concentrations rise into the micromolar range. Examples of chemicals that can cause a sustained rise in cytoplasmic Ca^{2+} levels are listed in Table 3–3.

TABLE 3–3 Agents Causing Sustained Elevation of Cytosolic Ca $^{2+}$

A. **Chemicals inducing Ca^{2+} influx into the cytoplasm**
   - I. Via ligand-gated channels in neurons
     1. Glutamate receptor agonists ("excitotoxins"): glutamate, kainate, domoate
     2. TRPV1 receptor (capsaicin receptor) agonists: capsaicin
     3. TRPA1 receptor agonists: SH-reactive electrophiles, such as lacrimators (e.g., chlorobenzalmalonitrile), acrolein, methyl isocyanate, phosgene, chloropicrin
   - II. Via voltage-gated channels: maitotoxin, $HO^{\cdot}$
   - III. Via "newly formed pores": maitotoxin, amphotericin B, chlordecone, methylmercury, alkyltins
   - IV. Across disrupted cell membrane
     1. Detergents: exogenous detergents, lysophospholipids, free fatty acids
     2. Hydrolytic enzymes: phospholipases in snake venoms, endogenous phospholipase A_2
     3. Lipid peroxidants: carbon tetrachloride
     4. Cytoskeletal toxins (by inducing membrane blebbing): cytochalasins, phalloidin
   - V. From mitochondria
     1. Oxidants of intramitochondrial NADH: alloxan, t-BHP, NAPBQI, divicine, fatty acid hydroperoxides, menadione, MPP^+
     2. Others: phenylarsine oxide, gliotoxin, $^{\cdot}NO$, $ONOO^-$
   - VI. From the endoplasmic reticulum
     1. IP_3 receptor activators: γ-HCH (lindane), IP_3 formed during "excitotoxicity"
     2. Ryanodine receptor activators: δ-HCH

B. **Chemicals inhibiting Ca^{2+} export from the cytoplasm (inhibitors of Ca^{2+}-ATPase in cell membrane and/or endoplasmic reticulum)**
   - I. Covalent binders: acetaminophen, bromobenzene, CCl_4, chloroform, DCE
   - II. Thiol oxidants: cystamine (mixed disulfide formation), diamide, *t*-BHP, and HOOH generators (e.g., menadione, diquat)
   - III. Others: vanadate, Cd^{2+}, thapsigargin (specific SERCA inhibitor)
   - IV. Chemicals impairing mitochondrial ATP synthesis (see Table 3-5)

Abbreviations: DCE, 1,1-dichloroethylene; HCH, hexachlorocyclohexane; MPP^+, 1-methyl-4-phenylpyridinium; NAPBQI, N-acetyl-p-benzoquinoneimine; SERCA, sarco/endoplasmic reticulum calcium ATPase; t-BHP, t-butyl hydroperoxide.

Sustained elevation of intracellular Ca^{2+} is harmful because it will deplete energy reserves, alter microfilament structure, activate hydrolytic enzymes, and generate ROS and RNS. Increased intracellular Ca^{2+} levels alter cellular energy balance because high cytoplasmic Ca^{2+} concentrations increase mitochondrial Ca^{2+} uptake by the Ca^{2+} uniporter. In doing so, the mitochondrial membrane potential ($\Delta\Psi m$) is dissipated, and ATP synthesis is reduced. Ca^{2+} may also impair ATP synthesis by causing oxidative injury to the inner membrane by mechanisms described later. Furthermore, a sustained rise in cytoplasmic Ca^{2+} increases ATP consumption by forcing Ca^{2+}-ATPases to work to eliminate the excess Ca^{2+}.

A second mechanism by which an uncontrolled rise in cytoplasmic Ca^{2+} causes cell injury is microfilamental dissociation. The cell-wide network of actin filaments maintains cellular morphology by attachment of the filaments to actin-binding proteins in the plasma membrane.

An increase of cytoplasmic Ca^{2+} causes dissociation of actin filaments from α-actinin and fodrin, proteins involved in anchoring the filaments to the plasma membrane. This mechanism leads to bleb formation in plasma membranes, a condition that predisposes the membrane to rupture.

A third event whereby high Ca^{2+} concentrations are deleterious to cells is activation of hydrolytic enzymes that degrade proteins, phospholipids, and nucleic acids. Many integral membrane proteins are targets for Ca^{2+}-activated neutral proteases, or calpains, that mediate hydrolysis of actin-binding proteins leading formation of membrane blebs.

Overproduction of ROS and RNS— A number of xenobiotics can directly generate ROS and RNS (refer to Fig. 3–3). In addition, overproduction of ROS and RNS can be secondary to intracellular hypercalcemia, as Ca^{2+}activates enzymes that generate ROS and/or RNS by activating dehydrogenases in the citric acid cycle, as $Ca2^{+}$ activates dehydrogenases in the citric acid cycle to generate ROS and/or RNS, which accelerate hydrogen output and in turn (see Fig. 3–9). This action increases the formation of $O_2^{\bullet-}$ by the mitochondrial electron transport chain. Ca^{2+} also activates proteases that convert xanthine dehydrogenase into xanthine oxidase, which generates $O_2^{\bullet-}$ and HOOH. Finally, neurons and endothelial cells constitutively express NOS that is activated by Ca^{2+}. Given the extremely high reactivity of $^{\bullet}NO$ with $O_2^{\bullet-}$, coproduction of these radicals will inevitably lead to formation of $ONOO^{-}$, a highly reactive oxidant (Fig. 3–3).

An important mechanistic consideration in understanding cell death mechanisms is that there is interplay between the three primary derangements in biochemistry.

- Depletion of cellular ATP reserves that deprive the endoplasmic and plasma membrane Ca^{2+} pumps of fuel, causing elevation of Ca^{2+} in the cytoplasm. With the influx of Ca^{2+} into the mitochondria, the mitochondrial membrane potential ($\Delta\Psi m$) declines, hindering ATP synthase.
- Intracellular hypercalcemia that facilitates formation of ROS and RNS, which inactivates thiol-dependent Ca^{2+} pumps by oxidative damage to further exacerbate hypercalcemia.
- Formation of ROS and RNS inhibits ATP synthesis. $^{\bullet}NO$ is a reversible inhibitor of cytochrome oxidase; NO^{+} (nitrosonium cation, a product of $^{\bullet}NO$), *S*-nitrosylate and inactivate glyceraldehyde 3-phosphate dehydrogenase, impairing glycolysis, whereas $ONOO^{-}$ irreversibly inactivates respiratory chain complexes I, II, III, and aconitase (by reacting with their Fe-S center).
- Formation of $ONOO^{-}$ that induces DNA single-strand breaks, which activates poly (ADP-ribose) polymerase (PARP). As part of the repair strategy, activated PARP transfers multiple ADP-ribose moieties from NAD^{+} to nuclear proteins and PARP itself. This consumption of NAD^{+} severely compromises ATP synthesis (Fig. 3–9), increasing the need to resynthesize NAD^{+} further consumes ATP to ultimately cause a significant cellular energy deficit.

However, the chain of events that contribute to the worsening metabolic conditions are somewhat cell- and toxicant-specific (see items 8 and 2, respectively, in Fig. 3–8). The interplay of ATP depletion, intracellular hypercalcemia, and overproduction of ROS and RNS involves multiple cycles that progressively aggravate biochemical dysfunction until the cell dies.

Mitochondrial Permeability Transition and Necrotic Cell Death—Mitochondrial permeability transition (MPT) is an abrupt change in mitochondrial membranes, which results in formation of a nonspecific pore that opens when mitochondrial Ca^{2+} levels are high, especially

when accompanied by decreased ΔΨm, generation of ROS and RNS, and depletion of ATP. Formation of the pore, which results from misfolded proteins in both the inner and outer mitochondrial membranes, enables solutes <1500 Da, and protons to readily enter the mitochondrial matrix space, which in turn causes rapid and complete dissipation of ΔΨm, cessation of ATP synthesis, and osmotic influx of water. Collectively, these events produce biochemical dysfunction and mitochondrial swelling. Ca^{2+} in the mitochondrial matrix effluxes out of mitochondria through the pore to increase cytoplasmic Ca^{2+} concentrations. In the face of these changes, mitochondria are incapable of synthesizing ATP and the organelle further wastes any remaining ATP because depolarization of the inner membrane converts ATP synthase to an ATPase that hydrolyzes ATP. At this point, even glycolysis is compromised by the insufficient ATP supply to the ATP-requiring glycolytic enzymes (hexokinase and phosphofructokinase). If extensive, a complete bioenergetic catastrophe develops that culminates in necrotic cell death.

The nuclear changes noted in necrotic cell death include (1) pyknosis characterized by shrunken and abnormally dark basophilic staining of the nucleus; (2) karyolysis, in which nuclei appear swollen and abnormally pale basophilic staining, and (3) karyorrhexis, the rupture and fragmentation of the nucleus (Table 3–4).

TABLE 3–4 Morphological, Biochemical, and Molecular Features of Cell Death Mechanisms

	Morphological Features				Core Regulators	
Type	Cell Membrane	Nucleus	Cytoplasm	Biochemical Features	Positive	Negative
Autophagy	No change	No chromatin condensation	Accumulation of autophagic vacuoles	Substrate degradation	ATG5 ATG7 Beclin	
Necrosis	Blebbing and rupture	Pyknosis, karyorrhexia, and karyolysis	Swelling	Lysosomal enzyme release	None: Pathologic stimulation	
Apoptosis	Blebbing and rounding up	Pyknosis, fragmentation, and chromatin condensation	Reduction of cell volume	Phosphatidylserine exposure, activation of caspases, DNA fragmentation	p53, Bax, Bak	Bcl-2, Bcl-X_L
Necroptosis	Rupture	Moderate chromatin condensation	Swelling of cell and cytoplasmic organelles	Reduced ATP levels, activation of RIPK1, RIPK3, and MLKL, release of DAMPS	RIPK1 RIPK3 MLKL	Necrostatin Caspase-8
Ferroptosis	No blebbing or rupture	No chromatin changes and normal size maintained	Small mitochondria with reduction and vanishing of mitochondria cristae	Iron and ROS accumulation, GSH depletion and NADPH oxidation, release of 11-HETE and 15-HETE	VDAC2 Ras NOX TFR1 p53	Gpx4 SLC7A11 Nrf2

Abbreviations: ATG, autophagy protein; MLKL, mixed lineage kinase domain like; RIPK, receptor interacting protein kinase; VDAC2, voltage-dependent anion channel 2.

Other Mechanisms of Cell Death—In the case of necrosis, the common steps outlined above

occur in a somewhat uncontrolled cascade. However, other forms of cell death typically involve a program or more ordered cascade that ultimately triggers irreversible damage. These programs include apoptosis, necroptosis, ferroptosis, and autophagy, and the general morphologic, biochemical, and molecular features of these pathways are summarized in Table 3–4. Though the necrotic cell swells and lyses, the apoptotic cell shrinks with condensation of nuclear and cytoplasmic materials, and cell breakage into membrane-bound fragments (apoptotic bodies) that are phagocytosed.

Apoptosis—A scheme of the apoptotic pathways is presented in Fig. 3–10. A critical first step in apoptosis is the release of mitochondrial cytochrome *c* (cyt *c*) from the mitochondria into the cytoplasm. Cyt *c* is a small, positively charged heme protein that normally resides in the mitochondrial intermembrane space where it is attached electrostatically to cardiolipin. Peroxidation of cardiolipin by HOOH, a process catalyzed by cyt *c*, results in detachment of cyt *c* from the lipid, a critical first step for cyt *c* to be released into the cytoplasm. This release, which adversely affects ATP synthesis in the mitochondria, initiates the apoptotic cascade by causing the cytoplasmic adapter protein apoptotic protease activating factor (Apaf-1) to oligomerize and bind latent procaspase-9, forming a complex called the apoptosome, and facilitating its conversion to active caspase-9.

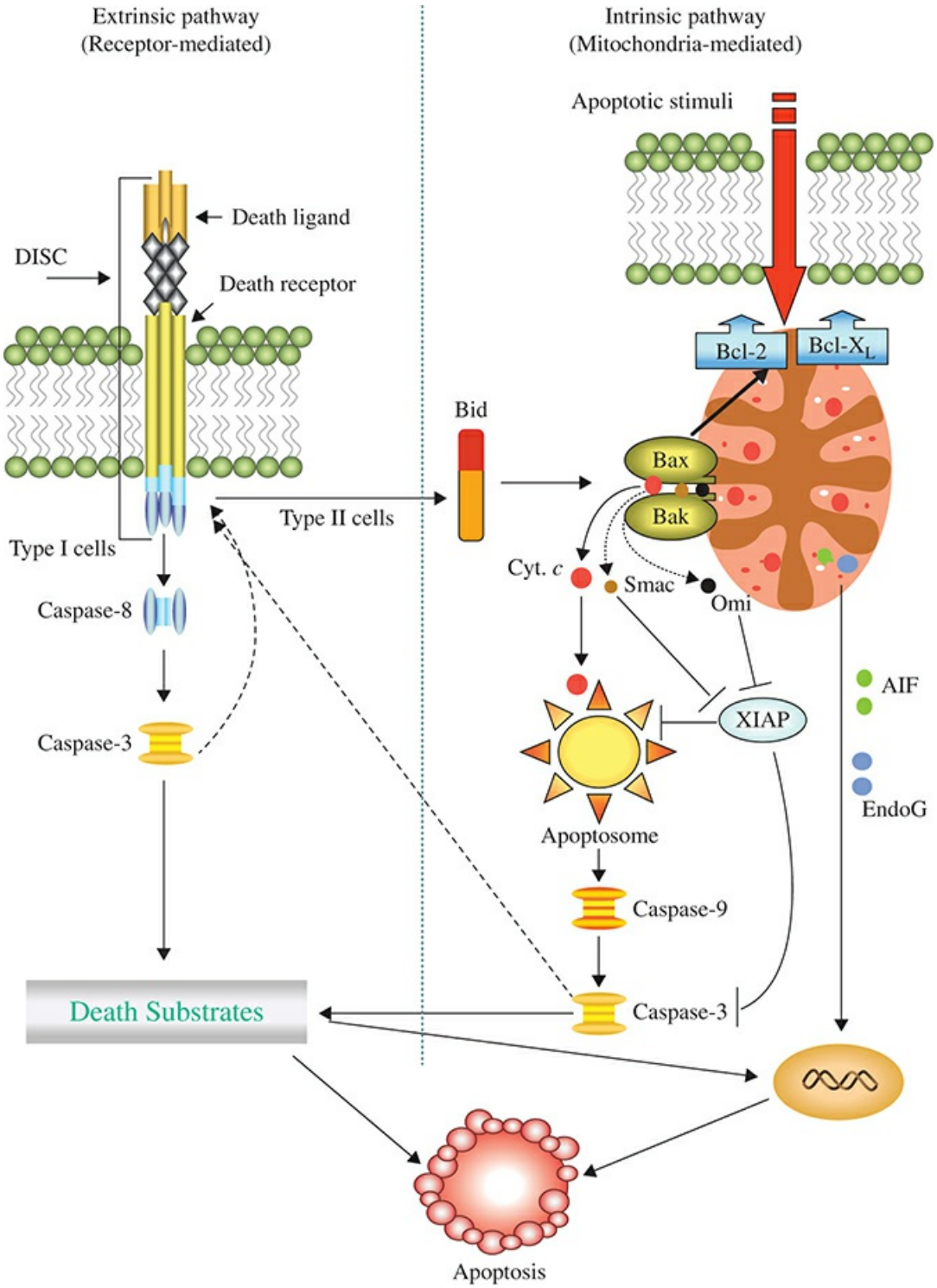

FIGURE 3–10 *The extrinsic and intrinsic pathways of apoptosis.* In the intrinsic pathway (right) mitochondrial-mediated changes open the permeability transition pore spanning both mitochondrial membranes and/or cause release of cytochrome *c* (Cyt *c*) and other proapoptotic and antiapoptotic proteins. Cyt *c* release is facilitated by Bax and opposed by Bcl-2 protein. The released Cyt *c* forms a multi-protein complex known as the apoptosome and initiates activation of the caspase cascade through caspase-9. In the extrinsic pathway, apoptosis is

initiated by receptor activation, and binding of TNF-α to TNF receptor 1 is the major extrinsic mediator of apoptosis. Additionally, activation of the Fas receptor (first apoptosis signal), which binds the Fas ligand (FasL), and TNF-related apoptosis-inducing ligand (TRAIL) receptor also activate the extrinsic pathway. The interaction between Fas and FasL results in the formation of the death-inducing signaling complex (DISC), which contains the Fas-associated death domain protein (FADD), caspase-8, and caspase-10. Caspase-8 is the major caspase involved in the extrinsic apoptotic pathway. The ligand-bound death receptors and the released Cyt *c* interact with specific adapter proteins (i.e., FADD, TRADD, and Apaf-1) through which specific initiator procaspases (PC, e.g., PC-8 and PC-9) become active caspases (C). The latter in turn cleave and activate other proteins, including Bid and the main effector procaspase-3 (PC-3). The active effector caspase-3 activates other effector procaspases (PC-6 and PC-7). Finally, C-3, C-6, and C-7 cleave specific cellular proteins, leading to morphological and biochemical features of apoptosis. (Reprinted with permission from Orrenius et al., Cell death mechanisms and their implications in toxicology. *Toxicol Sci.* 2011;119:3-19.)

Two major pathways that lead to apoptosis in mammalian cells are referred to as the extrinsic and intrinsic pathways (Fig. 3–10), and caspase activation is the hallmark of both pathways. The intrinsic pathway is initiated by signals for cell death acting directly on mitochondria and stimulate the release of proapoptotic proteins including cyt *c*, with subsequent activation of procaspase-9 and activation of caspase-3. The extrinsic pathway is a receptor-mediated pathway in which activation of caspases, particularly procaspase-8, occurs after ligation of membrane receptors (Fas receptor [CD95] or tumor necrosis factor receptor-1 [TNFR1]). Procaspase-8 is activated directly from receptor ligation through formation of the death-inducible signaling complex (DISC), and caspase-8 activates caspase-3, which in turn cleaves target proteins leading to apoptosis. As such, although the intrinsic and extrinsic pathways may be activated by different signals, they converge on caspase-3 activation.

Caspases are cysteine-aspartic proteases with a cysteine in the active site that cleaves target proteins only after an aspartate residue. They reside mostly in the cytoplasm in inactive forms (procaspases), which are activated by either dimerization (initiators) or proteolytic cleavage. Caspase activity is controlled by cytoplasmic proteins termed inhibitors of apoptosis proteins (IAPs). The best characterized IAP is XIAP (X-linked inhibitor of apoptosis), which binds caspase-9, caspase-3, and caspase-7 to inhibit their activation.

Caspases-8 and 9 are initiator caspases that carry the activation wave to the downstream effector caspases (caspases-3, 6, and 7), which cleave specific cellular proteins. It is the hydrolysis of these specific proteins that accounts directly or indirectly for the morphological and biochemical alterations in apoptotic cells.

DNA damage can also activate apoptotic processes through activation of the tumor suppressor gene *p53*. In the face of DNA damage, p53 is translocated to the nucleus where it regulates gene expression in a pleiotropic manner. In the case of apoptosis, p53 increases expression of proapoptotic members of the Bcl-family of proteins. Cytoplasmic p53 can also abrogate the function of antiapoptotic proteins to favor apoptotic cell death. DNA damage can cause mutations and cancer such that apoptotic death in the face of DNA damage is an important self-defense mechanism against cancer. Furthermore, many antitumor drugs damage nuclear DNA and induce apoptosis primarily via a p53-dependent mechanism. At the same time, drug-induced apoptosis is responsible for the cytotoxicity in rapidly dividing cells, which is observed with

cancer chemotherapeutic regimens.

Necroptosis— Another form of programmed cell death is necroptosis, which is an inflammation-related cell death. It is a programmed form of necrosis triggered by ligands of the death receptor family, particularly members of the TNF receptor superfamily, and its development is regulated by receptor-interacting serine/threonine protein kinases 1 and 3 (RIPK1 and RIPK3) and the mixed lineage kinase domain-like protein (MLKL). Necroptosis is distinguished from necrosis in that it is a programmed process and although the cell membrane is disrupted, this process is also tightly regulated (Table 3–4). Furthermore, necroptosis can be initiated by the same death receptors that trigger the extrinsic apoptotic pathway, with a major distinction being that necroptosis occurs when caspase-8 is inhibited. In fact, basal levels of caspase-8 can suppress necroptosis.

Necroptosis is initiated by activation of TNFR1, Fas, and toll-like receptors. In the case of TNFα, binding to TNFR1 initiates assembly of the molecular complex consisting of the TNF receptor–associated death domain (TRADD) along with TNF receptor–associated factor 2 (TRAF2) and cIAP1/2 that is associated with the intracellular domain of TNFR, and this in turn recruits RIPK1 (Fig. 3–11). Ultimately, the kinase activity of RIPK1 recruits and phosphorylates RIPK3 into the signaling complex and this dimer subsequently phosphorylates the pronecroptotic protein MLKL to form the necrosome. Necroptosis is ultimately mediated by the complex of phosphorylated RIPK3 and oligomers of MLKL, with phospho-MLKL the most downstream effector of necroptosis. MLKL induces cell death by inserting into the lipid bilayer of the plasma membrane leading to permeabilization, rupture, and the release of damage-associated molecular patterns (DAMPs) that can elicit immune responses. Activated MLKL can also translocate into intracellular membranes to cause similar rupture of the endoplasmic reticulum, lysosomes, or mitochondria. Although cell death is mediated by MLKL, the activity of RIPK1 is required for necroptosis to occur because necrostatin-1, an endogenous protein that specifically inhibits the kinase activity of RIPK1, can completely block necroptotic cell death.

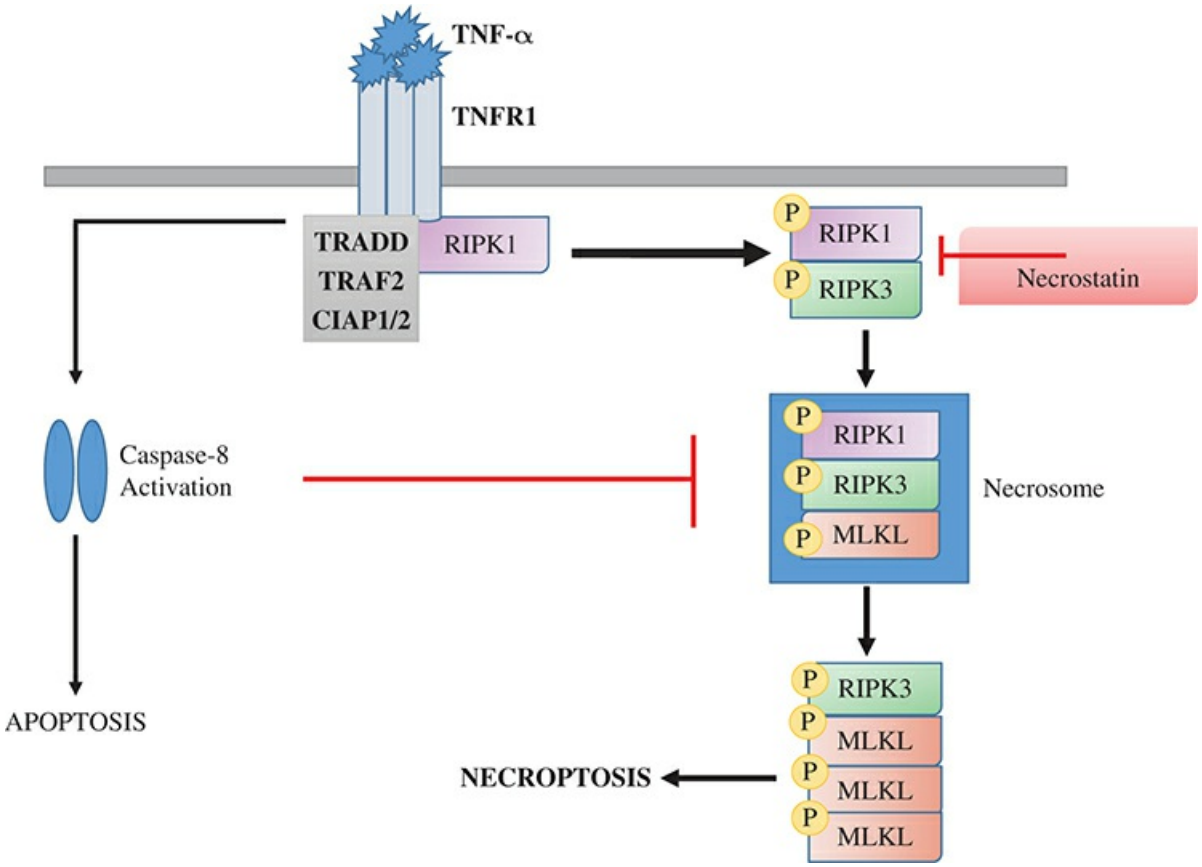

FIGURE 3–11 *The pathway regulating necroptosis.* TNF-α-mediated necroptosis is initiated by binding of TNF-α to TNFR1. Under conditions of reduced or absent caspase-8 activity, phosphorylation of RIPK1 recruits RIPK3, which undergoes auto-phosphorylation, and then recruits and phosphorylates MLKL to form an active complex termed the necrosome. RIPK3 and MLKL ultimately translocate to and destabilize the plasma membrane and initiate necroptotic cell death. Necroptosis has some features of necrosis, but this process causes release of intracellular contents, including damage-associated molecular patterns (DAMPs) that produce pro-inflammatory responses. It is distinguished from apoptosis because it is caspase-independent. The endogenous small molecule necrostatin is an inhibitor of necroptosis. Although a TNFα-mediated pathway is illustrated, other stimuli, including Fas ligand, lipopolysaccharide (LPS), Toll-like receptors (TLRs), and genotoxic stress, can induce necroptosis through the activation of RIPK1. *Abbreviations*: RIPK, receptor-interacting protein kinase; MLKL, mixed lineage kinase domain-like protein.

Ferroptosis—Another recently recognized form of regulated cell death is ferroptosis, a process characterized by the accumulation of lipid peroxidation products and ROS that are derived specifically from iron metabolism. Accordingly, along with activation of the mitogen-activated protein kinase (MAPK) pathway, iron metabolism and lipid peroxidation signaling are the central mediators of ferroptosis. Excessive iron generates ROS by the Fenton reaction (Fig. 3–3), whereas other sources of ROS, including NADPH-dependent lipid peroxidation (Fig. 3–6) and depletion of GSH, contribute to ferroptotic cell death. Ferroptotic cell death is distinguished from necrosis, apoptosis, and necroptosis in that there is no change in nuclear size and no chromatin condensation, nor does the plasma membrane show frank rupture. Rather, ferroptosis is

characterized by small mitochondria with reduction or complete loss of mitochondrial cristae along with rupture of the outer mitochondrial membrane (Table 3–4). Ferroptosis is recognized as a nonapoptotic form of cell death for which toxicants that inhibit glutathione biosynthesis or the glutathione-dependent antioxidant enzyme glutathione peroxidase 4 (GPX4) are integrally involved. This process is also characterized by activation of the mitochondrial voltage-dependent anion channel (VDAC), particularly VDAC2/3, increased activity of NADPH oxidase activity that forms superoxide $(O_2^{\bullet})$, and inhibition of the cysteine/glutamate antiporter (SLC7A11). These three effects act individually or in concert to increase sensitivity to ferroptosis. In contrast, GPX4 activity has been shown to protect against ferroptosis.

What Determines the Form of Cell Death?—The mode of cell death has consequences for the surrounding tissue. Moreover, many toxicants exert metabolic disturbances that involve MPT and progress to apoptotic, necroptotic, and ferroptotic changes. However, in general, the severity of the insult determines the mode of cell death. In particular, it appears that a severe toxic insult causes necrotic cell death rather than apoptosis because it incapacitates the cell to prevent it from a more deliberate form of programmed cell death. This incapacitation may result from the increasing number of mitochondria undergoing MPT, depletion of ATP, and lack of caspase activation.

Lack of ATP can prevent execution of the apoptotic program because apoptosis involves ATP-requiring steps such as activation of procaspase-9 in the apoptosome complex. Failure of caspase activation can also result from direct action of reactive toxicants on these enzymes. The active site of caspases is composed of a pentapeptide with a reactive cysteine in it (QACXG). At high concentrations, soft electrophiles, disulfides (e.g., glutathione disulfide), and oxidants can react with this cysteine, causing caspase inactivation and ablation of the apoptotic program, thereby favoring necrosis as the final outcome. The number of mitochondria undergoing MPT (which probably depends on the degree of chemical exposure) determines the fate of the cell. According to this model, when only a few mitochondria develop MPT, they are removed by selective autophagy (mitophagy; see the section “Cellular Repair”) and the cell survives. When the autophagic mechanism becomes overwhelmed, and proapoptotic factors are released to initiate caspase activation, and when MPT involves virtually all mitochondria, necrosis occurs.

Induction of Cell Death by Unknown Mechanisms—Some toxicants cause cell death by mechanisms other than necrosis or programmed cell death pathways. Chemicals that directly damage the plasma membrane, such as lipid solvents, detergents, and venom-derived hydrolytic enzymes, will cause direct cell rupture. Additionally, xenobiotics that damage the lysosomal membrane lead to tissue degradation by release of lysosomal hydrolases. Other mechanisms of cell death include destruction of the cytoskeleton and toxicants that disrupt protein synthesis.

Impairment of External Cellular Maintenance— Toxicants may also interfere with cells that are specialized to provide support to other cells, tissues, or the whole organism. Chemicals acting on specific organs illustrate this type of toxicity.

STEP 4: INAPPROPRIATE REPAIR AND ADAPTATION

The final steps in the development of toxicity are inappropriate repair and adaptation (Fig. 3–1). Progression of toxic lesions can be intercepted by repair mechanisms operating at molecular, cellular, and tissue levels. Cells and organisms also adapt to toxicity, which renders them more resistant to anticipated adverse effects.

Mechanisms of Repair

Molecular Repair—Damaged molecules may be repaired in numerous ways. Some chemical alterations, such as oxidation of protein thiols and methylation of DNA, are simply reversed. Hydrolytic removal of the damaged unit and insertion of a newly synthesized, correct unit often occur with chemically altered DNA or protein and peroxidized lipids. In some instances, the damaged molecule is totally degraded and resynthesized. This process is time-consuming but unavoidable in cases such as the regeneration of cholinesterase after organophosphate intoxication.

Repair of Proteins: Thiol Groups— Thiol groups are essential for the function of many proteins. Oxidation of protein thiols, protein disulfides, protein–glutathione mixed disulfides, and protein sulfenic acids is reversed by reduction. Thioredoxins and glutaredoxins, small proteins that feature active cysteine residues, are the major endogenous protein-reducing enzymes. There are two isoenzymes of these proteins and thioredoxin reductase that are distinguished by intracellular location (cytosolic and mitochondrial, respectively). When the catalytic thiol groups in these proteins are oxidized, they are reduced by NADPH generated by isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase. ROS may also oxidize methionines in proteins into sulfoxides (protein-Met-S=O), forming both the S and R epimers, which can be reduced by methionine sulfoxide reductase (Msr) A and B enzymes, respectively. Msr enzymes reduce oxidized methionines with concurrent oxidation of their catalytic cysteine to sulfenic acid (Msr-Cys-S-OH), and the modified Msr reacts with a neighboring thiol to form an intramolecular disulfide or with glutathione to form a protein–glutathione disulfide. The activity of the oxidized enzyme is restored by reduction catalyzed by thioredoxin or glutaredoxin.

Repair of Proteins: Heat Shock Chaperones— Soluble intracellular proteins such as cytosolic enzymes are typically folded into a globular form with hydrophobic amino acid residues folded internally, hydrophilic residues located externally, and a ligand (substrate) binding site that resides in a hydrophobic cleft. Physical or chemical insults may evoke an unduly large opening of this cleft, leading to protein denaturation and aggregation. Molecular chaperones such as the heat-shock proteins (Hsp; e.g., Hsp90, Hsp70, and Hsp40) can prevent protein unfolding by "clamping down" onto the exposed hydrophobic region of their client protein, using energy of ATP hydrolysis to execute a conformational change that is required for this maneuver.

Hsp90 and Hsp70 have opposing effects on protein stability. Hsp90 stabilizes proteins and prevents ubiquitination, whereas Hsp70 promotes ubiquitination and proteosomal degradation. Ubiquitin (Ub) is a small (8.6 kDa) protein that directs target proteins to proteosomal degradation when covalently linked to the target. In general, mono-ubiquitination triggers endocytosis or autophagy of the protein, whereas substrates that are tagged with Lys48-linked Ub chains are recognized and degraded by the 26S proteasome into small peptides. In contrast, proteins with Lys63-linked Ub chains are essential components of signaling pathways,

functioning as scaffolds to assemble signaling complexes, as is the case for NF-κB.

Repair of Lipids— Phospholipids containing fatty acid hydroperoxides are preferentially hydrolyzed by phospholipase A2, with the peroxidized fatty acids replaced by normal fatty acids. Peroxidized lipids (e.g., fatty acid hydroperoxides and phospholipid-associated hydroperoxides) may be reduced by the glutathione peroxidase–glutathione–glutathione reductase system or by the peroxiredoxin–thioredoxin–thioredoxin reductase system. NADPH is needed to repair the reductants that are oxidized in the process.

Repair of DNA— Damage to DNA is inevitable, as it occurs spontaneously during replication and it is frequently targeted by electrophiles and free radicals. However, nuclear DNA is remarkably stable, in part because it is packaged tightly in chromatin and because complex repair mechanisms correct adverse alterations. Mitochondrial DNA lacks histones and efficient repair mechanisms and therefore is more prone to damage.

Direct Repair—Certain covalent DNA modifications are directly reversed by enzymes such as DNA photolyase, which cleaves adjacent pyrimidines dimerized by UV light. The enzyme uses the energy of visible light to correct damage, so its function is restricted to light-exposed cells. Minor DNA adducts, such as methyl groups attached to the O^6 position of guanine, are cleaved by O^6-methyguanine-DNA-methyltransferase (MGMT). While repairing the DNA, this alkyltransferase inactivates itself by transferring the adduct onto one of its cysteine residues, after which the enzyme is subjected to ubiquitination and proteasomal degradation.

Excision Repair—Base excision and nucleotide excision are two major mechanisms for removing damaged bases from DNA (see Chapters 8 and 9). Lesions that do not distort the helix typically are removed by base excision, in which the altered base is recognized by DNA glycosylase that hydrolyzes the *N*-glycosidic bond, releasing the modified base and creating an apurinic or apyrimidinic (AP) site in the DNA.

DNA adducts are bulkier lesions that are removed by the nucleotide excision repair system. In this mechanism, proteins that recognize the distorted double helix at the lesion unwind the DNA and excise a number of intact nucleotides on both sides of the lesion along with the one containing the adduct. The excised section of the strand is restored by insertion of nucleotides into the gap of the actions of DNA polymerase and ligase, using the complementary strand as a template. Excision repair has a remarkably low error rate of less than one mistake in 10^9 bases repaired.

PARP (poly-[ADP-ribose] polymerase), another important contributor to excision repair, binds to damaged DNA caused by base damage or single-strand breaks (SSBs) and becomes activated. The active PARP cleaves NAD^+ to use the ADP-ribose moiety of this cofactor for attaching long chains of polymeric ADP-ribose to nuclear proteins, such as histones. The ADP-ribose unit contains two negative charges, so the poly(ADP-ribosyl)ated proteins are also negatively charged, and the resultant electrorepulsive force between the charged proteins and DNA causes decondensation of the chromatin structure. This opens tightly packed chromatin and allows the repair enzymes to access and repair the damaged DNA. Thereafter, poly(ADP-ribose) glycohydrolase gains access to the nucleus from its perinuclear localization and reverses the PARP-mediated modification of nuclear proteins.

Nonhomologous End Joining—This process repairs double-strand breaks (DSBs) that may be

formed when two SSBs occur in close proximity, or when DNA with SSBs undergoes replication. This repair system directly ligates the broken ends without the need for a homologous template. DSBs are recognized by the Ku protein (a heterodimer of Ku70 and Ku80) that binds to the DNA end. Ku then binds and activates DNA-dependent protein kinase catalytic subunit (DNA-PKcs), leading to recruitment and activation of DNA polymerases and DNA ligase.

Recombination (or Postreplication) Repair—Homologous recombination can be used to repair postreplication gaps and DSBs. Postreplication gaps form when excision of a bulky adduct or an intrastrand pyrimidine dimer does not occur before DNA replication. Proliferating cell nuclear antigen (PCNA) is a central player in postreplication repair. PCNA binds to chromatin and slides along the DNA strand to tether the polymerase to the DNA template. PCNA is a platform for a wide variety of proteins that participate in DNA replication and damage. In general, there is a limited role for RNA in DNA repair processes. However, there is emerging evidence that there are novel miRNAs that appear to be derived from the vicinity of DSBs. Moreover, small RNAs, termed DNA damage-induced RNAs (diRNAs), are generated only after DNA damage and are required for the proficient repair of such damage.

Cellular Repair—Autophagic removal of damaged cell organelles may be viewed as a universal mechanism of cellular repair, whereas clearance and regeneration of damaged axons are mechanisms specific for nerve cells. Autophagy is a lysosomal process in which intracellular substrates are degraded within the lysosomal compartment. It is a type of cell death mechanism, but one that is unique in its characteristic presence of cytoplasmic vacuoles resulting from engulfment of cellular components (Table 3–4). Autophagy is a constitutive and rapid process, and most autophagosomes are short-lived, with a half-life of no more than 10 minutes. The clearance of “debris” is an intracellular process, and there is no involvement of other phagocytic cells. Autophagy occurs by the engulfment of cellular constituents (macroautophagy), membrane uptake of smaller amounts of cytoplasm and organelles (microautophagy), or chaperone-mediated events that enable specific protein substrates to be taken into the lysosome for degradation. Autophagy is also an important mechanism for survival in the fasting state.

Autophagy is an important mechanism for removing misfolded proteins from cells resulting from oxidative stress or ER stress. Autophagic removal of damaged cell organelles supports quality control, disposal, and recycling in cells. For example, deletion of depolarized mitochondria occurs through selective autophagy or mitophagy in cells exposed to mitochondrial poisons (see Table 3–3).

Regeneration of Damaged Axons— It is generally recognized that neurons in the central nervous system have limited capacity to regenerate. In contrast, axonal damage in peripheral neurons can be repaired by axonal regeneration, a process that requires macrophages and Schwann cells. Macrophages remove debris by phagocytosis and produce cytokines and growth factors, which activate Schwann cells to proliferate and transdifferentiate to facilitate and support axonal regrowth. Distal to the injury, Schwann cells play an indispensable role in promoting axonal regeneration by increasing their synthesis of cell adhesion molecules (e.g., N-CAM), elaborating proteins for basement membrane reconstruction, and by producing an array of neurotrophic factors (e.g., brain-derived neurotrophic factor, glial cell line–derived neurotrophic factor, and nerve growth factor) and their receptors.

Tissue Repair—In tissues with cells capable of multiplying, damage is reversed by deletion of the injured cells and regeneration of the tissue by proliferation. However, it is important to remove the damaged cells, and often, apoptotic processes contribute. However, apoptosis may support tissue restoration only for tissues that are made up of constantly renewing cells (e.g., the bone marrow, the respiratory and GI epithelium, and the epidermis of the skin), or of conditionally dividing cells (e.g., hepatic and renal parenchymal cells). The role of apoptosis as a tissue repair strategy is markedly lessened in organs containing nonreplicating and nonreplaceable cells, such as the neurons, cardiac muscle cells, and female germ cells.

Proliferation: Regeneration of Tissue— Repair of tissue injury involves regeneration of lost cells, reconstitution of the extracellular matrix (ECM), and reintegration of the newly formed elements. In organs such as liver, kidney, and lung, various types of cells are involved in the process of tissue restoration. Nonparenchymal cells of mesenchymal origin residing in the tissue, such as resident macrophages and endothelial cells, and those migrating to the site of injury, such as monocytes, produce factors that stimulate parenchymal cells to divide and stimulate some specialized cells (e.g., the stellate cells in the liver) to synthesize ECM molecules.

Replacement of Lost Cells by Mitosis—After tissue injury, repair is frequently mediated by cell proliferation. DNA synthesis is increased, and this response is detected experimentally as an increase in the expression of proteins associated with S-phase of the cell cycle.

The regenerative process is initiated by the release of numerous chemical mediators from damaged cells. Nonparenchymal cells, such as resident macrophages and endothelial cells, release cytokines and growth factors that promote and propagate the regenerative process. In the partial hepatectomy model, the initial or priming phase of liver regeneration is controlled by TNF-α and IL-6, whose hepatic mRNA and serum levels increase. TNF, secreted from Kupffer cells, acts on the macrophages in an autocrine manner, activating its receptor and the coupled signal transduction network. This in turn activates NF-κB, which increases IL-6 expression. Secreted IL-6 exerts its action on the hepatocytes and through its receptor (Fig. 3–7) activates Janus kinase (JAK) and induces TFs (e.g., Stat3 and C/EBPβ) to activate several downstream target genes. This cytokine network promotes transition of the quiescent liver cells (G_0) into cell cycle (G_1) and makes them receptive to growth factors (“priming”). Growth factors, especially hepatocyte growth factor (HGF), transforming growth factor-α (TGF-α), and heparin-binding epidermal growth factor–like growth factor (HB-EGF), initiate the progression of the primed cells in the cycle toward mitosis. Despite its name, neither the formation nor the action of HGF is restricted to the liver. It is produced by resident macrophages and endothelial cells of various organs, including liver, lung, and kidney, and in a paracrine manner activates receptors on neighboring parenchymal cells.

Replacement of the Extracellular Matrix—The ECM is a biochemical and structural support composed of a variety of proteins (such as collagen and elastin), proteoglycans (such as chondroitin sulfate), and glycosaminoglycans (such as hyaluronic acid). Each tissue has its own specific ECM composition. Remodeling of the ECM is aided by matrix metalloproteinases, which hydrolyze specific components of the matrix, as well as by tissue inhibitors of matrix metalloproteinases. How tissue regeneration is terminated after repair is unclear, but the gradual dominance of TGF-β, a potent anti-mitogen, is a contributing factor in the termination of cell proliferation.

Other Reactions to Tissue Injury— In addition to mediators that aid in the replacement of lost cells and the ECM, resident macrophages and endothelial cells activated by cell injury produce other mediators that induce ancillary reactions with uncertain benefit or harm to tissues. Such reactions include inflammation, altered production of acute-phase proteins, and generalized reactions such as fever.

Inflammation—Leukocyte Invasion—Alteration of the microcirculation and accumulation of inflammatory cells are largely initiated by resident macrophages secreting cytokines, such as TNF-α and interleukin-1 (IL-1), which increase capillary permeability through release of mediators from endothelial cells and fibroblasts. Activated endothelial cells facilitate the egress of circulating leukocytes into the injured tissue by releasing chemoattractants. A strong interaction develops between intercellular adhesion molecules (ICAM-1) expressed on endothelial cell membranes and integrins expressed on leukocyte membranes. Ultimately, all types of cells near the injury express ICAM-1, thus promoting leukocyte invasion, and the invading leukocytes synthesize additional chemotactic mediators to propagate the inflammatory response.

Inflammation: ROS and RNS Production—Macrophages, as well as leukocytes, recruited to the site of injury undergo a respiratory burst, discharging free radicals and enzymes into the damaged tissue. The respiratory burst is a robust production of ROS in the inflamed tissue, with NADPH oxidase (NOX2) responsible for the production of superoxide anion from NADPH to increase ROS. Nitric oxide synthase (NOS) also contributes to the respiratory burst by catalyzing the formation of the nitric oxide free radical ($^{\bullet}NO$) via oxidation of arginine to citrulline. Finally, myeloperoxidase (MPO) in granulocytes catalyzes for the formation of HOCl, a powerful oxidizing agent, from HOOH and chloride ion. All these reactive intermediates exert beneficial antimicrobial activity at the site of microbial invasion, but because they are so reactive, they can also damage the adjacent healthy tissues and thus contribute to tissue injury.

Altered Protein Synthesis: Acute-Phase Proteins—Interleukins, including IL-6, IL-1, and IL-8 along with TNF-α, alter the transcriptional activity of genes encoding a group of proteins referred to as acute-phase reactants, and these can be increased or decreased (positive and negative acute-phase proteins, respectively. Many of the hepatic acute-phase proteins, such as C-reactive protein and hepcidin, are secreted into the circulation, where elevated levels in serum are diagnostic of tissue injury, inflammation, or neoplasm. Downregulated acute-phase proteins include albumin, transthyretin, transferrin, several forms of cytochrome P450 and glutathione *S*-transferases, ligand-activated TFs (e.g., PPARα and FXR), and many transporters.

Mechanisms of Adaptation

Adaptation is a biological process by which an organism develops increased tolerance to the harm itself. It involves responses acting to preserve or regain the biological homeostasis in the face of increased harm. Theoretically, adaptation to toxicity may result from biological changes causing (1) diminished delivery of the causative chemical(s) to the target, (2) decreased size or susceptibility of the target, (3) increased capacity of the organism to repair itself, and (4) mechanisms to compensate for the toxicant-inflicted dysfunction. Mechanistically, adaptation involves sensing the noxious chemical and/or the initial damage or dysfunction, with a response

that limits the toxicity that typically occurs through altered gene expression.

Adaptation by Decreasing Delivery to the Target—Given that the first step in the development of toxicity is delivery of the ultimate toxicant to the target, some adaptive processes include those that decrease absorption, increase sequestration by intracellular binding proteins, enhance their detoxification, or promote cellular export.

Induction of Ferritin and Metallothionein— Adaptive cellular accumulation of the binding proteins ferritin and metallothionein (MT) is protective against iron and cadmium ions, respectively. Like DMT1, ferritin is regulated by iron regulatory protein 1 in iron overload. However, apoIRP1 acts oppositely on ferritin mRNA to block its translation. Therefore, in iron overload, ferritin mRNA is markedly increased. In this manner, ferritin is protective as it acts to bind and remove Fe^{2+} systemically.

MT is induced by cadmium, and elevated levels of MT protect the liver by restricting distribution of this toxic metal ion to sensitive intracellular targets. Induction of MT by Cd^{2+} is likely mediated by displacement of Zn ions from the protein, which then activate metal-responsive transcription factor 1 (MTF-1) to increase transcription of the MT gene.

The Electrophile Stress Response: Induction of Detoxification— Adaptive increases in detoxification and cellular export have a major role in limiting toxicity. An important contributor to such adaptation is the Keap1-Nrf2 pathway, a cytosolic complex that senses thiol reactivity. The response is initiated by Nrf2 (NF-E2-related factor-2), a TF that activates genes with an electrophile response element (EpRE) in their regulatory region (Fig. 3–12). Normally, Nrf2 is retained in the cytoplasm by Keap1, a cysteine-rich homodimeric protein. Keap1 keeps Nrf2 inactive by anchoring it to the cytoskeleton and maintains low intracellular levels by linking it to cullin 3, a component of E3 ubiquitin ligase that directs it to proteasomal degradation. Many different thiol-sensitive compounds bind to Keap1 at its reactive cysteine thiol groups, thereby forcing Keap1 to release Nrf2. On disruption of the Keap1–Nrf2 complex, active Nrf2 translocates into the nucleus, forms a heterodimer with small Maf proteins, and activates genes through binding to EpREs.

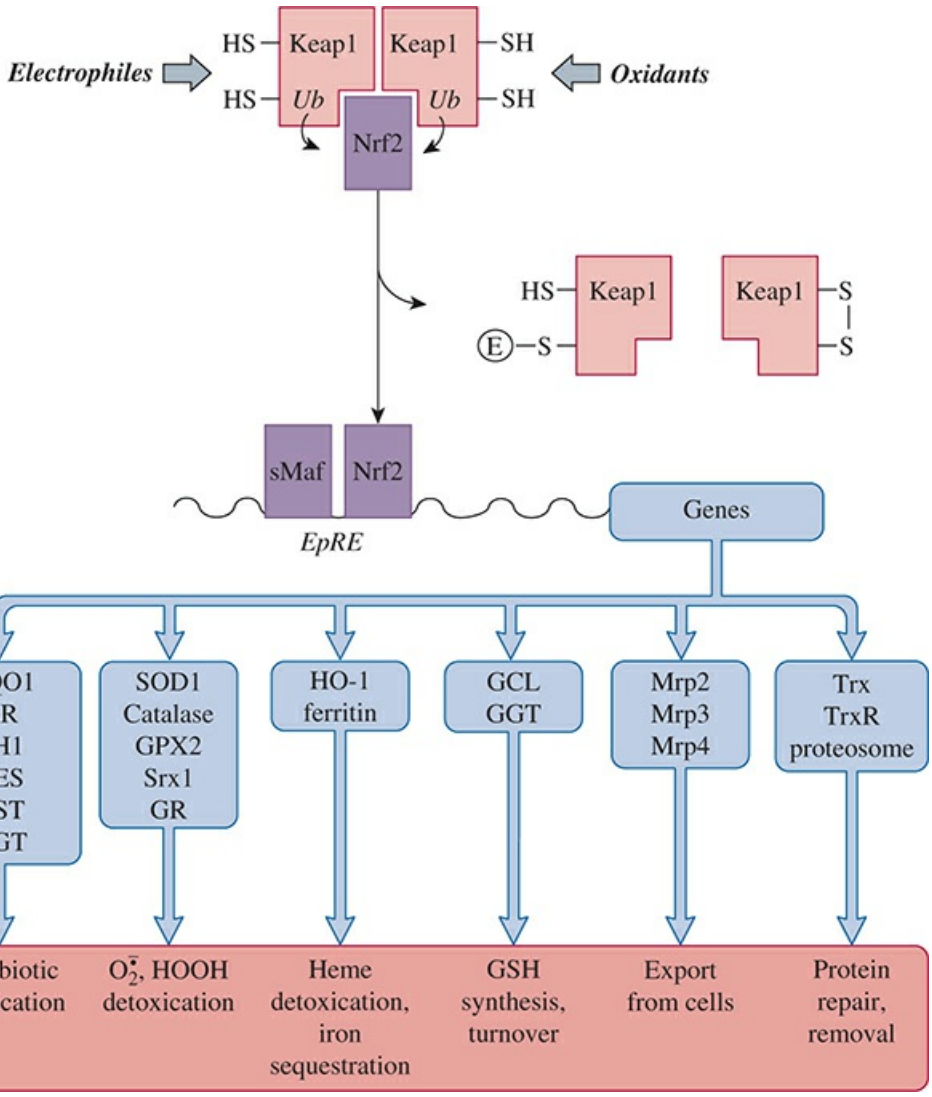

FIGURE 3–12 *Signaling by Keap1/Nrf2 mediates the electrophile stress response.* Normally, NF-E2-related factor 2 (Nrf2) is kept inactive and at a low intracellular level by interacting with Keap1 that promotes its ubiquitination and proteasomal degradation. Electrophiles covalently bind to, whereas oxidants oxidize, the reactive thiol groups of Keap1, causing Keap1 to release Nrf2. Alternatively, Nrf2 release may follow its phosphorylation by protein kinases. After being released from Keap1, active Nrf2 accumulates in the cell, translocates into the nucleus, and forms a heterodimer with small Maf proteins to activate genes that contain electrophile response element (EpRE) in their promoter region. These include enzymes, binding proteins, and transporters functioning in detoxication and elimination of xenobiotics, ROS, and endogenous reactive chemicals, as well as some proteins that can repair or eliminate oxidized proteins (highlighted in blue). Induction of such proteins represents an electrophile stress response that provides protection against a wide range of toxicants. *Abbreviations*: AR, aldose reductase; CES, carboxylesterase; EH1, microsomal epoxide hydrolase; GCL, glutamate–cysteine ligase; GGT, gamma-glutamyl transpeptidase; GPX2, glutathione peroxidase 2; GR, glutathione reductase; GST, glutathione *S*-transferase; HO-1, heme oxygenase 1; NQO1, NAD(P)H:quinone oxidoreductase; Mrp2, Mrp3, and Mrp4,

multidrug resistance protein 2, 3, and 4; SOD1, superoxide dismutase 1; Srx1, sulfiredoxin 1; UGT, UDP-glucuronosyltransferase; Trx, thioredoxin; TrxR, thioredoxin reductase.

There are many genes with EpRE motifs. The broad response is illustrated in Fig. 3–12 and includes (1) enzymes that detoxify xenobiotics, (2) enzymes that eliminate $O_2^{\bullet-}$, (3) proteins that detoxify heme, (4) enzymes involved in the synthesis of GSH and its regeneration from GSSG, and (5) efflux transporters. The list of Nrf2-regulated genes provided here is incomplete.

Adaptation by Decreasing the Target Density or Responsiveness—Decreasing the density and sensitivity of the xenobiotic target is an adaptation mechanism for several receptors. Such alterations underlie the tolerance induced by opioids and drugs of abuse. There are several opioid receptors, but the major receptor for drugs like morphine or heroine is the μ-opioid receptor. This is a G*i* protein–coupled inhibitory receptor, and stimulation by an agonist inhibits adenyl cyclase to decrease levels of cyclic AMP levels and PKA activity. Brief stimulation induces adaptation manifested as receptor downregulation by loss of the receptor from the plasma membrane. Receptor downregulation is mediated by receptor internalization that facilitates lysosomal degradation rather than recycling back to the membrane.

Adaptation by Increasing Repair—There are several repair mechanisms that can be induced after toxicant exposure. Some of these may aid in repairing damaged molecules, proteins, and DNA, others support regeneration of injured tissue.

The Electrophile Stress Response: Induction of Enzymes Repairing Oxidized Protein— Nrf2 activation also induces proteins that mediate protein repair. These include thioredoxin 1 (Trx1) and thioredoxin reductase 1 (TR1), which catalyze reduction of oxidized proteins, and several subunits of the proteasome complex. Trx1 and TR1 are also reduction partners for ribonucleotide reductase, thereby supporting de novo synthesis of deoxyribonucleotides necessary for DNA synthesis. In this manner, induction of Trx1 and TR1 mediate repair of both protein and DNA.

Induction of Chaperones Repairing Misfolded Protein: The Heat-Shock Response— Regulation of these heat-shock proteins to support adaptive responses is mediated by heat-shock transcription factors (HSF), mainly HSF1, which transactivate genes that encode Hsp through heat-shock response elements (HSE). HSF1, like Nrf2, normally resides in the cytoplasm, where it associates with Hsp90, Hsp70, and Hsp40. On heat- or chemical-induced protein damage, Hsps are sequestered by damaged proteins, allowing HSF1 to translocate to the nucleus, where it forms a trimer and is phosphorylated. The chaperones Hsp90 and Hsp70, together with co-chaperone proteins, are important in maintaining the integrity of hundreds of proteins including those performing housekeeping functions, signaling and apoptosis. Therefore, induction of Hsps has pleiotropic effects beyond increased protection from cytotoxicity.

Endoplasmic Reticulum Stress and the Unfolded Protein Response— The endoplasmic reticulum (ER) is the primary site where transmembrane and secretory proteins are folded. In this Ca^{2+}-rich, oxidative environment, protein modifications including *N*-glycosylation at asparagine residues, formation of disulfide bonds, and folding assisted by EPR-resident chaperones occur. When there is damage to proteins being processed in the EPR by reactive metabolites generation of ROS, and/or depletion of Ca^{2+} in the EPR lumen, there is accumulation of unfolded or misfolded proteins, a condition known as EPR stress. EPR stress can be triggered by high

secretory activity, conditions that perturb protein maturation, calcium homeostasis, redox balance, or induced by compounds that disturb different aspects of EPR homeostasis (Table 3–3). When the load of unfolded or misfolded proteins in the EPR exceeds the capacity of EPR-resident chaperones, a complex adaptive response called the unfolded protein response (UPR) reduces the load of unfolded proteins and restores EPR homeostasis.

In the adaptive mode, UPR includes mechanisms to (1) reduce mRNA translation to decrease the functional load on the EPR, (2) increase transcription of EPR chaperones to boost the folding capacity, and (3) remove aberrant proteins via translocon peptide channels into the cytosol for proteasomal degradation. The final option is to eliminate the affected cell via apoptosis.

Induction of Enzymes Repairing DNA: The DNA Damage Response— Maintaining the fidelity of DNA is critical to cell survival, and DNA damage initiates numerous pathways for repair. The tumor suppressor p53, regarded as the "guardian of the genome," is also critical to DNA repair processes. Normally, p53 is kept inactive and at low levels in the cytoplasm by binding protein mdm2 (mouse double minute homolog 2), an E3 ubiquitin ligase that ubiquitinates p53 and facilitates its proteasomal degradation. However, it is activated by several kinase pathways that signal in response to double-stranded DNA breaks.

Adaptive Increase in Proliferative Responses to Promote Tissue Repair— In association with toxic injury, there is often a resulting signaling cascade to initiate tissue repair via cell replacement. Phosphatases such as PTP and PTEN, which normally serve as brakes on growth factor receptor-initiated mitogenic signaling, play an active role (refer to Fig. 3–7). These enzymes, which contain reactive cysteine thiols in their active sites, are covalently modified and thereby inactivated by electrophiles. In this manner, the loss of their inhibitory activity on proliferative signaling cascades serves to amplify intracellular signaling for mitosis and survival.

NF-κB is also a converging pathway for proliferative signaling, as it transactivates genes producing cell cycle acceleration (e.g., cyclin D1 and c-Myc) and inhibitors of apoptosis (e.g., antiapoptotic Bcl proteins and the caspase IAPs). NF-κB also transactivates ferritin, GST, SOD1, HO-1, a proteasome subunit, and gadd45, facilitating detoxification and molecular repair.

Proliferating cells also need to increase protein synthesis, a process controlled by the protein kinase mTOR (mammalian target of rapamycin). mTOR, a major regulator of general metabolic activity, is activated by the MAPK pathway through phosphorylation of the MAPK isoform Erk and by the PI3K pathway through phosphorylation of Akt (Fig. 3–7). These pathways are also activated in response to oxidant or electrophile exposure as they are held in check by controlled PTPs and PTEN such that inhibition of these phosphatases increases signaling through Erk and Akt. In the cascade, Erk and Akt activate mTOR, which phosphorylates and regulates effectors of protein synthesis, such as the translation repressor protein 4EBP1 and the protein kinase S6K, which modifies ribosomes increasing their translational efficiency. In contrast to its activation during cell proliferation, it is shutdown in conditions such as hypoxia to reduce ATP synthesis.

Adaptation to Hypoxia: The Hypoxia Response— Hypoxia-inducible factor 1α (HIF-1α) is maintained at very low constitutive intracellular levels because of continuous hydroxylation of its two proline residues by HIF-prolyl hydroxylases. Hydroxylation of the proline residues permits a ubiquitin ligase subunit (von Hippel Lindau protein [VHL]) to initiate proteasomal degradation. When O_2 delivery is impaired and hypoxia persists for more than a few minutes, HIF-1α increases markedly. Moreover, HIF hydroxylases have a K*M* for O_2 that is very close to

ambient O_2 concentration, so that when the O_2 concentration falls, there is decreased hydroxylation of HIF-1α as well as its VHL-mediated ubiquitination for proteasomal degradation, which increase the abundance and transcriptional activity of HIF-1α.

In hypoxic conditions, HIF-1α dimerizes with HIF-1β (also called Arnt, which is the dimerization partner for AhR). The HIF complex transactivates a vast array of genes containing the hypoxia response element (HRE). These include (1) erythropoietin (EPO) produced largely in kidney to activate erythropoiesis in bone marrow, (2) proteins involved in iron homeostasis (e.g., transferrin, TFR, ceruloplasmin, and heme oxygenase) to increase availability of iron for erythropoiesis, (3) vascular endothelial growth factor (VEGF) and angiopoietin-2 to stimulate blood vessel growth, (4) proteins facilitating glycolysis including the glucose transporter GLUT1 and some glycolytic enzymes, (5) proteins that correct acidosis caused by glycolytic overproduction of lactate (e.g., a monocarboxylate transporter and a Na^+/H^+ exchanger for export of lactate and H^+), (6) the REDD1 signal transducer protein that initiates a complex signaling pathway that leads to suspension of the ATP-consuming protein synthesis via inactivation of mTOR, and (7) a generalized response to promote ECM remodeling (e.g., matrix metalloproteinase-2) and cell migration and to induce autophagy and/or apoptosis.

The Energy Stress Response to Adapt to Energy Depletion— Cells try to maintain ATP levels at all times. When the ratio of AMP to ATP increases, AMP-activated protein kinase (AMPK) responds to boost ATP production and limit ATP consumption. The kinase is strongly activated by AMP, and it is phosphorylated by protein kinase LKB1 (or by calmodulin-dependent protein kinase kinase [CaMKK] in neurons). To increase ATP levels AMPK increases (a) glucose uptake (via glucose transporters GLUT4 and GLUT1), (b) glycolysis (via phosphorylation and activation of 6-phosphofructo-2-kinase [PFK-2]), and (c) fatty acid oxidation in mitochondria (via phosphorylation and inactivation of acetyl-CoA-carboxylase). Simultaneously, AMPK reduces ATP consumption by broadly inhibiting (a) glycogen synthesis (by phosphorylation and inactivation of glycogen synthase), (b) lipid synthesis (by phosphorylating and inactivating acetyl-CoA-carboxylase), (c) cholesterol synthesis (by phosphorylating and inactivating HMG-CoA reductase), (d) glucose synthesis (via decreased expression of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase), and (e) protein synthesis (by inhibiting mTOR). AMPK-mediated modulation of cellular energy supply and consumption occurs largely through kinase-mediated phosphorylation reactions rather than transcriptional regulation, rendering this pathway a rapid, adaptive response.

In addition to the rapid AMPK-dependent reprogramming of cellular intermediary metabolism, cells may consume cellular constituents (lipid droplets, glycogen particles, proteins, and organelles) by nonselective or bulk autophagy with lysosomal hydrolysis providing essential metabolic intermediates (amino acids, fatty acids, nucleosides, and carbohydrates). When nutrients are abundant, mTOR phosphorylates and inactivates ULK-1 and Atg13, thereby inhibiting autophagy. In conditions of nutrient shortage, mTOR is inactivated, which releases inhibition of the ULK-1–Atg13–FIP200 complex, and initiates autophagosome formation. The important role that mTOR serves in switching autophagy on and off is further evidence of its role as key regulator of cellular energy homeostasis.

Final and Irreversible Actions of Repair and Adaptation Failure

The most important consequences of inappropriate repair are irreversible organ failure, as is the

case with necrosis or tissue changes that advance to fibrosis or carcinogenesis.

When Repair Fails—Although repair mechanisms operate at molecular, cellular, and tissue levels, they can fail to provide protection against injury. Toxicity is manifested when repair of the initial injury fails because the repair mechanisms become overwhelmed, exhausted, or impaired. Sometimes toxicant-induced injury adversely affects the repair process itself. Also, repair may contribute to toxicity.

When Adaptation Fails—Moderate oxidative stress activates NF-κB, AP-1, and Nrf2 to initiate adaptive protection. However, extensive oxidant exposure aborts these programs because it leads to oxidation of thiol groups in the DNA-binding domain of these TFs. Similarly, some otherwise adaptive and protective mechanisms may be harmful under extreme conditions. It is also possible that an adaptive mechanism that is beneficial in the short term may become harmful when forced to operate for a prolonged time.

Toxicity Resulting from Inappropriate Repair and Adaptation

Failure to repair involves molecular, cellular, and tissue dysfunction and can show species- and age-related differences. Some toxicities involve dysrepair at an isolated level. Necrosis, fibrosis, and carcinogenesis may involve inappropriate repair and adaptation.

Tissue Necrosis—Although cell death ensues when repair mechanisms are inefficient or the molecular damage is not readily reversible, progression to tissue necrosis can be interrupted by apoptosis and cell proliferation. Injured cells can initiate apoptosis, which counteracts the progression of the toxic injury. Apoptosis does this by preventing necrosis of injured cells and the consequent inflammatory response, which may cause additional injury by releasing cytotoxic mediators. Cell proliferation also serves to negate the loss of organ function when necrosis occurs. Early cell division is instrumental in the rapid and complete restoration of injured tissue and the prevention of necrosis. The dose–response relationship that describes toxicant-induced injury also contributes to the dose response for irreversible injury. In general, tissue repair increases up to a threshold dose that can limit or prevent irreversible injury. However, once that threshold is reached, toxicity and cell death progress rapidly.

Fibrosis

Fibrosis is characterized by excessive formation and deposition of connective tissue with abnormal composition of the ECM. In general, fibrotic conditions can develop in any organ, but liver, lung, kidney and heart are noteworthy. Physiologically, fibrosis is an exaggerated wound healing response which interferes with normal organ architecture and function. The epithelial-mesenchymal transition (EMT) is characterized by the phenotypic transformation of an epithelial cell into a fibroblast or myofibroblast. Although EMT is necessary for organ development and wound healing, it is uncontrolled in fibrosis, and cells that manufacture the ECM during tissue repair overproduce the matrix in an uncontrolled manner.

As an end-stage disease, fibrosis is detrimental because (1) scars can compress and ultimately obliterate parenchymal cells and blood vessels; (2) deposition of basement membrane components between the capillary endothelial cells and the parenchymal cells presents a

diffusional barrier that contributes to malnutrition of the tissue cells; (3) an increased amount of ECM alters the elasticity and flexibility of the whole tissue, compromising the mechanical function of organs such as the heart and lungs, and (4) fibrotic disease can change basic aspects of cell behavior, including cell polarity, motility, and gene expression.

TGF-β is a central mediator of fibrotic disease, but additional factors including connective tissue growth factor (CTGF), platelet-derived growth factor (PDGF), and IL-4 also contribute to the process. The fibrotic action of TGF-β is due to increased production and decreased degradation of ECM components. There are also changes to the composition of the ECM in fibrotic diseases. Basement membrane components, such as collagen IV and laminin, as well as the fibrillar-type collagens (collagens I and III), which confer rigidity to tissues, increase disproportionately during fibrogenesis. miRNAs contribute to fibrosis by affecting the EMT, and numerous miRNAs contribute to this process. Three major miRNAs involved in fibrotic disease are miR-21, miR-29, and miR-200.

Carcinogenesis

A central feature of carcinogenic mechanisms is altered expression of proteins that function as proto-oncogenes and/or suppressor proteins (see Chapter 8). These important proteins can be altered by mutation resulting from direct DNA damage or by altered transcription leading to overexpression of proto-oncogenes or decreased expression of tumor suppressors. Also, DNA-reactive or genotoxic mechanisms bring about a *qualitative* change in gene expression, with expression of an altered gene product, a mutant protein or miRNA, and gain or loss in activity. In contrast, epigenetic mechanisms are non-DNA reactive (or non-genotoxic) and do not alter gene sequence, but rather, cause *quantitative* change in gene expression resulting in more or less gene product. Regardless of mechanism, cancer ultimately develops from cellular failures in apoptosis and/or from uncontrolled cell proliferation.

Genotoxic Mechanisms of Carcinogenesis— Chemicals that react with DNA may cause damage such as adduct formation, oxidative alteration, and strand breakage. As described above, these lesions are repaired or the injured cells are eliminated. If neither event occurs, a lesion in the parental DNA strand may induce a mutation in the daughter strand during replication. The mutation may remain silent if it does not alter the protein encoded by the mutant gene or if the mutation causes an amino acid substitution that does not affect the function of the protein. Alternatively, the genetic alteration may be incompatible with cell survival. The worst scenario occurs when the altered genes express mutant proteins that reprogram cells for multiplication and escaping apoptosis (i.e., immortalization). This trait is passed to daughter cells during mitosis, enabling sustained proliferation. Moreover, enhanced DNA replication and cell division increases the likelihood of additional mutations that may further augment their growth advantage. Ultimately, tumors arise from the nidus of rapidly proliferating transformed cells.

Mutation of Proto-oncogenes—Proto-oncogenes are highly conserved genes that encode proteins that stimulate the progression of cells through the cell cycle or oppose apoptosis. Under normal conditions, these important proteins are required for regulating growth, with essential roles in supporting embryogenesis, tissue regeneration, and growth factor responses. Mutations in proto-oncogenes can cause permanent activation and/or overexpression of these proteins to favor neoplastic transformation. The altered gene is then referred to as an oncogene. Figure 3–13

depicts several proto-oncogene products that are involved in initiating the cell division cycle.

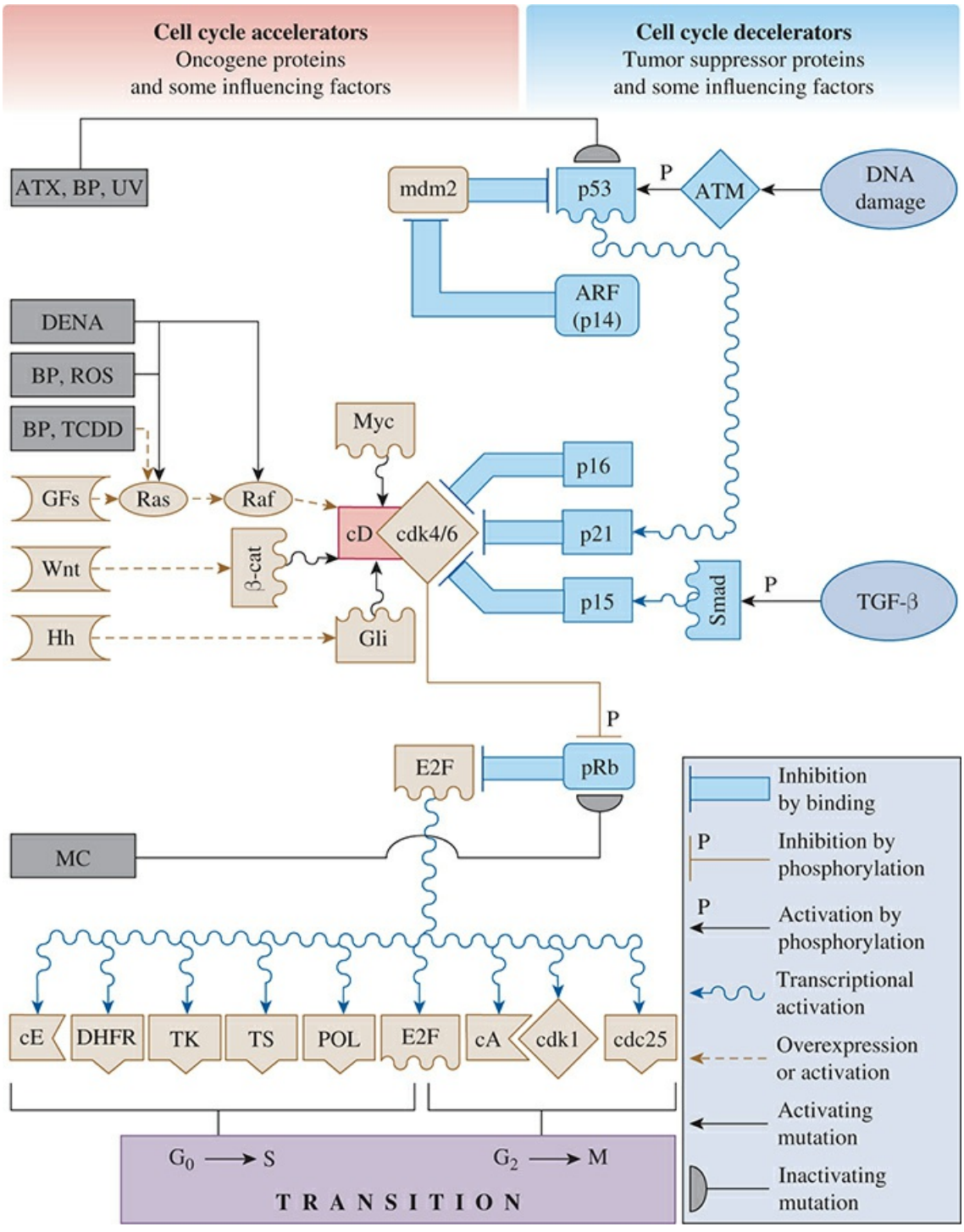

FIGURE 3–13 *Key regulatory proteins controlling the cell division cycle along with relevant effects in the signaling cascades that regulate the cell cycle.* The left side of the figure highlights proteins that accelerate the cell cycle and are oncogenic if permanently active or expressed at high level. In contrast, proteins on the right, represented by blue symbols, decelerate or arrest the cell cycle to suppress oncogenesis, unless they are inactivated (e.g., by

mutation). Expression of cyclin D (cD) is increased, for example, by growth factors signaling through Ras proteins and the MAPK pathway as well as by Wnt and Hedgehog (Hh) ligands that ultimately signal through β-cat and Gli transcription factors, respectively. Carcinogens such as benzo[a]pyrene (BP), reactive oxygen species (ROS), and diethylnitrosamine (DENA) may cause mutation of the Ras or Raf gene that results in permanently active mutant proteins.

An example of mutational activation of an oncogene is the Ras family of proteins (H-Ras, K-Ras, and N-Ras). Ras proteins are G-coupled proteins with GTP/GDP-binding capacity as well as GTPase activity. Ras is activated downstream from growth factor receptors and protein tyrosine kinases and upstream from the mitogen-activated protein kinase (MAPK) cascade (Fig. 3–7). Ras functions as a molecular switch that is only active in the GTP-bound form. Mutations in Ras genes lock the protein in the permanently active GTP-bound form to drive uncontrolled cell division and transformation (Fig. 3–13). Activating mutations in the proto-oncogene b-Raf is another important example of genotoxic mechanisms of carcinogenesis. Raf proteins are protein kinases just downstream from Ras and are the first signal transducers in the MAP kinase pathway. While constitutive activation of oncogenes is a common initiator of chemical carcinogenesis, overexpression resulting from gene amplification also can contribute to neoplastic cell transformation. Such an event can occur spontaneously or may be initiated by DNA strand breaks.

Mutation of Tumor Suppressor Genes—Tumor suppressor genes encode proteins that inhibit the cell cycle progression or promote DNA repair or apoptosis when there is DNA damage. Figure 3–13 depicts such proteins, which include cyclin-dependent protein kinase inhibitors (e.g., p15, p16, and p21), TFs including p53 and Smads, ATM and ATR that sense DNA damage to signal p53 activation, BRCA1 and BRCA2 that contribute to recombinational DNA repair, PTEN phosphatase that turns off the PI3K–Akt pathway-mediated proliferative signaling, and the tuberous sclerosis complex-2 (TSC2) that prevents activation of mTOR. Uncontrolled proliferation occurs when the mutant tumor suppressor gene can no longer suppress cell division.

Non-genotoxic Mechanisms in Carcinogenesis—Non-genotoxic carcinogens often stimulate sustained cell proliferation. This can occur by direct activity on proliferative signaling, or be evoked by endogenous mitogens and hormones.

Role of Signaling in Carcinogenesis—Cell injury may evoke the release of mitogenic growth factors such as HGF and TGF-α from tissue macrophages and endothelial cells. Although these growth factors are instrumental in tissue repair after acute cell injury, their continuous presence is potentially harmful because they may ultimately transform affected cells into neoplastic cells.

Role of DNA Methylation and Histone Modification in Carcinogenesis—Some non-genotoxic carcinogens alter DNA methylation. Promoter methylation silences genes by weakening TF binding to the promoter and triggering secondary alterations in histone proteins, which renders the histone more compact, thereby reducing access of TFs and other proteins involved in transcription initiation at the gene promoter. It is well documented that the normal methylation pattern of DNA is disrupted in cancer cells. Both global hypomethylation and tumor suppressor gene hypermethylation intensify with increased malignancy of the tumor.

Role of MicroRNAs in Non-genotoxic Mechanisms of Carcinogenesis—miRNAs can affect

carcinogenic mechanisms through effects on DNA repair as well as on pathways considered to be non-genotoxic. The miR-17-92 cluster is considered oncogenic because its members promote mitosis by repressing the translation of PTEN and p21, and they inhibit apoptosis by repressing the proapoptotic protein Bim.

Genotoxic and non-genotoxic mechanisms of carcinogenesis may not be mutually exclusive, they can complement or amplify each other. Increased mitotic activity, regardless of mechanism or pathway involved, increases the likelihood of carcinogenicity for two major reasons. First, increased mitotic activity increases the probability that mutations may occur because the shortened G_1 phase allows less time for DNA repair. Although repair still may be feasible after replication, postreplication repair is error-prone. Second, increased proliferation enables clonal expansion of the initiated cells, and this facilitated growth contributes to tumor formation and progression. Thus, epigenetic alterations evoked by genotoxic carcinogens may contribute to carcinogenesis after the initiating genotoxic event. Conversely, global DNA hypomethylation and tumor suppressor gene hypermethylation may increase the occurrence of mutations in cells exposed to non-genotoxic carcinogens.

Failure to Execute Apoptosis Promotes Mutation and Clonal Growth—In many cases, initiated preneoplastic cells have much higher apoptotic activity than do normal cells, a mechanism designed to counteract clonal expansion. It follows then that inhibition of apoptosis is detrimental because it facilitates the fixing of mutations and allows for clonal expansion of preneoplastic cells.

CONCLUSION

Mechanisms of Toxicity and Adverse Outcome Pathways

A “real world” application of mechanisms of toxicity is the development of adverse outcome pathways (AOPs) to inform and facilitate toxicity testing and hazard identification. Basically, an AOP is a systematic approach that organizes and integrates a series of biological events that are likely to lead to an adverse outcome. AOPs place emphasis on the key events, often called molecular initiating events that link a causal chain of endpoints to the final toxicity. These events are also organized into biological levels ranging from macromolecular effects to those observed at the cellular, organ, and organism level. The AOP approach integrates existing knowledge on chemical mode of action, starting with a molecular initiating event and continuing through numerous key events that culminate in a toxic effect. Additionally, integration of mechanisms of toxicity into AOPs may be beneficial for prioritizing compounds for more defined toxicity testing in animals or as a stand-alone system for predicting toxicity. Currently, the proposed applications and utility of AOPs focus on their potential to (1) advance the use of in vitro, nonanimal methods in safety evaluation; (2) extrapolate across species, with emphasis on increasing confidence of human safety; and (3) identify potential mechanism-based biomarkers of toxicity. While these approaches may bring more integrated systems biology approaches to assessing mechanisms of toxicity, the tools must be able to broadly integrate all aspects of toxic mechanisms presented in this chapter. That is, a useful tool will have to encompass the broad aspects of chemical disposition and biotransformation, and inform quantitative relationships describing when cellular protective mechanisms are overwhelmed, thereby initiating or

propagating the chain of key events that lead to toxicity. The application of AOPs in mechanistic and predictive toxicology is an emerging area, but it is not yet clear whether, and if so how, these approaches will advance the application of mechanism of toxicity into hazard identification and risk assessment.

The Importance of Mechanisms of Toxicity

In this chapter, a simplified scheme has been used as an overview of the development of toxicity (Fig. 3–1 A and B), and the potential events that follow toxicant exposure and contribute to toxicity have been presented. In completing the chapter, it should be readily apparent that the route to toxicity can be considerably diverse and complicated, as described by the many examples throughout the chapter and by the comprehensive case study of d-limonene. This chapter is a compendium of the fundamental processes that determine toxicity and provides the foundation for more in-depth understanding of specific toxicities that are discussed in detail throughout other chapters. The basic concepts of any toxic mechanism are essential to identifying key events associated with toxic outcome and inform species differences in toxicity, with particular emphasis on informing the human relevance of toxicity. Moreover, understanding mechanisms of toxicity provides opportunities and direction to identifying biomarkers of toxicity, tools to predict toxicity, and potential ways to mitigate adverse effects.

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QUESTIONS

1. The severity of a toxicant depends, in large part, on the concentration of the toxicant at its site of action. Which of the following will decrease the amount of toxicant reaching its site

of action?

a. absorption across the skin.
b. excretion via the kidneys.
c. toxication.
d. reabsorption across the intestinal mucosa.
e. discontinuous endothelial cells of hepatic sinusoids.

2. Toxication (or metabolic activation) is the biotransformation of a toxicant to a more toxic and reactive species. Which of the following is not a reactive chemical species commonly formed by toxication?

a. electrophiles.
b. nucleophiles.
c. superoxide anions.
d. hydroxy radicals.
e. hydrophilic organic acids.

3. Which of the following is not an important step in detoxication of chemicals?

a. formation of redox-active reactants.
b. reduction of hydrogen peroxide by glutathione peroxidase.
c. formation of hydrogen peroxide by superoxide dismutase.
d. reduction of glutathione disulfide (GSSG) by glutathione reductase (GR).
e. conversion of hydrogen peroxide to water and molecular oxygen by catalase.

4. Regarding the interaction of the ultimate toxicant with its target molecule, which of the following is false?

a. Toxicants often oxidize or reduce their target molecules, resulting in the formation of a harmful by-product.
b. The covalent binding of a toxicant with its target molecule permanently alters the target's function.
c. The noncovalent binding of a toxicant to an ion channel irreversibly inhibits ion flux through the channel.
d. Abstraction of hydrogen atoms from endogenous compounds by free radicals can result in the formation of DNA adducts.
e. Several toxicants can act enzymatically on their specific target proteins.

5. All of the following are common effects of toxicants on target molecules EXCEPT:

a. blockage of neurotransmitter receptors.
b. interference with DNA replication due to adduct formation.
c. cross-linking of endogenous molecules.
d. opening of ion channels.
e. mounting of an immune response.

6. Which of the following proteins functions to prevent the progression of the cell cycle?

a. NF-κB.

b. MAPK.
c. CREB.
d. c-Myc.
e. IκB.

7. Which of the following would have the largest negative impact on intracellular ATP levels?
   a. moderately decreased caloric intake.
   b. interference with electron delivery to the electron transport chain.
   c. inability to harvest ATP from glycolysis.
   d. increased synthesis of biomolecules.
   e. active cell division.

8. What happens when a toxicant induces elevation of cytoplasmic calcium levels?
   a. Mitochondrial uptake of calcium dissipates the electrochemical gradient needed to synthesize ATP.
   b. Formation of actin filaments increases the strength and integrity of the cytoskeleton.
   c. It decreases the activity of intracellular proteases, nucleases, and phospholipases.
   d. The cell becomes dormant until the calcium is actively pumped from the cell.
   e. The generation of reactive oxygen species slows because of calcium-induced decrease in activity of the TCA cycle.

9. Cytochrome *c* is an important molecule in initiating apoptosis in cells. All of the following regarding cytochrome *c* are true EXCEPT:
   a. The release of cytochrome *c* into the cytoplasm is an important step in apoptosis initiation.
   b. The loss of cytochrome *c* from the electron transport chain blocks ATP synthesis by oxidative phosphorylation.
   c. Loss of cytochrome *c* from the inner mitochondrial membrane results in increased formation of reactive oxygen species.
   d. Bax proteins mediate cytochrome *c* release.
   e. Caspases are proteases that increase cytoplasmic levels of cytochrome *c*.

10. All of the following regarding DNA repair are true EXCEPT:
   a. In a lesion that does not cause a major distortion of the double helix, the incorrect base is cleaved and the correct base is inserted in its place.
   b. Base excision repair and nucleotide excision repair are both dependent on a DNA polymerase and a DNA ligase.
   c. In nucleotide excision repair, only the adduct is cleaved, and the gap is then filled by DNA polymerase.
   d. Pyrimidine dimers can be cleaved and repaired directly by DNA photolyase.
   e. Recombinational repair requires that a sister strand serves as a template to fill in missing nucleotides.

11. Apoptosis can serve as a tissue repair process in a number of cell types. In which of the

following cell types would this be a plausible mechanism of tissue repair?

a. female germ cells.
b. gastrointestinal epithelium.
c. neurons.
d. retinal ganglion cells.
e. cardiac muscle cells.

12. Which of the following is NOT associated with carcinogenesis?

a. mutation.
b. normal p53 function.
c. Ras activation.
d. inhibition of apoptosis.
e. DNA repair failure.

CHAPTER 4

Risk Assessment

Elaine M. Faustman

KEY POINTS

- *Risk assessment* is the systematic scientific characterization of potential adverse health effects resulting from human exposures to hazardous agents or situations.
- *Risk* is defined as the probability of an adverse outcome under specified conditions.
- *Risk management* refers to the process by which policy actions are chosen to control hazards.

INTRODUCTION AND HISTORICAL CONTEXT

Toxicologic research and toxicity testing conducted and interpreted by toxicologists constitute the scientific core of an important activity known as *risk assessment*. In 1983, the National Research Council detailed the steps of hazard identification, dose–response assessment, exposure analysis, and characterization of risks in *Risk Assessment in the Federal Government: Managing the Process* (widely known as *The Red Book*). The scheme shown in Figure 4–1 provides a consistent framework for risk assessment across agencies with bidirectional arrows showing an ideal situation where mechanistic research feeds directly into assessments and critical data uncertainty drives research. Often, public policy objectives require extrapolations that go far beyond the observation of actual effects and reflect different tolerances for risks, generating controversy.

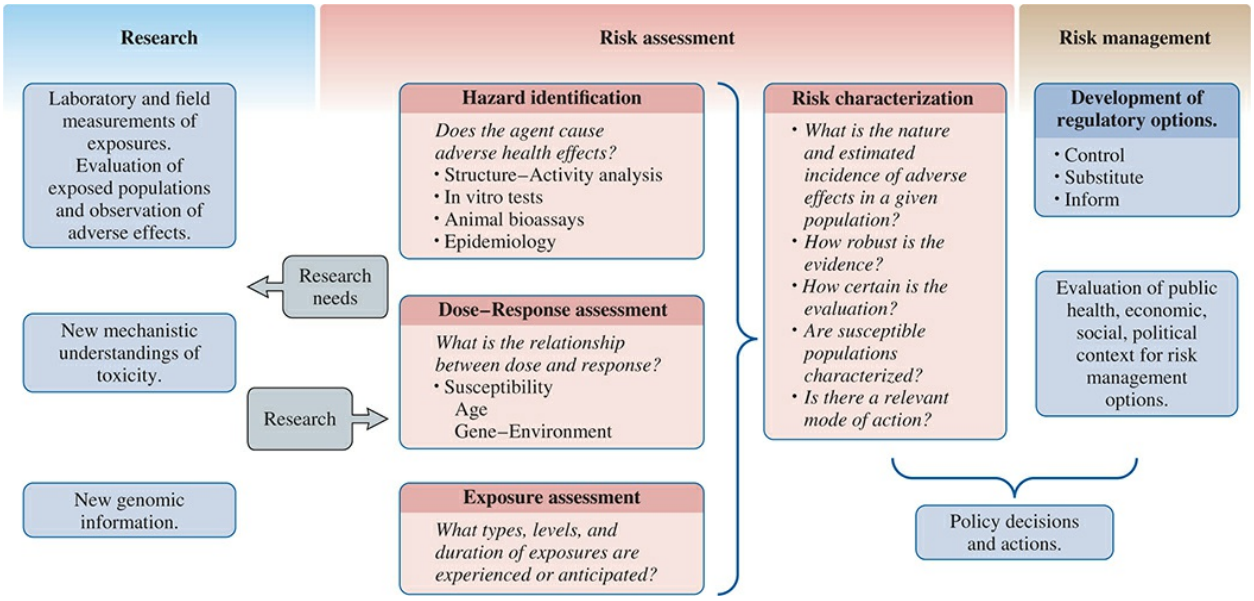

FIGURE 4–1 *Risk assessment/risk management framework.* This framework shows, under the red highlight, the four key steps of risk assessment: hazard identification, dose–response assessment, exposure assessment, and risk characterization. It shows an interactive, two-way process where research needs from the risk assessment process drive new research, and new research findings modify risk assessment outcomes. At the start of the risk assessment process, a risk problem is formulated and provides context for the assessment (Ecology RA). (Reprinted with permission from Risk Assessment in the Federal Government: Managing the Process. Washington, DC: National Academies Press, National Academy of Sciences, 1983.)

A comprehensive framework that applies two crucial concepts: (1) putting each environmental problem or issue into public health and/or ecological context and (2) proactively engaging the relevant stakeholders, including affected or potentially affected community groups, from the very beginning of the six-stage process shown in Figure 4–2. Particular exposures and potential health effects must be evaluated across sources and exposure pathways and in light of multiple end points, and not the current general approach of evaluating one chemical in one environmental medium (air, water, soil, food, and products) for one health effect at a time.

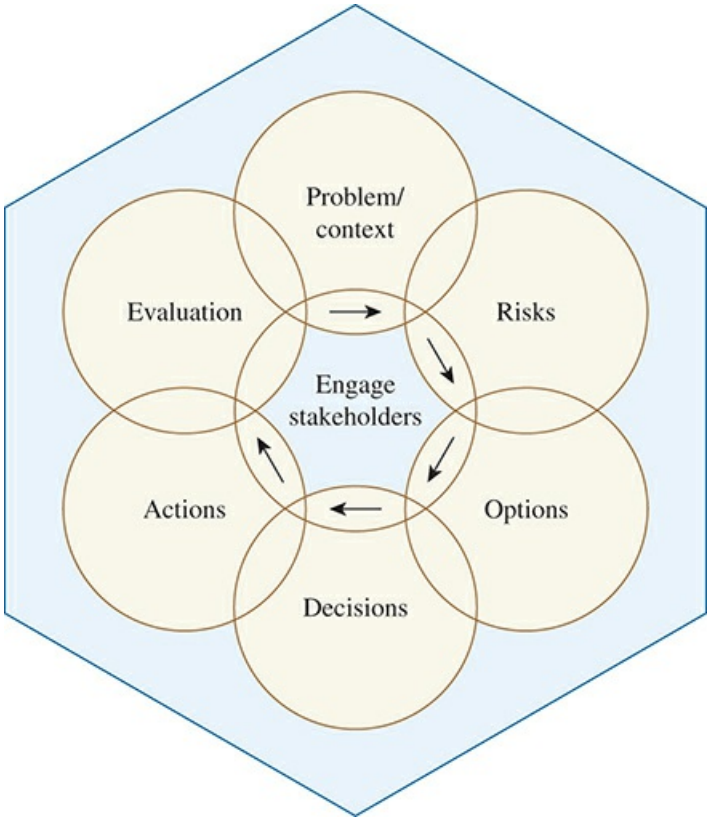

FIGURE 4–2 *Risk management framework for environmental health from the U.S. Commission on Risk Assessment and Risk Management.* The framework comprises six stages: (1) formulating the problem in a broad public health context; (2) analyzing the risks; (3) defining the options; (4) making risk reduction decisions; (5) implementing those actions; and (6) evaluating the effectiveness of the actions taken. Interactions with stakeholders are critical across all the stages and thus have been put at the center of the framework.

DEFINITIONS

The phrase *characterization of risk* reflects the combination of qualitative and quantitative analyses. Unfortunately, many users tend to equate risk assessment with quantitative risk assessment, generating a number for an overly precise risk estimate, while ignoring crucial information about the uncertainties of risk assessment, mode of action (MOA), and type of effect across species or context.

Risk management refers to the process by which policy actions are chosen to control hazards identified in the risk assessment/risk characterization stage of the framework (Figure 4–2). Risk managers consider scientific evidence and risk estimates—along with statutory, engineering, economic, social, and political factors—in evaluating alternative options and choosing among those options.

Risk communication is the challenging process of making risk assessment and risk management information comprehensible to community groups, lawyers, local elected officials, judges, business people, labor, environmentalists, etc. A crucial, too-often neglected requirement

for communication is listening to the fears, perceptions, priorities, and proposed remedies of these "stakeholders."

DECISION MAKING

Risk management decisions are reached under diverse statutes in the United States and many other countries. Some statutes specify reliance on risk alone, whereas others require a balancing of risks and benefits of the product or activity (Table 4–1). Risk assessments provide a valuable framework for priority setting within regulatory and health agencies, in the chemical development process within companies, and in resource allocation by environmental organizations. Currently, there are significant efforts toward a global harmonization of testing protocols and the assessment of risks and standards.

TABLE 4–1 Objectives of Risk Assessment

1. Protect human and ecological health
   - Toxic substances
2. Prioritize testing needs
3. Balance risks and benefits
   - Drugs
   - Pesticides
4. Set target levels of risk
   - Food contaminants
   - Water pollutants
5. Set priorities for program activities
   - Regulatory agencies
   - Manufacturers
   - Environmental/consumer organizations
6. Inform green chemistry, life cycle analysis, and clinical alternatives
7. Estimate residual risks and extent of risk reduction after steps are taken to reduce risks

A major challenge for risk assessment, risk communication, and risk management is to work across disciplines to demonstrate the biological plausibility and clinical significance of the conclusions from studies of chemicals thought to have potential adverse effects. Biomarkers of exposure, effect, or individual susceptibility can link the presence of a chemical in various environmental compartments to specific sites of action in target organs and to host responses. Individual behavioral and social risk factors may be critically important to both the characterization of risk and the reduction of risk. Finally, public and media attitudes toward local polluters, other responsible parties, and relevant government agencies can greatly influence the communication process and the choices for risk management.

HAZARD IDENTIFICATION

Throughout this book, a common toxicology paradigm (Fig. 4–3) forms the basis for our toxicity

assessments. In order to assess the toxicity of chemicals, information from four types of studies is used: structure–activity relationships (SARs), in vitro or short-term studies, in vivo animal bioassays, and human epidemiologic studies. In many cases, toxicity information for chemicals is limited.

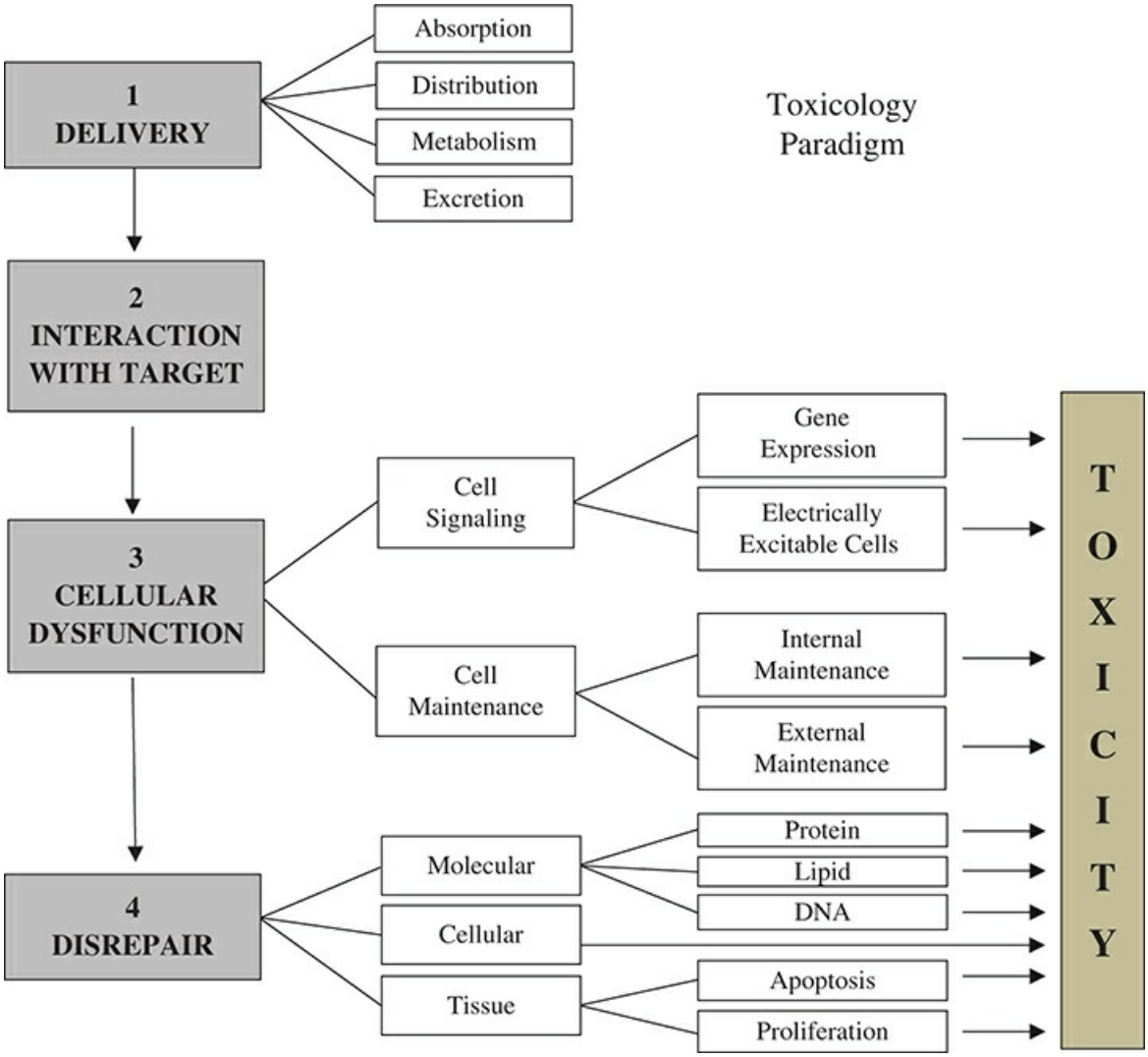

FIGURE 4–3 *Toxicology paradigm.* A common paradigm forms the basis for our toxicology assessments and subsequent translation for assessment of health risks. For risk assessment this paradigm is a critical underpinning where delivery represents exposure and absorption, distribution, metabolism, and elimination (ADME) determining the amount, the rate, the form, and distribution of the chemical and its metabolites. The key initial impacts of the chemical interacting with the target link the early cellular dysfunction with early biomarkers effect. Disrepair is identified as the endpoint, which impacts across levels of biological investigation and observation at the molecular, cellular, and tissue levels.

Assessing Toxicity of Chemicals: Approaches

Structure–Activity Relationships—Given the cost of $2 to $4 million and the 3 to 5 years

required for testing a single chemical in a lifetime rodent carcinogenicity bioassay, initial decisions on whether to continue development of a chemical, to submit a premanufacture notice (PMN), or to require additional testing may be based largely on results from SARs and limited short-term assays. A chemical's structure, solubility, stability, pH sensitivity, electrophilicity, volatility, and chemical reactivity can be important information for hazard identification.

SARs have been used for assessment of complex mixtures of structurally related compounds. Prominent applications have been the assessment of risks associated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), related chlorinated and brominated dibenzo-*p*-dioxins, dibenzofurans, and planar biphenyls, and chemicals generally present as mixtures in the environment. Toxicity equivalence factors (TEFs) are used to evaluate health risks associated with closely related chemicals. Computerized SAR methods have, in general, given disappointing results. More successful are the efforts of pharmaceutical companies using combinatorial chemistry and three-dimensional (3D) molecular modeling approaches to design ligands (new drugs) that can sterically fit into the "receptors of interest." A renewed interest in quantitative SAR (QSAR) approaches has also resulted from the need to evaluate engineered nanomaterials where the tremendous number of unique new products has highlighted the necessity of using QSAR approaches to handle the avalanche of novel untested materials.

Efforts within Registration, Evaluation, Authorisation, and Restriction of Chemicals (REACH) have also emphasized the potential for use of SARs as similar chemicals are collectively evaluated using a concept of "read-across." Substances whose physicochemical, toxicological, and ecotoxicological properties are similar can be grouped as a "category" of substances when they have a common functional group, common precursor or breakdown product, or a common pattern of potency.

In Vitro and Short-Term Tests—Additional biological information obtained within the hazard identification process includes assessment of the test chemical in in vitro or short-term tests, ranging from bacterial mutation assays performed entirely in vitro to more elaborate short-term tests, such as skin painting studies in mice or altered rat liver foci assays conducted in vivo. Other assays evaluate developmental, reproductive, neuro- and immuno-toxicity.

The validation and application of short-term assays are particularly important to risk assessment because such assays can be designed to provide information about mechanisms of effects, and, moreover, they are fast and inexpensive compared with lifetime bioassays. Validation requires determination of their sensitivity (e.g., ability to identify true carcinogens), specificity (e.g., ability to recognize noncarcinogens as noncarcinogens), and predictive value for the toxic endpoint under evaluation. Efforts to improve our ability to utilize short-term tests for carcinogenicity prediction include increased attention to improving the mechanistic basis of short-term testing. Mechanistic information from short-term in vitro assays can also be used to extend the range of biological observations available for dose–response assessment. In addition, for developmental toxicity assessment, assay methods that acknowledge the highly conserved nature of developmental pathways across species have accelerated the use of a broader range of model organisms and assay approaches for noncancer risk assessments.

Animal Bioassays—Animal bioassays are a key component of the hazard identification process. A basic premise of risk assessment is that chemicals that cause tumors in animals can cause tumors in humans. All human carcinogens that have been adequately tested in animals produce positive results in at least one animal model. Although this association cannot establish that all

chemicals and mixtures that cause cancer in experimental animals also cause cancer in humans, nevertheless, in the absence of adequate data on humans, it is biologically plausible and prudent to regard chemicals and mixtures for which there is sufficient evidence of carcinogenicity in experimental animals as if they presented a carcinogenic risk to humans—a reflection of the "precautionary principle." In general, the most appropriate rodent bioassays are those that test exposure and biological pathways of most relevance to predicted or known human exposure. Bioassays for reproductive and developmental toxicity and other noncancer endpoints have a similar rationale.

Standard cancer bioassays include testing in two species and both sexes, with 50 animals per dose group and near-lifetime exposure. Important choices include the strains of rats and mice, the number of doses, and dose levels (typically 90%, 50%, and 10–25% of the maximally tolerated dose [MTD]), and details of the required histopathology (number of organs to be examined, choice of interim sacrifice pathology, etc.). Positive evidence of chemical carcinogenicity can include increases in number of tumors at a particular organ site, induction of rare tumors, earlier induction (shorter latency) of commonly observed tumors, and/or increases in the total number of observed tumors.

The cancer bioassay, originally designed for hazard identification, is frequently used to evaluate dose–response. The relatively limited number of evaluated doses and the use of high doses have caused issues for low-dose extrapolations and have limited the use of cancer bioassays as a "gold standard" for prediction of human carcinogenicity risk. Tumors may be increased only at the highest dose tested, which is usually at or near a dose that causes systemic toxicity. Second, even without toxicity, the high dose may trigger different events than do low-dose exposures and high doses can saturate important metabolism and elimination pathways. Also, because rats and mice give concordant positive or negative results in approximately 70% of bioassays, it is unlikely that rodent/human concordance would be higher.

Lifetime bioassays have been enhanced with the collection of such additional mechanistic data and with the assessment of multiple noncancer endpoints. It is feasible and desirable to integrate such information together with data from mechanistically oriented short-term tests and biomarker and genetic studies in epidemiology. Such approaches may allow for an extension of biologically observable phenomena to doses lower than those leading to frank tumor development and help to address the issues of extrapolation over multiple orders of magnitude to predict response at environmentally relevant doses.

To improve the prediction of cancer risks to humans, transgenic mouse models have been developed as possible alternatives to the standard 2-year cancer bioassay. By using mice that incorporate or eliminate a gene that is linked to human cancer, these transgenic models have the power to improve the characterization of key cellular and mode of action responses. Transgenic models have been shown to reduce cost and time as compared with the standard 2-year assay, but they have also been shown to be somewhat limited in their sensitivity.

Use of Epidemiologic Data in Risk Assessment— The most convincing lines of evidence for human risk are well-conducted epidemiologic studies in which a positive association between exposure and disease has been observed. Table 4–2 shows examples of epidemiologic study designs and provides clues on types of outcomes and exposures evaluated. There are important limitations inherent in epidemiologic studies. Robust exposure estimates are often difficult to obtain as they are frequently done retrospectively (e.g., through retrospective job history records). Also, because many important health effects have long latency before clinical

manifestations appear, reconsideration of relevant populations can be challenging. Another challenge for interpretation is that there are often exposures to multiple chemicals, especially when a lifetime exposure period is considered. There is frequently a trade-off between detailed information on relatively few persons and very limited information on large numbers of persons. Contributions from lifestyle factors, such as smoking and diet, are important to assess as they can have a significant impact on cancer development. Human epidemiologic studies can provide both useful information for hazard assessment and quantitative information for data characterization.

TABLE 4–2 Example of Three Types of Epidemiologic Study Designs

Methodological Attributes	Type of Study		
	Cohort	Case–Control	Cross-Sectional
Initial classification	Exposure–nonexposure	Disease–nondisease	Either one
Time sequence	Prospective	Retrospective	Present time
Sample composition	Nondiseased individuals	Cases and controls	Survivors
Comparison	Proportion of exposed with disease	Proportion of cases with exposure	Either one
Rates	Incidence	Fractional (%)	Prevalence
Risk index	Relative risk–attributable risk	Relative odds	Prevalence
Advantages	Lack of bias in exposure; yields incidence and risk rates	Inexpensive, small number of subjects, rapid results, suitable for rare diseases, no attrition	Quick results
Disadvantages	Large number of subjects required, long follow-up, attrition, change in time of criteria and methods, costly, inadequate for rare diseases	Incomplete information, biased recall, problem in selecting control and matching, yields only relative risk—cannot establish causation, population of survivors	Cannot establish causation (antecedent consequence), population of survivors, inadequate for rare diseases

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Three examples of epidemiologic study designs—cross-sectional studies, cohort studies, and case–control studies—are detailed in Table 4–2. Cross-sectional studies survey groups of humans to identify risk factors (exposure) and disease but are not useful for establishing cause and effect. Cohort studies can evaluate individuals selected because of their exposure to a chemical under study. Thus, based on exposure status, these individuals are monitored for development of disease. These prospective studies monitor over time individuals who initially are disease-free to determine the rates at which they develop disease. In case–control studies, subjects are selected based on disease status: disease cases and matched cases of disease-free individuals. Exposure histories of the two groups are compared to determine key consistent features in their exposure histories. All case–control studies are retrospective studies.

In risk assessment, epidemiologic findings can be judged by the following criteria: strength of association, consistency of observations (reproducibility in time and space), specificity (uniqueness in quality or quantity of response), appropriateness of temporal relationship (did the exposure precede responses?), dose–response, biological plausibility and coherence, verification, and analogy (biological extrapolation). Epidemiologic study designs should also be evaluated for their power of detection, appropriateness of outcomes, verification of exposure assessments, completeness of assessing confounding factors, and general applicability of the outcomes to

other populations at risk. Power of detection is calculated using study size, variability, accepted detection limits for endpoints under study, and a specified significance level. Meta-analysis is used with epidemiologic studies to combine results from different studies using weighting of results to account for sample size across studies.

Advances from the human genome project have increased sophistication of molecular biomarkers and have improved the mechanistic bases for epidemiologic hypotheses. Molecular epidemiology integrates molecular biology into traditional epidemiologic research. Epidemiologists can now include the contribution of potential genetic factors with environmental risk factors for the determination of the etiology, distribution, and prevention of disease. The range of biomarkers has grown dramatically and includes identification of single-nucleotide polymorphisms (SNPs), genomic profiling, transcriptome analysis, and proteomic analysis. These improvements for risk assessment provide an improved biological basis for extrapolation across the diversity of human populations and allow for improved cross-species comparisons with rodent bioassay information. In addition, genomics allows for "systems-based" understanding of disease and response, moving risk assessment away from a linear, single-event-based concept and improving the biological plausibility of epidemiologic associations.

Integrating Qualitative Aspects of Risk Assessment

Qualitative assessment of hazard information should include a consideration of the consistency and concordance of findings, including a determination of the consistency of the toxicological findings across species and target organs, an evaluation of consistency across duplicate experimental conditions, and a determination of the adequacy of the experiments to consistently detect the adverse endpoints of interest. Many agencies use similar evidence classifications for both the animal and human evidence categories by both agencies. These classifications include levels of "sufficient, limited, inadequate, and no evidence," or "evidence suggesting lack of carcinogenic, probably not carcinogenic to humans, and not likely to be carcinogenic to humans." An overall weight of evidence approach considers both the quality and quantity of data as well as any underlying assumptions.

Mode of Action and Adverse Outcome Pathways— Building on MOA analyses, a structured framework for ecological risk assessment entitled *Adverse Outcome Pathways* (AOP) links biological events with adverse outcomes and disease. Figure 4–4 shows how the initial exposure to a chemical is linked through a series of early molecular initiating events (MIEs) through responses at the cellular, organ, and ultimately to organism and population level adverse outcomes. These approaches allow the identification of a temporal series of events that can be evaluated in vivo and in vitro and across species using related biomarkers of exposure, biological effect, and response. It also can incorporate biomarkers of susceptibility. Figure 4–4 also illustrates how information from methods such as structure–activity relationship assessments can link high- and medium-throughput assays with high content assay outcomes across in vitro assessments with biomarkers of effect seen at the organ, organism, and population levels with adverse outcomes and disease. These useful concepts can provide both a qualitative and quantitative frame for risk assessment.

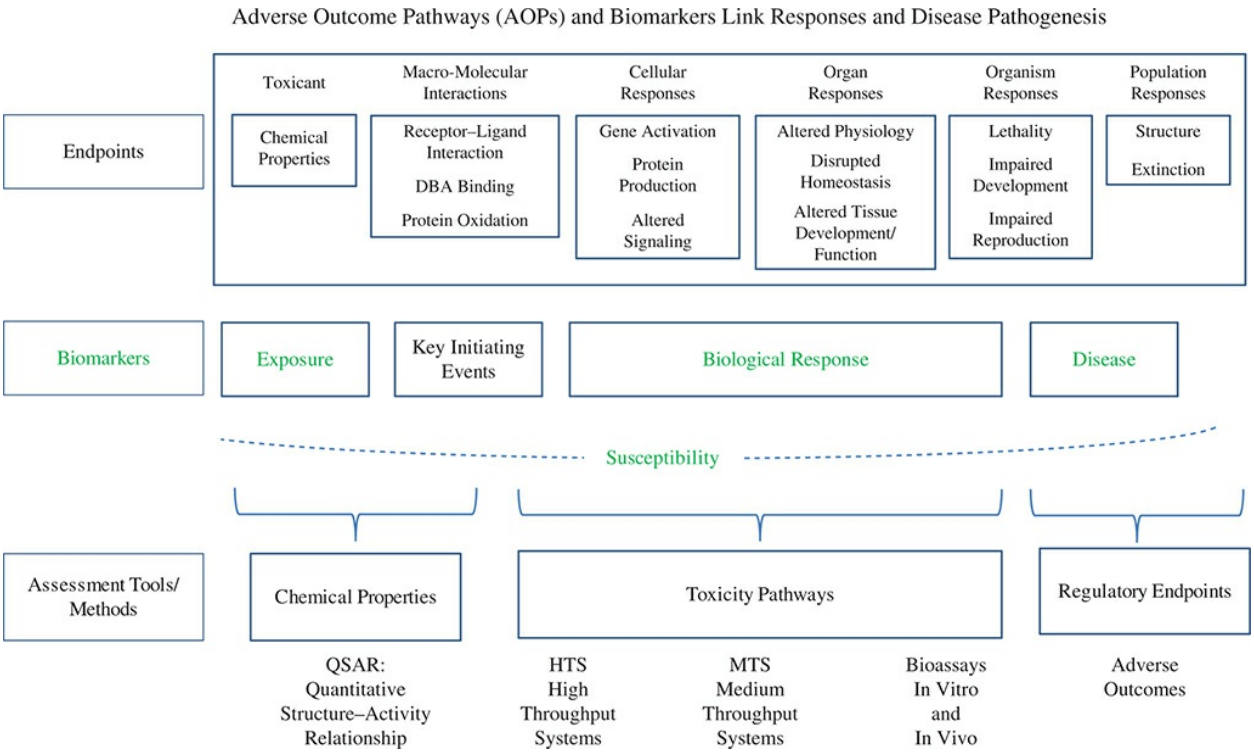

FIGURE 4–4 *Adverse outcome pathways (AOPs) and biomarkers link responses and describe disease pathogenesis.* Relationships between AOPs and biomarkers of exposure, effect, susceptibility, and disease are illustrated across disease response. Methods used to collect information along the disease continuum include quantitative SAR (QSAR), high and medium throughput in vitro systems, and bioassays (other in vivo and in vitro assessments).

DOSE–RESPONSE ASSESSMENT

Integrating Quantitative Aspects of Risk Assessment

Quantitative considerations in risk assessment include dose–response assessment, exposure assessment, variation in susceptibility, and characterization of uncertainty. For dose–response assessment, varying approaches have been proposed including threshold versus nonthreshold endpoints. The fundamental basis of the quantitative relationships between exposure to a chemical and the incidence of an adverse response is the dose–response assessment. Analysis of dose–response relationships must start with the determination of the critical effects to be quantitatively evaluated. It is usual practice to choose the most robust data sets with adverse effects occurring at the lowest levels of exposure from studies using the most relevant exposure routes. The "critical" adverse effect is defined as the significant adverse biological effect that occurs at the lowest exposure level.

Threshold Approaches— Approaches for characterizing threshold dose–response relationships include identification of "no observed adverse effect level" (NOAEL) or "lowest observed adverse effect level" (LOAEL). On the dose–response curve illustrated in Fig. 4–5, the doses tested in the bioassay are given as F, G, H, and I. The statistical significance of points G, H, and I

is indicated using an asterisk (*). The NOAEL (F) is identified as the highest nonstatistically significant dose tested; in this example, the NOAEL occurs at approximately 2 mg/kg body weight. Point G is the LOAEL (~2.3 mg/kg body weight), as it is the lowest dose tested with a statistically significant effect. Lines A to D represent possible extrapolations below the point of departure (POD), which is represented on this figure as a square (■) and is labeled as point E. The POD is used to specify the estimated dose near the lower end of the observed dose range, below which extrapolation to lower exposures is necessary. In Fig. 4–5, the POD occurs at 10% effective dose or ED_{10}. The type of extrapolation below the POD depends on the type of data available. From these modeled approaches, model D shows a model where the threshold (T) represents the dose below which no additional increase in response is observed.

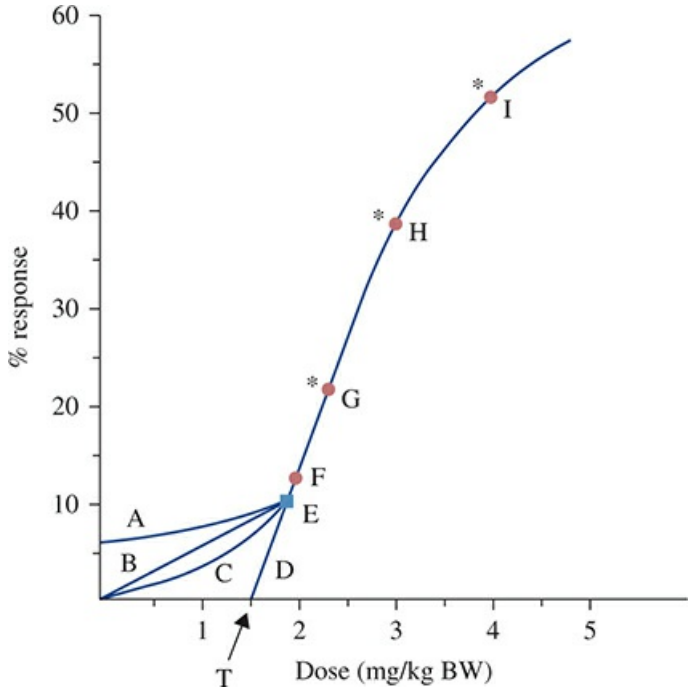

FIGURE 4–5 *Dose–response curve.* This figure is designed to illustrate a typical dose–response curve with points F to I indicating the biologically determined responses. Statistical significance of these responses is indicated with the symbol "*." Point E (■) represents a dose near the lower end of the observed dose–response range, below which extrapolation to lower doses can occur for cancer risk estimates. Point F is the highest nonstatistically significant response point; hence, it is the "no observed adverse effect level" (NOAEL) for this example. Point G is the "lowest observed adverse response level" (LOAEL). Point T represents a threshold for response curve D. Curves A to D show some options for extrapolating the dose–response relationship below the range of biologically observed data points and POD.

Generally, most animal bioassays are constructed with sufficient numbers of test animals to detect biological responses at the 10% response range. Significance thus usually refers to both biological and statistical criteria and depends on the number of dose levels tested, the number of animals tested at each dose, and background incidence of the adverse response in the nonexposed control groups. The NOAEL should not be perceived as risk-free.

As described in Chapter 2, approaches for characterizing dose–response relationships include

identification of effect levels such as LD_{50}, LC_{50}, ED_{10} as well as NOAELs. NOAELs have traditionally served as the basis for risk assessment calculations, such as reference doses (RfDs) or acceptable daily intake (ADI) values. RfDs or concentrations (RfCs) are estimates of daily exposure (oral or inhalation, respectively) to a chemical that is assumed to be without adverse health impact in humans. ADI values may be defined as the daily intake of chemical taken over a lifetime that appears to be without appreciable risk on the basis of all known facts at that time. RfDs (first introduced in Chapter 2) and ADI values typically are calculated from NOAEL values by dividing by uncertainty factor (UF) and/or modifying factor (MF):

$$\mathrm{RfD} = \frac{\mathrm{NOAEL}}{\mathrm{UF} \times \mathrm{MF}},$$

$$\mathrm{ADI} = \frac{\mathrm{NOAEL}}{\mathrm{UF} \times \mathrm{MF}}.$$

Tolerable daily intakes (TDIs) can be used to describe intakes for chemicals that are not "acceptable" but are "tolerable" as they are below levels thought to cause adverse health effects. These are calculated in a manner similar to ADI. Dividing by the UFs allows for interspecies (animal-to-human) and intraspecies (human-to-human) variability with default values of 10 each. An additional UF is used to account for experimental inadequacies—for example, to extrapolate from short-exposure-duration studies to a situation more relevant for chronic study or to account for inadequate numbers of animals or other experimental limitations. If only a LOAEL value is available, then an additional 10-fold factor may be used to arrive at a value more comparable to a NOAEL. Traditionally, a safety factor of 100 would be used for RfD calculations to extrapolate from a well-conducted animal bioassay (10-fold factor for animal-to-human variability) and to account for human variability in response (10-fold factor for human-to-human variability).

MFs are values that can be used to adjust the UFs if data on mechanisms, pharmacokinetics, or relevance of the animal response to human risk are available. To reduce uncertainty in calculating RfDs and ADIs, there has been a transition from the use of traditional 10-fold UFs to the use of data-derived and chemical-specific adjustment factors. Such efforts have included reviewing the human pharmacologic literature from published clinical trials and developing human variability databases for a large range of exposures and clinical conditions. Intra- and interspecies UFs have two components: toxicokinetics (TK) and toxicodynamic (TD) aspects (Fig. 4–6). This approach may provide a structure for incorporating scientific information on specific aspects of the overall toxicological process into the RfD calculations; thus, relevant data can replace a portion of the overall "uncertainty" surrounding these extrapolations.

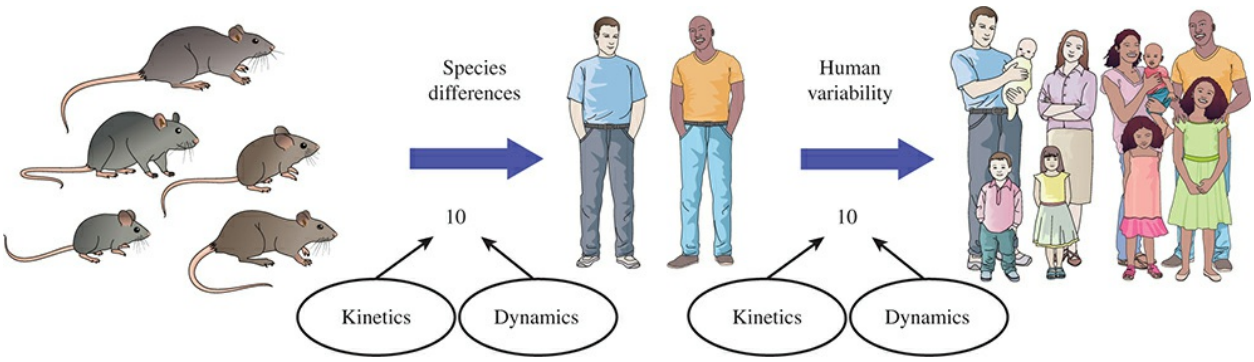

FIGURE 4–6 *Toxicokinetic (TK) and toxicodynamic (TD) considerations inherent in interspecies and interindividual extrapolations. TK* refers to the processes of absorption, distribution, elimination, and metabolism of a toxicant. *TD* refers to the actions and interactions of the toxicant within the organism and describes processes at organ, tissue, cellular, and molecular levels. This figure shows how uncertainty in extrapolation both across and within species can be due to two key factors: a kinetic component and a dynamic component. Challenges remain for extrapolating information on human variation to specific populations.

NOAEL values have also been utilized for risk assessment by evaluating a "margin of exposure" (MOE), where the ratio of the NOAEL determined in animals and expressed as mg/kg per day is compared with the level to which a human may be exposed. Low values of MOE indicate that the human levels of exposure are close to levels for the NOAEL in animals. There is usually no factor included in this calculation for differences in human or animal susceptibility or animal-to-human extrapolation; thus, MOE values of less than 100 have been used by regulatory agencies as flags for requiring further evaluation.

The NOAEL approach has been criticized because (1) the NOAEL must, by definition, be one of the experimental doses tested and (2) once identified, the rest of the dose–response curve is ignored. In an alternative to the NOAEL approach, the benchmark dose (BMD) method models the dose–response and the lower confidence bound for a dose at a specified response level (benchmark response [BMR]) is calculated. The BMR is usually specified at 1%, 5%, or 10%. The BMD*x* (with *x* representing the percent BMR) is used as an alternative to the NOAEL value for RfD calculations. Thus, the RfD would be:

$$\text{RfD} = \frac{\text{BMD}_x \text{ or } \text{BMDL}_x}{\text{UF} \times \text{MF}}$$

Advantages of the BMD approach include (1) the ability to consider the dose–response curve; (2) the inclusion of a measure of variability (confidence limit); and (3) the use of a consistent BMR level for RfD calculations across studies. In the animal bioassays, a minimal number of test doses for evaluation, shallow dose responses, and use of widely spaced test doses limit the utility of these assays for any type of quantitative assessments, whether NOAEL- or BMD-based approaches.

Nonthreshold Approaches— As Fig. 4–5 shows, numerous dose–response curves can be proposed in the low-dose region of the dose–response curve if a threshold assumption is not made. Because the risk assessor may need to extrapolate beyond the region of the dose–response curve for which experimentally observed data are available, the choice of models to generate curves in this region has received lots of attention. For nonthreshold responses, methods for dose–response assessments have models for extrapolation to de minimis (10^{-4} to 10^{-6}) risk levels at very low doses, far below the biologically observed response range and far below the effect levels evaluated for threshold responses. Risk estimates using the linear model (biological response increases proportionally with level of exposure) are generally higher than those using nonlinear models. Increased attention has been given to the use of mechanistic data (such as biomarker data) to extend the POD to lower regions of the dose–response curve.

Statistical or Probability Distribution Models— There are two general types of dose–response

models for extrapolation: statistical (or probability distribution) and mechanistic models. The distribution models assume that each individual has a tolerance level for a test chemical and that this response level is a variable following a specific probability distribution function. These responses can be modeled using a cumulative dose–response function. However, extrapolation of the experimental data from 50% response levels to a "safe," "acceptable," or "de minimus" level of exposure—for example, one in a million risk above background—illustrates the huge gap between scientific observations and highly protective risk limits (sometimes called virtually safe doses [VSDs] or those corresponding to a 95% upper confidence limit on adverse response rates).

Models Derived from Mechanistic Assumptions— This approach designs a mathematical equation to describe dose–response relationships that are consistent with postulated biological mechanisms of response. These models are based on the idea that a response (toxic effect) in a particular biological unit (animal, human, pup, etc.) is the result of the random occurrence of one or more biological events (stochastic events).

A hit for cancer modeling is defined as a critical cellular event that must occur before a toxic effect is produced. The simplest mechanistic model is the one-hit (one-stage) linear model in which only one hit or critical cellular interaction is required for a cell to be altered. Thus a single molecule of a genotoxic carcinogen would have a minute but finite chance of causing a mutational event. As theories of cancer have grown in complexity, multihit models have been developed that can describe hypothesized single-target multihit events, as well as multitarget, multihit events in carcinogenesis.

Toxicological Enhancements of the Models— Research areas that have improved the models used in risk extrapolation are time to tumor information, physiologically based toxicokinetic modeling, and biologically-based dose-response (BBDR) modeling. BBDR modeling aims to make the generalized mechanistic models more clearly reflect specific biological processes. Measured rates are incorporated into the mechanistic equations to replace default or computer-generated values. Development of BBDR models for endpoints other than cancer is limited. However, several approaches have been explored in developmental toxicity, utilizing MOA information on cell cycle kinetics, enzyme activity, and cytotoxicity as critical endpoints.

EXPOSURE ASSESSMENT

Designed to determine source, type, magnitude, and duration of contact with the chemical(s) of interest, exposure assessment is a critical element of the risk assessment process. However, quality exposure data are frequently identified as the key area of uncertainty in the overall risk determination. The primary goal of exposure calculations is to determine the type and amount of total exposure and to find out specifically who may be exposed and how large a dose may be reaching target tissues. Determination of exposure pathways relevant for the risk scenario under development, quantitation of each pathway identified as a relevant exposure, and summarizing these pathway-specific exposures are required for calculation of overall exposure.

Exposure assessments also consider how time and duration of exposure are evaluated in risk assessment. Estimates for cancer risk use average exposure over a lifetime, as lifetime average daily dose (LADD). In a few cases, short-term exposure limits (STELs) are required and

characterization of brief but high levels of exposure is significant. In these cases, exposures are not averaged over the lifetime and the effects of high, short-term doses are directly estimated. With developmental toxicity, a single exposure can be sufficient to produce an adverse developmental effect if exposures occur during a window of developmental susceptibility; thus, daily doses are used, rather than lifetime weighted averages.

RISK CHARACTERIZATION

Risk characterization is a summary of the risk assessment components that outlines the key findings and informs the risk manager in public health decisions. It is an analysis and integration of the conclusions from the hazard assessment, the dose–response assessment, and the exposure assessment. Risk characterization considers the nature, estimated incidence, and reversibility of adverse effects in a given population; how robust the evidence is; how certain the evaluation is; if susceptible populations are characterized; and if there is a relevant MOA.

Uncertainty analysis is an essential component in our final risk characterization and includes factors such as variability and lack of knowledge. Variability refers to true differences such as is reflected in temporal, spatial, or interindividual differences. It usually cannot be reduced with further study. Lack of knowledge can be discussed as a source of uncertainty and may be reduced with further study.

Variation in Susceptibility

Risk assessment methodologies incorporating human variability have been slow to develop. Generally, assay results utilize means and standard deviations to measure variation, or even standard errors of the mean. This ignores variability in response due to specific differences in age, sex, health status, and genetics.

Ecogenetics has been defined as the study of critical genetic determinants that define susceptibility to environmentally influenced adverse health effects. Ecogenetic variation can affect biotransformation systems that activate and detoxify chemicals or alter the response in target tissues. The identification of human polymorphisms has greatly expanded our potential for understanding how genetic variability can impact biological response and susceptibility. There have been numerous activities initiated with the goal of understanding the linkage between genes and the environment. Next-generation sequencing has revealed rare copy number variants that can be linked to disease and provide another level of understanding concerning human variation.

A key challenge in risk assessments is the interpretation and linking of observations from highly sensitive molecular and genome-based methods with the overall process of toxicity. Early, subtle, and possibly reversible effects can generally be distinguished from irreversible disease states. Interpretation of early and highly sensitive response biomarkers in the complicated data from gene expression arrays (toxicogenomics) is also challenging.

INFORMATION RESOURCES

There are numerous information resources available for risk assessment and a few are listed

below to provide the reader with examples of risk assessment resources and databases. Such resources include the Toxicology Data Network from the National Library of Medicine (http://toxnet.nlm.nih.gov/); the National Toxicology Program (http://ntp.niehs.nih.gov/); and the World Health Organization (http://who.int/). The NCBI portfolio of comprehensive data and tools (http://www.ncbi.nlm.nih.gov/) contains information on chemicals and bioassays, data and software, DNA and RNA, protein domains and structures, genes and expression, genetic information databases linked with decision outcomes, genomes and gene maps, and cross-species homology. ACToR (https://actor.epa.gov/actor/home.xhtml), the EPA's online database on chemical toxicity data and potential chemical risks to human health and the environment, is another useful resource for risk assessments. These databases can be especially useful for hazard identification and mechanistic information; few emphasize exposure.

RISK PERCEPTION AND COMPARATIVE ANALYSES OF RISK

Because individuals respond differently to information about hazardous situations and products, understanding behavioral responses at the individual, community, and population levels is critical in stimulating constructive risk communication and evaluating potential risk management options. In a classic study, students, League of Women Voters members, active club members, and scientific experts were asked to rank 30 activities or agents in order of their annual contribution to deaths. Club members ranked pesticides, spray cans, and nuclear power as safer than did other laypersons. Students ranked contraceptives and food preservatives as riskier and mountain climbing as safer than did others. Experts ranked electric power, surgery, swimming, and x-rays as riskier, but nuclear power and police work as less risky than did laypersons. There are cultural and gender differences in perception of risks. Group differences in perceptions of risk from chemicals among toxicologists correlated with their employment in industry, academia, or government. Recent risk perception research must know the balance between "affect" and analytical thinking. Our immediate emotional reaction can shape our initial risk perceptions ("affect"), while our thoughtful, deliberate "analytical" thinking impacts our risk understanding as a slower response. Understanding this balance can help us explain why there is a complex relationship between perceived risk and benefits.

Psychological factors such as dread, perceived uncontrollability, and involuntary exposure interact with factors that represent the extent to which a hazard is familiar, observable, and "essential" for daily living. Figure 4–7 presents a grid on the parameters controllable/uncontrollable and observable/not observable for many risky activities; for each of the two-paired main factors, highly correlated factors are described in the boxes.

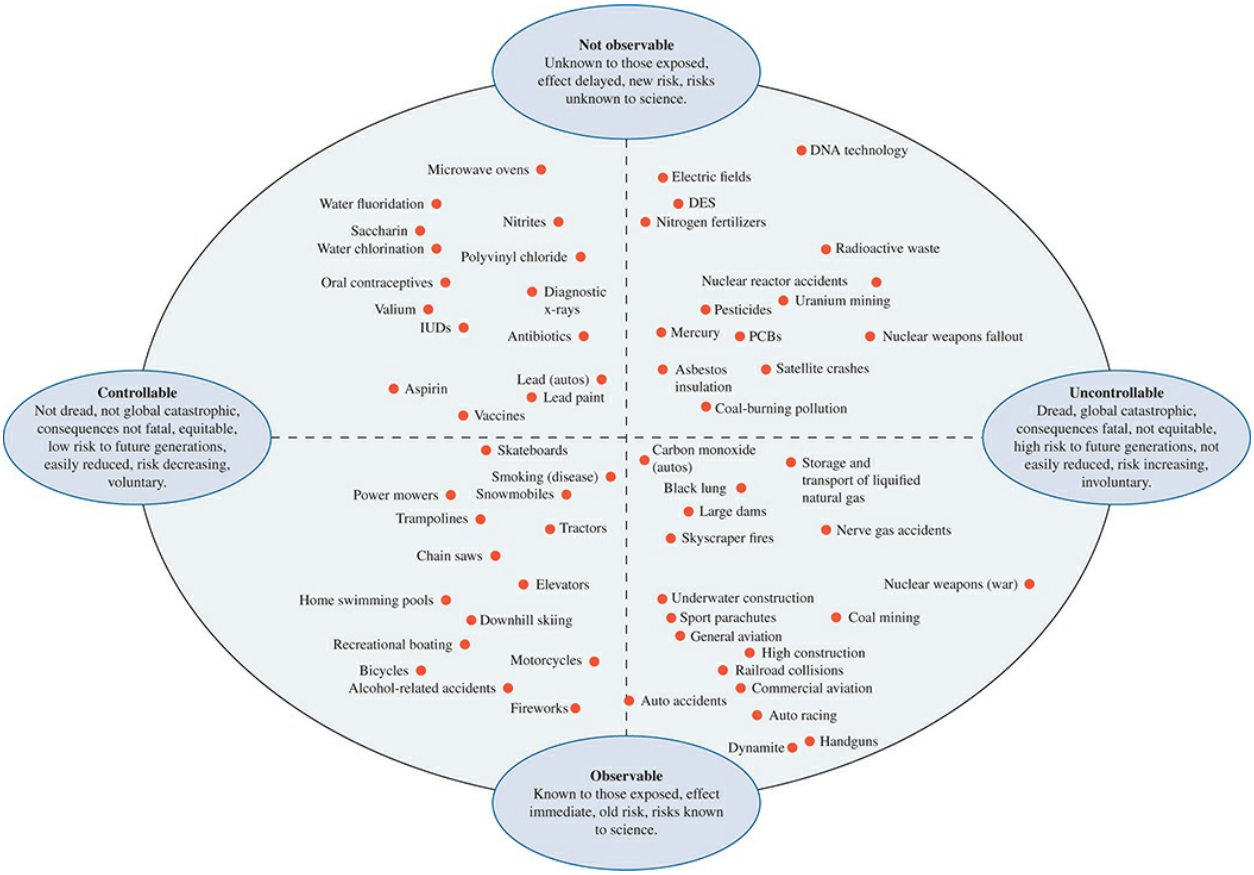

FIGURE 4–7 *Perceptions of risk illustrated using a "risk space" axis diagram.* Risk space has axes that correspond roughly to a hazard's perceived "dreadedness" and to the degree to which it is familiar or observable. Risks in the upper right quadrant of this space are most likely to provoke calls for government regulation.

Public demand for government regulations often focuses on involuntary exposures (especially in the food supply, drinking water, and air) and unfamiliar hazards, such as radioactive waste, electromagnetic fields, asbestos insulation, and genetically modified crops and foods. The public can respond negatively when they perceive that information about hazards has been withheld or under-rated. Engineering-based "as low as reasonably achievable" (ALARA) approaches also reflect the general "precautionary principle," which is strongly favored by those who believe we are far from knowing all risks given frequently limited toxicity testing data.

EMERGING CONCEPTS

For risk assessment to inform environmental risk management, there is a need to ensure that the initial "problem formulation" of the risk question(s) is succinctly framed to answer questions in the real world. Environmental health is dynamic and many divergent emerging environmental challenges such as climate change, energy shortages, and engineered nanoparticles will require an expansion of our context beyond single-chemical, single-exposure scenarios. To accomplish this goal, several factors including defining not only health but also well-being and sustainability

will require a global and international context.

Well-being is being used to describe human health and sustainable environmental risk management. Well-being goes beyond “disease-free” existence to freedom from want (including food and water security) and fear (personal safety) and sustainable futures. Concepts such as food security (abundance and quality of foods), water security (plentiful supplies and high quality of water), and sustainability form internationally recognized environmental and developmental goals. Recognition that environmental problems are global is essential to our understanding of how we manage risks and how we address sustainability. Ocean health and air pollution exemplify the need for understanding the global context where pollutants do not honor country and national borders. Applications for alternatives assessment, life cycle analysis, and green chemistry open new opportunities to enlarge the framing of our assessments.

PUBLIC HEALTH RISK MANAGEMENT

Associated with concepts of well-being and sustainability is a public health orientation to risk assessment and risk management. There are three stages of prevention: *primary*, whose goal is prevention and risk or hazard avoidance; *secondary*, whose goal is mitigation or preparedness including risk or vulnerability reduction and risk transfer; and *tertiary*, where prompt response or recovery is an approach for decreasing residual risk or risk reduction. These three prevention stages frame many risk management actions. In occupational health, a prevention hierarchy is applied that includes 1) substitution where the hazardous chemical or activities are removed and substituted with a safer chemical or process option, 2) engineering controls with a focus on reducing workplace exposures is a subsequent action, 3) administrative controls can be used to change job tasks and methods, and 4) the use of personal protective equipment (PPE) such as gloves, hats, protective gloves, and work clothes is usually considered after all other management efforts fail to meet occupationally safe levels.

CONCLUSION

The objectives of risk assessments vary with the issues, risk management needs, and statutory requirements. Hence, setting the context and problem formation for risk evaluation is essential. The frameworks are sufficiently flexible to address various objectives and to accommodate new knowledge while also providing guidance for priority setting in industry, environmental organizations, and governmental regulatory and public health agencies. Risk assessment analyzes the science and, if incomplete, identifies uncertainty and provides approaches for moving forward with decisions. Toxicology, epidemiology, exposure assessment, and clinical observations can be linked with biomarkers, cross-species investigations of mechanisms of effects, and systematic approaches to risk assessment, risk communication, and risk management. Advances in toxicology are certain to improve the quality of risk assessments for a broad array of health endpoints as scientific findings substitute data for assumptions and help to describe and model uncertainty more credibly.

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QUESTIONS

1. Which of the following is NOT important in hazard identification?
   - **a.** structure–activity analysis.
   - **b.** in vitro tests.
   - **c.** animal bioassays.
   - **d.** susceptibility.
   - **e.** epidemiology.

2. The probability of an adverse outcome is defined as:
   - **a.** hazard.
   - **b.** exposure ratio.
   - **c.** risk.
   - **d.** susceptibility.
   - **e.** epidemiology.

3. The systematic scientific characterization of adverse health effects resulting from human exposure to hazardous agents is the definition of:
   - **a.** risk.
   - **b.** hazard control.
   - **c.** risk assessment.
   - **d.** risk communication.
   - **e.** risk estimate.

4. Which of the following is not an objective of risk management?
   - **a.** setting target levels for risk.
   - **b.** balancing risks and benefits.
   - **c.** calculating lethal dosages.
   - **d.** setting priorities for manufacturers.
   - **e.** estimating residual risks.

5. Which of the following is NOT a feature in the design of standard cancer bioassays?
   a. more than one species.
   b. both sexes.
   c. near lifetime exposure.
   d. approximately 50 animals per dose group.
   e. same dose level for all groups.

6. Which of the following types of epidemiologic study is always retrospective?
   a. cohort.
   b. cross-sectional.
   c. case–control.
   d. longitudinal.
   e. exploratory.

7. Which of the following is defined as the highest nonstatistically significant dose tested?
   a. ED_{50}.
   b. ED_{100}.
   c. NOAEL.
   d. ADI.
   e. COAEL.

8. Which of the following represents the dose below which no additional increase in response is observed?
   a. ED_{10}.
   b. LD_{10}.
   c. RfC.
   d. threshold.
   e. significance level.

9. Which of the following is NOT needed to calculate the reference dose using the BMD method?
   a. MF.
   b. percent benchmark response.
   c. NOAEL.
   d. UF.
   e. benchmark dose.

10. Virtually safe doses are described at which confidence level?
    a. 90 percent.
    b. 95 percent.
    c. 99 percent.
    d. 99.9 percent.
    e. 99.99 percent.

UNIT 2 DISPOSITION OF TOXICANTS

CHAPTER 5

Absorption, Distribution, and Excretion of Toxicants

Angela L. Slitt

KEY POINTS

- Absorption is the transfer of a chemical from the site of exposure, usually an external or internal body surface, into the systemic circulation.
- Toxicants are removed from the systemic circulation by biotransformation, excretion, and storage at various sites in the body.
- Excretion is the removal of xenobiotics from the blood and their return to the external environment via urine, feces, or exhalation, etc.

INTRODUCTION

The disposition of a chemical or xenobiotic is defined as the composite actions of its absorption, distribution, biotransformation, and elimination. The quantitative characterization of xenobiotic disposition is termed pharmacokinetics or toxicokinetics. Figure 5–1 is a pictoral overview of the processes that determine disposition, which are likely to occur simultaneously. The toxicity of a substance in most circumstances directly depends on the dose, where "dose" is defined as the amount that ultimately reaches the site or sites of action (tissue, cell, or molecular target). Therefore, the disposition of a chemical determines its concentration at the site of action such that the concerted actions of absorption, distribution, and elimination also determine the potential for adverse events.

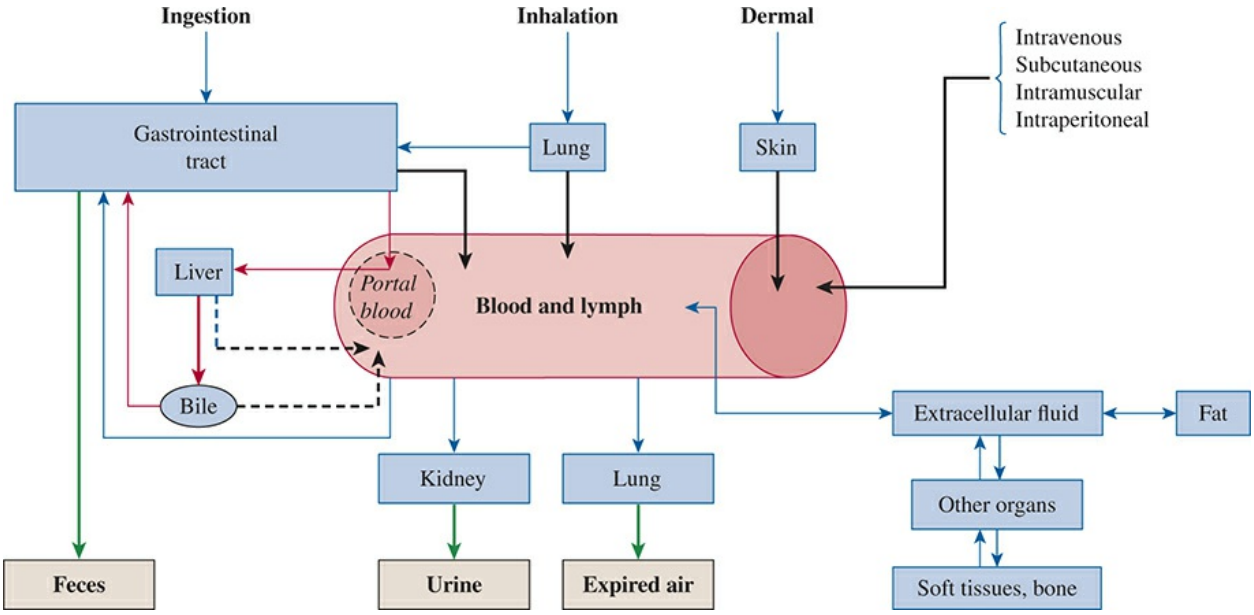


FIGURE 5–1 *Summary of the disposition of toxicants as determined by absorption, distribution, and excretion in the body.* Black lines represent major pathways of absorption into the body, blue designates distribution, and green lines identify pathways of final excretion (elimination) from the body, with the exception of enterohepatic circulation, which is designated in red.

The skin, lungs, and alimentary canal are the main barriers that separate higher organisms from an environment containing many chemicals (Fig. 5–1). Toxicants must cross one or several of these incomplete barriers to exert deleterious effects, except for caustic and corrosive chemicals (acids, bases, salts, oxidizers) that act directly at the point of contact. A chemical absorbed into the bloodstream or lymphatics through any of the major barriers is distributed, at least to some extent, throughout the body, including the target organ or target tissue where it produces damage. A chemical may have one or several target organs, and, in turn, several chemicals may have the same target organ or organs. However, it cannot be assumed that the tissue of highest concentration is also the target organ where toxicity will be observed.

Poor absorption of a toxicant resulting from a low amount absorbed or from a low rate of absorption will limit or prevent toxicity because a chemical may never attain a sufficiently high

concentration at a potential site of action to cause toxicity. A well absorbed chemical that is rapidly biotransformed or eliminated from an organism is less likely to cause toxicity because rapid excretion prevents it from reaching a sufficiently high concentration at a site of action.

CELL MEMBRANES

Toxicants usually pass through cells, such as the stratified epithelium of the skin, the thin cell layers of the lungs or the gastrointestinal (GI) tract, capillary endothelium, and ultimately the cells of the target organ. The plasma membranes surrounding all these cells are remarkably similar. The basic unit of the cell membrane is a lipid bilayer composed primarily of phospholipids, glycolipids, and cholesterol. Mammalian cell plasma membranes contain four major phospholipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and sphingomyelin) that are amphiphilic, consisting of a hydrophilic polar head and a hydrophobic lipid tail. The polar head groups are oriented toward the outer and inner surfaces of the membrane, whereas the hydrophobic tails are oriented inward and face each other to form a continuous hydrophobic inner space. The thickness of the cell membrane is about 7 to 9 nm. Numerous proteins are inserted or embedded in the bilayer, and some transmembrane proteins traverse the entire lipid bilayer, functioning as important biological receptors, aqueous pores, ion channels, and transporters (Fig. 5–2). The membrane fatty acids are semifluid at physiological temperatures. Overall, hydrophobic interactions are the major driving force in the formation of membrane lipid bilayers, and the fluid character of membranes is determined largely by the structure and relative abundance of unsaturated fatty acids. The more unsaturated fatty acids the membranes contain, the more fluid-like they are, facilitating more rapid active or passive transport.

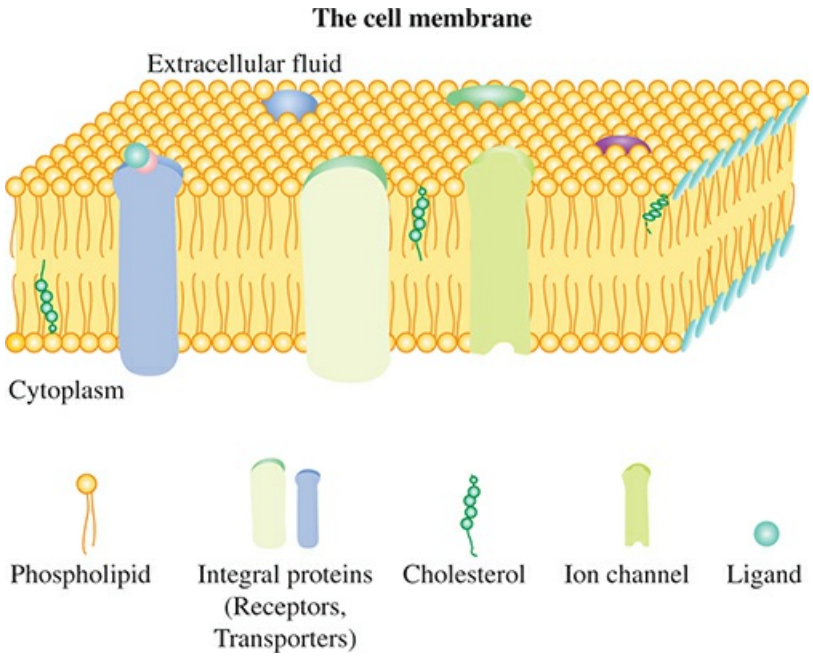


FIGURE 5–2 *Schematic model of a biological membrane.*

The membrane barrier is differentially permeable, which regulates what enters or exits the cells. Toxicants cross membranes either by passive processes in which the cell expends no energy or by mechanisms in which the cell provides energy to translocate the toxicant. Physicochemical properties such as molecular weight, hydrophilicity, and polarity (total polar surface area) are determinants of cell membrane permeability.

Passive Transport

Simple Diffusion—Many toxicants cross membranes by simple diffusion following the principles of Fick's law, which establishes that chemicals move from regions of higher concentration to regions of lower concentration without any energy expenditure. Small hydrophilic molecules (up to about 600 Da) permeate membranes through aqueous pores in a process termed paracellular diffusion, whereas hydrophobic molecules diffuse across the lipid domain of membranes (transcellular diffusion). The smaller a hydrophilic molecule is, the more readily it traverses membranes by simple diffusion through aqueous pores. Consequently, a small, water-soluble compound is absorbed rapidly from the GI tract and is distributed rapidly throughout the body.

For larger organic molecules with differing degrees of lipid solubility, the rate of transfer across membranes correlates with lipophilicity. Lipid solubility is generally determined by the octanol/water partition coefficient, *P*, which is defined as the ratio of the concentration of compound in organic and aqueous phases under equilibrium conditions. Usually expressed in logarithmic form, log *P* is an extremely informative physicochemical parameter relative to assessing potential membrane permeability.

Many chemicals are weak organic acids or bases, which in solution are ionized according to Arrhenius' theory. The ionized form usually has low lipid solubility and thus does not permeate readily through the lipid domain of a membrane. Some transfer of organic anions and cations (depending on their molecular weight) through the aqueous pores is a slow and inefficient process. In contrast, the nonionized form of weak organic acids and bases is lipid soluble to some extent, and the rate of movement of the nonionized form is proportional to its lipid solubility.

The molar ratio of ionized to nonionized molecules of a weak organic acid or base in solution depends on the ionization constant, which is defined as the pH at which a weak organic acid or base is 50% ionized (denoted as pK_a or pK_b for acids and bases, respectively). Like pH, pK_a and pK_b are defined as the negative logarithm of the ionization constant of a weak organic acid or base. An organic acid with a low pK_a is relatively a strong acid, and one with a high pK_a is a weak acid. The opposite is true for bases. The numerical value of pK_a does not indicate whether a chemical is an organic acid or a base. Knowledge of the chemical structure is required to distinguish between organic acids and bases.

The Henderson–Hasselbalch equation describes the derivation of pH as a function of the pK_a in a biological system. Two equivalent forms of this equation are as follows:

$$\text{pH} = \text{p}K_a + \log\frac{[\text{ionized}]}{[\text{nonionized}]}$$

$$\text{pH} = \text{p}K_a - \log\frac{[\text{nonionized}]}{[\text{ionized}]}$$

Rearranging these equations enables determination of the extent to which a weak acid or base is ionized or nonionized at any pH as follows:

$$\text{For acids: } \text{p}K_a - \text{pH} = \log\frac{[\text{nonionized}]}{[\text{ionized}]}$$

$$\text{For bases: } \text{p}K_a - \text{pH} = \log\frac{[\text{nonionized}]}{[\text{ionized}]}$$

The effect of pH on the degree of ionization of an organic acid (benzoic acid) and an organic base (aniline) is illustrated in Fig. 5–3. According to the Brönsted–Lowry acid–base theory, an acid is a proton (H^+) donor and a base is a proton acceptor. Thus, the ionized and nonionized forms of an organic acid represent an acid–base pair, with the nonionized moiety being the acid and the ionized moiety being the base. At a low pH, a weak organic acid such as benzoic acid is largely nonionized. At pH 4, exactly 50% of benzoic acid is ionized and 50% is nonionized because this is the pK_a of the compound. As the pH increases, more and more protons are neutralized by hydroxyl groups, and benzoic acid continues to dissociate until almost all of it is ionized. For an organic base such as aniline, the inverse is true. At a low pH, when protons are abundant, almost all of aniline is ionized. This form of aniline is an acid because it can donate protons. As the pH increases, ions from aniline continue to dissociate until almost all the aniline is in the nonionized form, which is the aniline base. As transmembrane passage is largely restricted to the nonionized form, benzoic acid is more readily translocated through a membrane from an acidic environment, whereas more aniline is transferred from an alkaline milleu.

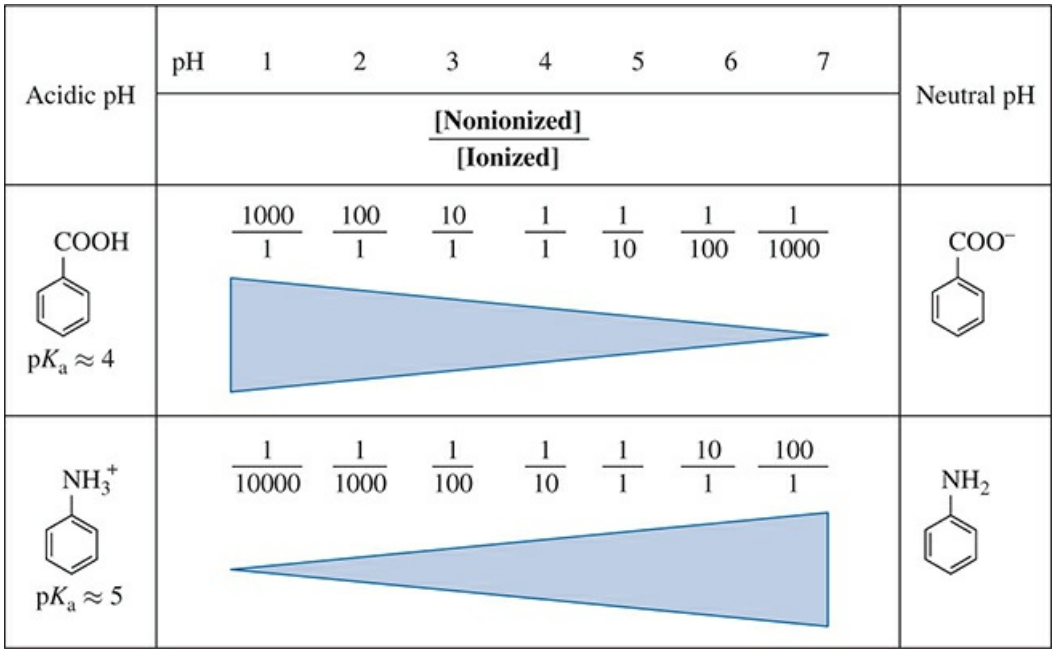


FIGURE 5–3 *Effect of pH on the ionization of benzoic acid (pKa = 4) and aniline (pKa = 5).*

Lipid solubility can also be expressed as the distribution coefficient, log *D*, which is the ratio of the sum of the concentrations of all forms of the compound (ionized plus un-ionized) in each of the two phases, one essentially always aqueous; as such, it depends on the pH of the aqueous phase, and log *D* = log *P* for nonionizable compounds at any pH. The major difference between log *P* and log *D* is that log *P* is a constant for the molecule under its neutral form, whereas log *D* considers all neutral and charged forms of the molecule. Because a charged form of the molecule might hardly enter the octanol phase, the distribution varies with pH. log *D* = log *P* in the pH region where the molecule is mostly unionized. However, a pH region where a significant fraction of the molecule is ionized, log *D* becomes a function of log *P*, pH, and pK_a. Overall, log *D* is a parameter commonly used for in silico prediction of absorption, distribution, metabolism, and excretion (ADME), as well as toxicity properties.

Filtration— When water flows in bulk across a porous membrane, any solute small enough to pass through the pores flows with it. Passage through these channels is called filtration. One of the main differences between various membranes is the size of these channels. In renal glomeruli, a primary site of blood filtration, these pores are relatively large (about 70 nm) allowing molecules smaller than albumin (approximately 60 kDa) to pass through. In contrast, there are no aqueous pores at cellular tight junctions, and channels in most cells are much smaller (3 to 6 Å), thereby only permitting substantial passage of molecules with molecular weights of no more than a few hundred daltons.

Special Transport

There are numerous compounds whose movement across membranes is not simple diffusion or filtration (Table 5–1). They are often transported rapidly across membranes, even against concentration gradients by specialized transport systems. Approximately 1500 genes encode for

transporters or transport-related proteins, not all of these genes contribute to the disposition of toxicants. The role of xenobiotic transporters in chemical disposition remains an expanding research field, and new information regarding their function, molecular regulation, and genetic polymorphisms continues to inform and modify traditional concepts in toxicology.

TABLE 5–1 Distribution of Specialized Transport Systems in the Small Intestine of Man and Animals

SUBSTRATES	UPPER	MIDDLE	LOWER	COLON
Sugar (glucose, galactose, etc.)	++	+++	++	0
Neutral amino acids	++	+++	++	0
Basic amino acids	++	++	++	?
Gamma globulin (newborn animals)	+	++	+++	?
Pyrimidines (thymine and uracil)	+	+	?	?
Triglycerides	++	++	+	?
Fatty acid absorption and conversion to triglyceride	+++	++	+	0
Bile salts	0	+	+++	
Vitamin B_{12}	0	+	+++	0
Na^+	+++	++	+++	+++
H^+ (and/or secretion)	0	+	++	++
Ca^{2+}	+++	++	+	?
Fe^{2+}	+++	++	+	?
Cl^-	+++	++	+	0

Facilitated Transport—Facilitated transport applies to carrier-mediated transport that exhibits the properties of active transport except that the substrate is not moved against an electrochemical or concentration gradient, and the transport process does not require the input of energy (e.g., ATP). Transport of glucose from the GI tract across the basolateral membrane of the intestinal epithelium, from plasma into red blood cells, and from blood into the central nervous system (CNS) occurs by facilitated diffusion via glucose transporters.

Active Transport—Active transport is characterized by (1) movement of chemicals against electrochemical or concentration gradients, (2) saturability at high substrate concentrations, (3) selectivity for certain structural features of chemicals, (4) competitive inhibition by chemical congeners or compounds that are carried by the same transporter, and (5) requirement for expenditure of energy, so that metabolic inhibitors block the transport process. Substances actively transported across cell membranes presumably form a complex with a membrane-bound macromolecular carrier on one side of the membrane. The complex translocates to the other side of the membrane, where the substance is released. Afterward, the carrier returns to the original surface to repeat the transport cycle.

Xenobiotic Transporters—It is estimated that at least 5% of all human genes are transporter related, indicating the importance of transport function in normal biological and toxicological outcomes. Transporters mediate the influx (uptake) or efflux of xenobiotics.

A large superfamily of transport proteins known as ATP-binding cassette (ABC) transporters has at least seven subfamilies (classified A to G) (Box 5–1). Many of these transporters play key roles in the homeostasis of endogenous substrates. Members of the ABC transport family are involved in absorption from the GI tract and elimination into bile or into urine for numerous xenobiotics. They are also critical to maintaining the barrier function of tissue sites including the blood–brain barrier (BBB), the blood–testis barrier, and the maternal–fetal barrier or placenta.

The second major family of xenobiotic transporters is the solute carriers (SLCs), which function predominantly as facilitative transporters. There are 43 SLC gene families identified, and many of the nearly 300 genes comprising the 43 distinct SLC families are involved in the disposition of endogenous compounds, including glucose, neurotransmitters, nucleotides, essential metals, and peptides. Additionally, several families that are vital to xenobiotic disposition are regulating the movement of many diverse organic anions and cations across cell membranes.

The major human solute carriers involved in xenobiotic disposition are summarized in Box 5–2. The organic anion–transporting peptides (OATPs, SLCO family) mediate the sodium-independent membrane transport of a wide range of compounds, including organic acids, bases, and neutral compounds. Although solutes can move bidirectionally, these proteins appear to be especially important in the hepatic uptake of xenobiotics. For OATPs, the mechanism of transport is anion exchange such that the cellular influx of an organic compound is thought to be coupled to the efflux of bicarbonate, glutathione, or glutathione conjugates. The multidrug and toxin extrusion transporters (MATEs; SLC47) are a unique gene family of solute carriers expressed predominantly in liver and kidney that function specifically as cation efflux pumps.

Additional Transport Processes— Other forms of specialized transport have been proposed, but their overall importance is not as well established as that of active transport and facilitated diffusion. Phagocytosis and pinocytosis are important for the removal of particulate matter from the alveoli by phagocytes and from blood by the reticuloendothelial system of the liver and spleen.

ABSORPTION

The process by which toxicants cross body membranes to enter the bloodstream is referred to as

absorption. Xenobiotics penetrate membranes during absorption by the same processes as do biologically essential substances such as oxygen, foodstuffs, and other nutrients. The main sites of absorption are the GI tract, lungs, and skin. Enteral administration includes routes pertaining to the alimentary canal (sublingual, oral, and rectal), whereas parenteral administration involves all other routes (intravenous, intraperitoneal, intramuscular, subcutaneous, etc.).

Absorption of Toxicants by the Gastrointestinal Tract

The GI tract may be viewed as a tube traversing the body. Although it is within the body, its contents remain outside of the body. Therefore, unless a noxious chemical has direct caustic or irritating properties, toxicants remaining in the GI tract may damage the cells that make up the organ, but usually do not produce systemic injury unless they are absorbed.

BOX 5–1 Human ABC Transporters: Gene Family Overview and Major Transporters Involved in Xenobiotic Disposition

ABC SUBFAMILY	GENES IN FAMILY	GENE SYMBOLS
A	12	*ABCA1-10, 12, 13*
B	**11**	***ABCB1-11***
C	**13**	***ABCC1-13****
D	4	*ABCD1-4*
E	1	ABCE1
F	3	*ABCF1-3*
G	5	***ABCG1, 2, 4, 5, 8***

Bolded subfamily designations are those with a major role in xenobiotic disposition.
**ABCC13 is reported to be a pseudogene.*

GENE SYMBOL	COMMON NAME	GENERAL FUNCTION	TOXICOLOGICAL IMPACT
ABCB1	Multidrug resistance protein/P-glycoprotein (MDR)	Efflux from gut, brain, and placenta; biliary excretion	Intestinal inhibition increases bioavailability Loss of expression increases penetration to brain and fetus
ABCB11	Bile salt export pump (BSEP)	Bile salt transport	Inhibition associated with drug-induced liver injury
ABCC1	Multidrug resistance–associated protein 1 (MRP1)	Multidrug resistance in many tissues; export pump	Inhibition may improve drug-refractory tumors
ABCC2	Multidrug resistance–associated protein 2 (MRP2)	Organic anion efflux, glucuronide and glutathione conjugates, and biliary excretion	Mediates efflux of drug-conjugates (i.e., NSAIDs) that can be deconjugated in intestine and cause intestinal injury. Loss or inhibition can cause hyperbilirubinemia
ABCC3	Multidrug resistance–associated protein 3 (MRP3)	Organic anion efflux, and glucuronide and glutathione conjugates	Some evidence for inhibition associated with drug-induced liver injury for TAK-875
ABCC4	Multidrug resistance–associated protein 4 (MRP4)	Nucleoside transport and organic anion efflux	Implicated in tenofovir disoproxil fumarate–induced renal proximal tubular toxicity, which is observed in humans
ABCG2	Breast cancer resistance protein (BCRP)	Organic anion efflux, many sulfate conjugates, and biliary excretion	Not well described Protection against protoporphyria and diet-dependent phototoxicity

Absorption of toxicants can take place along the entire GI tract, even in the mouth and the rectum. If a toxicant is an organic acid or base, it can be absorbed by simple diffusion in the part of the GI tract where it exists in its most lipid-soluble (nonionized) form. Because gastric juice is acidic (pH about 2) and the intestinal contents are nearly neutral, the lipid solubility of weak organic acids or bases can differ markedly in these two areas of the GI tract. It should also be noted that pH along the GI differs in the fed and fasting state. The Henderson–Hasselbalch equations determine the fraction of a toxicant that is in the nonionized (lipid-soluble) form and estimate the rate of absorption from the stomach or intestine. Other factors—including the mass action law, surface area, and blood flow rate—may influence the absorption of weak organic acids or bases. Absorption by simple diffusion is proportional to the surface area. The small intestine villi and microvilli increase the surface area approximately 600-fold, which greatly facilitates intestinal absorption.

Numerous xenobiotic transporters are expressed in the GI tract, where they function to increase or decrease absorption of xenobiotics. There are a handful of transporters in the intestine

that are considered to impact absorption—namely, PEPT1, OATP2B1, and MDR1 (*p*-glycoprotein; PgP).

Regarding uptake, several proteins in the SLC families are expressed in the intestine where they are predominantly localized on the apical brush-border membranes of the enterocytes and increase uptake from the lumen into the enterocytes. The efflux transporters are particularly relevant to the disposition of toxicants, as there will be a net reduction in the absorption of chemicals that are substrates for these transporters.

BOX 5–2 Major Members of the Human Solute Carrier Transporter Families Involved in Xenobiotic Disposition

TRANSPORTER	GENE FAMILY	HUMAN PROTEINS	GENE NAME	FUNCTION	TOXICOLOGICAL IMPACT
Organic anion transporting polypeptide (OATP)	*SLCO*	OATP1A2 OATP1B1 OATP1B3 OATP1C1 OATP2A1 OATP2B1 OATP3A1 OATP4A1 OATP4C1 OATP5A1 OATP6A1	SLCO1A2 SLCO1B1 SLCO1B3 SLCO1C1 SLCO2A1 SLCO2B1 SLCO3A1 SLCO4A1 SLCO4C1 SLCO5A1 SLCO6A1	Transport of organic anions, cations, and neutral compounds	1. OATP1B1: Decreased function due to polymorphisms can decrease hepatic extraction of substrates and leading to increased extrahepatic concentrations (example: statins); transport of chemicals of environmental concern (example: perfluoroalkyl substances) and microcystins 2. Oatp2b1: Transport of microcystins in rodents 3. OATP2B1: Expression in intestine and important bioavailability of organic anions
Organic cation transporter (OCT)	*SLC22*	OCT1 OCT2 OCT3	SLC22A1 SLC22A2 SLC22A3	Transport of organic cations	1. OCT1: Important for metformin uptake into liver and efficacy 2. OCT2: Cisplatin dose-limiting nephrotoxicity
Organic cation/ carnitine transporter (OCTN)	*SLC22*	OCTN1 OCTN2	SLC22A4 SLC22A5	Organic cations OCTN2 specific for carnitine	
Organic anion transporter (OAT)	*SLC22*	OAT1 OAT2 OAT3 OAT4 OAT5	SLC22A6 SLC22A7 SLC22A8 SLC22A11 SLC22A10	Transport of organic anions	OAT1: Antiviral drugs cidofovir and adefovir; methylmercury; ochratoxin A
Peptide transporter (PEPT)	*SLC15*	PEPT1 PEPT2	SLC15A1 SLC15A2	Transport of dipeptides and tripeptides, and xenobiotics	Aids in the renal excretion of beta-lactam antibiotics of the cephalosporin and penicillin classes, angiotensin-converting enzyme inhibitors (ACE inhibitors), and amino acid–conjugated antiviral drugs (e.g., valacyclovir)
Multidrug and toxin extrusion transporter (MATE)	*SLC47*	MATE1 MATE2K	SLC47A1 SLC47A2	Efflux of organic cations; MATE2K localized to kidney	Aids in metformin excretion

The number of toxicants actively absorbed by the GI tract is low; most enter the body by simple diffusion. Lipid-soluble substances are absorbed more rapidly and extensively than are water-soluble substances. Some organic ions of low molecular weight (<200 Da) can passively

cross the mucosal barrier by paracellular transfer through aqueous pores at the tight junctions.

Particles and particulate matter can also be absorbed by the GI epithelium. In this case, particle size is a major determinant of absorption, whereas factors such as the lipid solubility or ionization characteristics are less important. For particles, size is inversely related to absorption such that absorption increases with decreasing particle diameter. Large particles (greater than about 20 μm in diameter) enter intestinal cells by pinocytosis. There is increasing interest in nanoparticles or nanomaterials that are typically less than 100 nm in size and that may be used in various chemical and biological processes (see Chapter 28). Overall, the absorption of a toxicant from the GI tract depends on its physical properties, including lipid solubility and its dissolution rate. An increase in lipid solubility increases the absorption of chemicals.

Additional factors relating to the GI tract itself that influence the absorption of xenobiotics include pH, the presence of food, digestive enzymes, bile acids, and bacterial microflora in the GI tract, along with the motility and permeability of the GI tract. Chemical resistance or lack of resistance to alteration by the acidic pH of the stomach, enzymes of the stomach or intestine, or the intestinal microflora is extremely important. A toxicant may be hydrolyzed by stomach acid, biotransformed by enzymes in the GI tract, or modified by the resident microflora to new compounds with a toxicity different from that of the parent compound. Intestinal microflora can also influence absorption and toxicity of compounds.

Simple diffusion is proportional not only to the surface area and the permeability but also to the residency time within various segments of the GI tract. Therefore, the rate of absorption of a toxicant remaining for longer periods in the intestine increases, whereas that with a shorter residency time decreases. The oral toxicity of some chemicals is increased by diluting the dose. This phenomenon may be explained by more rapid stomach emptying induced by increased dosage volume, which in turn leads to greater absorption in the duodenum because of the larger surface area there. Furthermore, chemicals used as laxatives reduce absorption of xenobiotics by increasing intestinal motility, whereas agents used as antidiarrheals may increase absorption by slowing intestinal motility.

The amount of a chemical that enters the systemic circulation after oral administration depends on several factors. The amount absorbed into the cells of the GI tract is important, and transporters can influence this amount by affecting the uptake or efflux from the cells. Further, before a chemical enters the systemic circulation, it can be biotransformed by the cells in the GI tract or extracted by the liver and excreted into bile with or without prior biotransformation. This phenomenon of the removal of chemicals before entrance into the systemic circulation is referred to as presystemic elimination or first-pass effect. The lung can also contribute to the biotransformation or elimination of chemicals before passage into the systemic circulation. Chemicals that have a high first-pass effect will appear to have a lower absorption because they are eliminated as quickly as they are absorbed. For toxicants, a high first-pass effect will serve to limit exposure and typically minimize toxic potential.

Other factors may alter absorption. Although lead and many other heavy metal ions are not absorbed readily from the GI tract, ethylenediaminetetraacetic acid (EDTA) and other chelators increase the lipid solubility and absorption of complexed ions. Consumption of grapefruit juice can also influence GI absorption through the actions of naringin, a flavonoid that inhibits several transporters including MDR1 (Pgp). By reducing MDR1-dependent efflux, grapefruit juice increases GI absorption of numerous drugs, and, in some cases, this effect leads to toxic or adverse reactions resulting from increased drug exposure. Inhibition of OATPs reduces absorption and may limit toxicity.

Absorption of Toxicants by the Lungs

Toxic responses to chemicals can occur following inhalation exposure. Toxicants that are absorbed by the lungs are gases, vapors of volatile or volatilizable liquids, and aerosols (see Chapter 15).

Gases and Vapors—A vapor is the gas form of substance that can also exist as a liquid or solid at atmospheric pressure and normal temperature. Most organic solvents evaporate and produce vapors, and some solids can sublimate into a gaseous form. Vapor pressure is that exerted by a vapor above its own liquid in a closed system, such that liquids that have a high vapor pressure have a higher tendency to evaporate. A toxicant with a high vapor pressure at room temperature is considered volatile.

When inhaled, gases first pass through the nose, filtering through delicately scrolled, simple epithelial-lined turbinates, which serve to increase the surface area of exposure. Because the mucosa of the nose is covered by a film of fluid, gas molecules can be retained by the nose and do not reach the lungs if they are water soluble or react with cell surface components. Therefore, the nose acts as a "scrubber" for water-soluble and highly reactive gases, partially protecting the lungs from potentially injurious insults. These actions may increase the risk of adverse effects in the nose.

Once inhaled into the lungs, gas molecules diffuse from the alveolar space into the blood until equilibrium is reached. As the inspired gas remains in contact with blood in the alveoli, more molecules dissolve in blood until gas molecules in blood are in equilibrium with gas molecules in the alveolar space. Thus, gas molecules partition between two media, namely air and blood during the absorptive phase and blood and other tissues during the distributive phase. At this equilibrium, the ratio of the concentration of chemical in the blood and in the gas phase is constant. This solubility ratio is called the blood-to-gas partition coefficient, and it is unique for each gas. Note that although the ratio is constant, the concentrations achieved vary in accordance with Henry's law, which dictates that the amount of gas dissolved in a liquid is proportional to the partial vapor pressure of the gas in the gas phase at any given concentration before or at saturation. Thus, the higher the inhaled concentration of a gas (i.e., the higher the partial pressure), the higher the gas concentration in blood, but the blood:gas ratio does not change unless saturation has occurred. When equilibrium is reached, the rate of transfer of gas molecules from the alveolar space to blood equals the rate of removal by blood from the alveolar space.

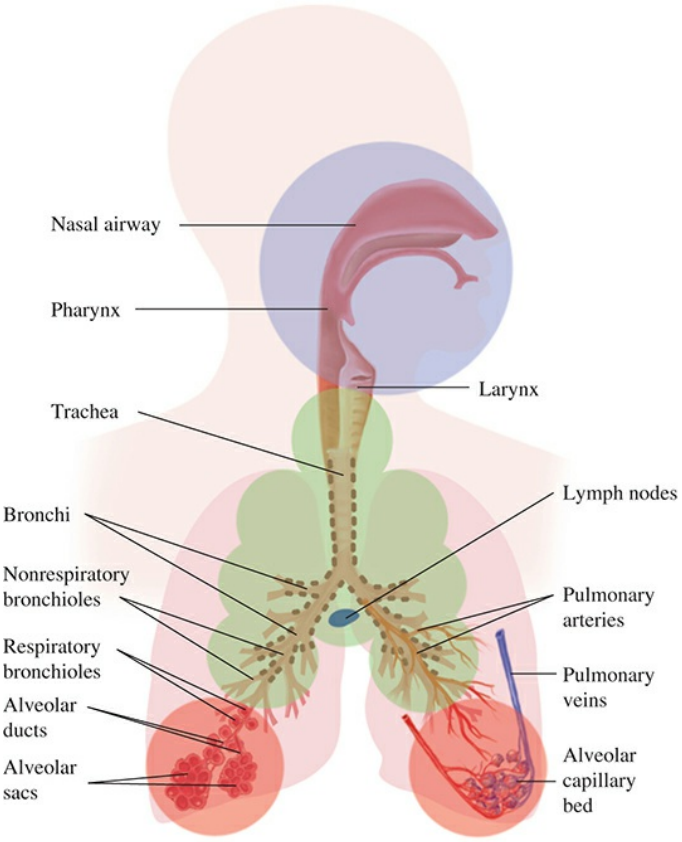
The more soluble a toxic chemical is in blood, the more of it will be dissolved in blood by the time equilibrium is reached. Consequently, the time required to equilibrate with blood is much longer for a gas with a high blood-to-gas partition coefficient than for a gas with a low ratio. With highly soluble gases, the principal factor limiting the rate of absorption is respiration. Because the blood is already removing virtually all gases with a high solubility ratio from the lungs, increasing the blood flow rate does not substantially increase the rate of absorption. However, the rate can be accelerated greatly by increasing the rate of respiration.

The blood carries the dissolved gas molecules to the rest of the body. In each tissue, the gas molecules are transferred from the blood to the tissue until equilibrium is reached at a tissue concentration dictated by the tissue-to-blood partition coefficient. After releasing part of the gas to tissues, blood returns to the lungs to take up more of the gas. The process continues until a gas reaches equilibrium between blood and each tissue according to the tissue-to-blood partition coefficients characteristic of each tissue. This equilibrium is referred to as steady state, and at

this time, no net absorption of gas takes place as long as the exposure concentration remains constant.

AEROSOLS AND PARTICLES

Absorption of aerosols (a colloid of solid particles and lipid droplets in air) and particles is determined by the aerosol size and water solubility of any chemical present in the aerosol. The site of deposition of aerosols and particulates depends largely on the size of the particles (see Chapter 15). In general, the smaller the particle, the further into the respiratory tree the particle will deposit (Fig. 5–4). Particles ranging from 5 μm or larger, described as "course particles," usually are deposited in the nasopharyngeal region. Those deposited on the unciliated anterior or rostral portion of the nose tend to remain at the site of deposition until they are removed by nose wiping, blowing, or sneezing. The mucous blanket of the ciliated nasal surface propels insoluble particles by the movement of the cilia. These particles and particles inhaled through the mouth are swallowed within minutes. Soluble particles may dissolve in the mucus and be carried to the pharynx or may be absorbed through the nasal epithelium into the blood.



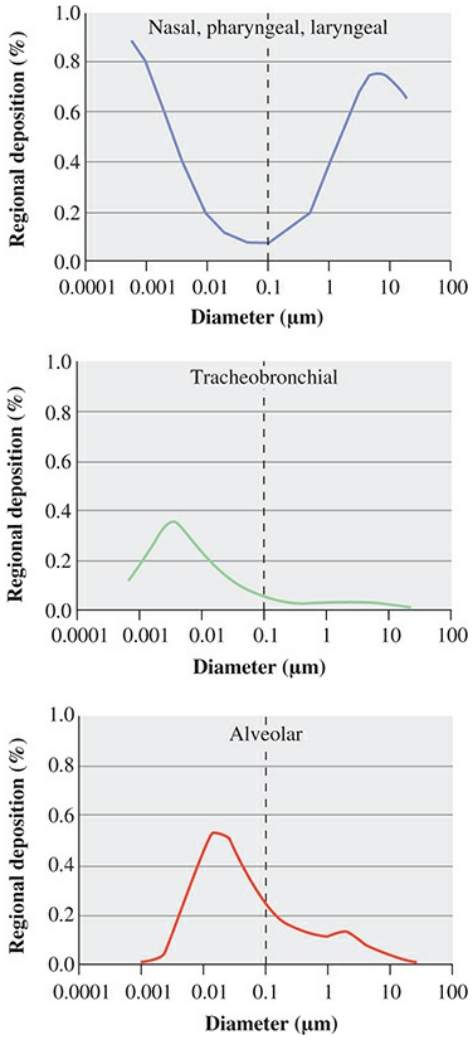


FIGURE 5–4 *Schematic diagram of the absorption and translocation of chemicals by lungs.* (Reprinted from Oberdorster G, Oberdorster E, Oberdorster J: Nanotoxicology: An emerging discipline evolving from studies of ultrafine particles. *Environ Health Perspect.* 2005;113:823–539.

Particulate matter with diameters of approximately 2.5 μm, referred to as "fine particles," are deposited mainly in the tracheobronchiolar regions of the lungs, from which they may be cleared by retrograde movement of the mucus layer in the ciliated portions of the respiratory tract (also known as the mucociliary escalator). Toxicants or viral infections that damage cilia may impair the efficiency of this process. Measurements have shown transport rates between 0.1 and 1 mm/min. Coughing and sneezing greatly increase the movement of mucus and particulate matter toward the mouth. Particles may be swallowed and absorbed from the GI tract or expectorated.

Particles 1 μm and smaller penetrate to the alveolar sacs of the lungs. Ultrafine particles or nanoparticles, particularly those that are approximately 10 to 20 nm in size, have the greatest likelihood of depositing in the alveolar region. These extremely small particles may be absorbed into blood or cleared through the lymphatics after being scavenged by alveolar macrophages.

Removal or absorption of particulate matter from the alveoli appears to occur by three major mechanisms. First, particles may be removed from the alveoli by a physical process. As described earlier, it is thought that particles deposited on the fluid layer of the alveoli are aspirated onto the mucociliary escalator of the tracheobronchial region. From there, they are transported to the mouth and may be swallowed. Second, particles from the alveoli may be removed by phagocytosis by the resident alveolar macrophages. These phagocytic cells are found in large numbers in normal lungs and contain many phagocytized particles of both exogenous and endogenous origins. They apparently migrate to the distal end of the mucociliary escalator and are cleared and eventually swallowed. Third, removal may occur via the lymphatics. The endothelial cells lining lymphatic capillaries are permeable to very large molecules and particles (molecular weight >1000 kDa), thereby collecting high-molecular-weight proteins leaked from cells or blood capillaries and particulate matter from the interstitium and the alveolar spaces. Particulate matter may remain in lymphatic tissue for long periods.

In general, the overall removal of particles from the alveoli is relatively inefficient. The rate of clearance by the lungs can be predicted by a compound's solubility in lung fluids. The lower the solubility, the lower the removal rate. Thus, removal of particles that enter the alveoli is largely due to dissolution and vascular transport. Some particles may remain in the alveoli indefinitely, which may occur when long-lived alveolar macrophages phagocytose indigestible dust particles and secrete cytokines that stimulate the development of a local network of type I and III collagen fibers to form an alveolar dust plaque or nodule.

Absorption of Toxicants Through the Skin

Skin is the largest body organ and provides a relatively good barrier for separating organisms from their environment. Overall, human skin contacts many toxic chemicals, but exposure is usually limited by its relatively impermeable nature. However, some chemicals can be absorbed by the skin in quantities sufficient to produce systemic effects.

The skin comprises two major layers, the epidermis and dermis (Fig. 5–5). The epidermis is the outermost layer and contains keratinocytes that are metabolically competent and able to divide. Proliferating keratinocytes in the stratum germinativum displaces maturing keratinocyte

layers upward until they reach the outermost layer, the stratum corneum. The stratum corneum contains densely packed keratinized cells that have lost their nuclei and are biologically inactive. The stratum corneum represents the single most important barrier to preventing fluid loss from the body while also serving as the major barrier to prevent the absorption of xenobiotics into the body.

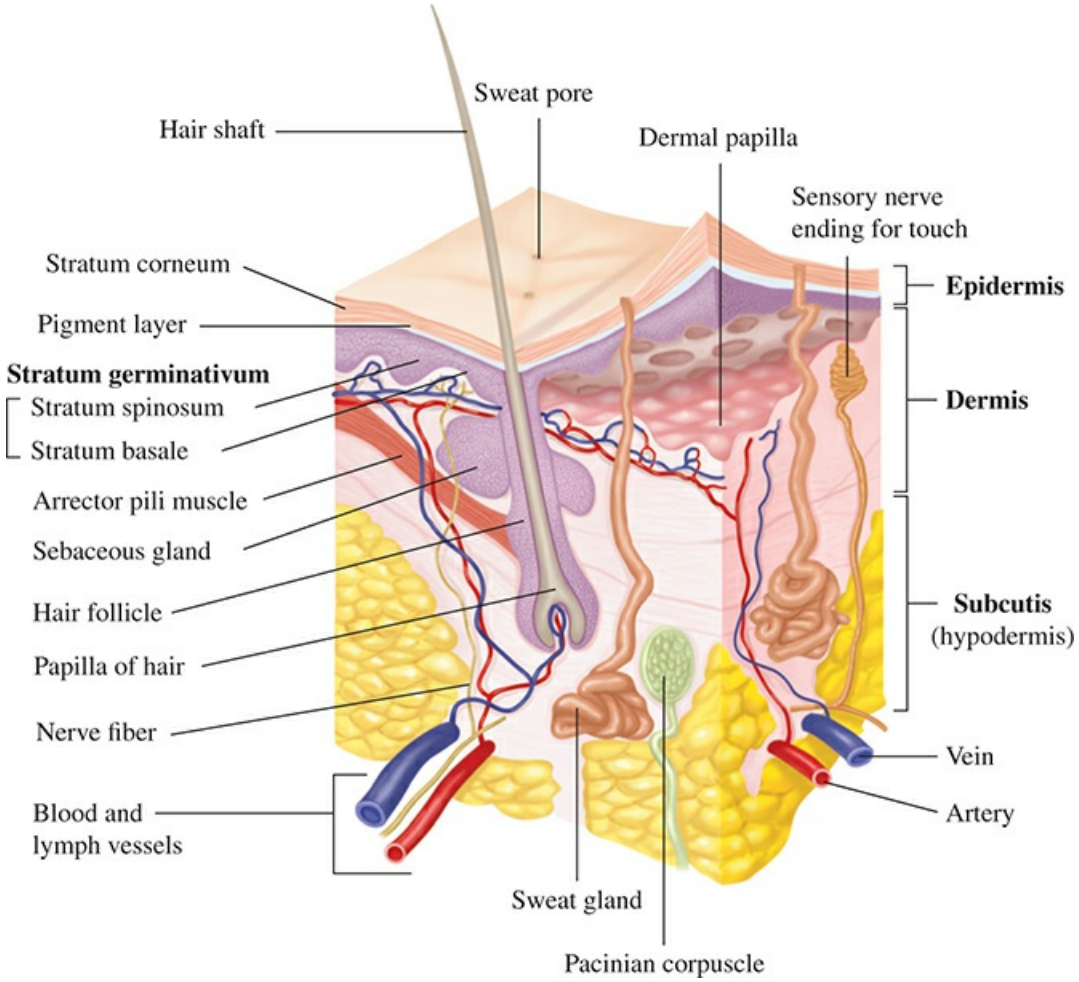


FIGURE 5–5 *Diagram of a cross-section of human skin.*

The dermis is situated beneath the epidermis and consists primarily of fibroblasts. This region contains the vascular network that provides the dermis and epidermis with blood supply and serves to carry absorbed compounds into the body. Chemicals may also be absorbed through dermal appendages, including sweat and sebaceous glands and hair follicles, found in the dermis. Sweat glands and hair follicles are scattered in varying densities on skin. These appendages account for no more than 1% of the total cross-sectional area of the total skin surface, and, in general, passage through these areas is much more rapid than passage through the stratum corneum. Ultimately, to be absorbed a chemical must pass the barrier of the stratum corneum and then traverse the other six layers of the skin. All toxicants move across the stratum corneum by passive diffusion. In general, lipophilic compounds are absorbed more readily across the stratum corneum, whereas the penetration of hydrophilic compounds is more limited. Nonpolar toxicants diffuse through the skin in a manner that is proportional to their lipid solubility and inversely

related to molecular weight. Although lipophilic compounds may pass more readily through the stratum corneum, their passage through the dermis may become rate-limiting. Hydrophilic compounds are more likely to penetrate the skin through appendages such as hair follicles.

The permeability of the skin also depends on both the diffusivity and the thickness of the stratum corneum. Although the stratum corneum is much thicker on the palms and soles (400 to 600 μm in callous areas) than on the arms, back, legs, and abdomen (8 to 15 μm), it has much higher diffusivity per unit thickness. In contrast, the skin of the scrotum is characterized by a thin stratum corneum and a high diffusivity. The second phase of percutaneous absorption consists of diffusion of the toxicant through the lower layers of the epidermis (stratum germinativum, spinosum, and granulosum) and the dermis. Despite possessing tight intercellular junctions, these cell layers contain a porous, nonselective, aqueous diffusion medium. Toxicants pass through this area by diffusion and enter the systemic circulation through the numerous venous and lymphatic capillaries in the dermis. The rate of diffusion depends on blood flow, interstitial fluid movement, and perhaps other factors, including interactions with dermal constituents.

There are several factors that can influence the absorption of toxicants through the skin, including (1) the integrity of the stratum corneum, (2) the hydration state of the stratum corneum, (3) temperature, (4) solvents as carriers, and (5) molecular size. Solvents used to dissolve compounds of interest can also influence dermal penetration. In general, lower absorption will be observed if a toxicant is highly soluble in the vehicle, whereas low solubility of the toxicant in the vehicle will tend to increase dermal penetration. With respect to molecular size, it is generally recognized that compounds above 400 Da exhibit poor dermal penetration.

Inter-species differences in dermal absorption of xenobiotics result from variance in (1) the composition and thickness of the stratum corneum along with the nature of dermal appendages, (2) cutaneous blood flow, (3) biotransformation reactions, and (4) the levels and patterns of xenobiotic transporters.

Absorption of Toxicants After Special Routes of Administration

Chemicals may be administered by other routes, including (1) intravenous, (2) intraperitoneal, (3) subcutaneous, and (4) intramuscular. The intravenous route introduces the toxicant directly into the bloodstream, eliminating the process of absorption. Intraperitoneal injection results in rapid absorption of xenobiotics because of the rich peritoneal and mesenteric blood supply and the relatively large surface area of the peritoneal cavity. In addition, this route of administration circumvents the delay and variability of gastric emptying. Subcutaneous and intramuscular injections usually result in slower absorption rates, but toxicants enter directly into the general circulation.

The toxicity of a chemical may or may not depend on the route of administration. If a toxicant is injected intraperitoneally, most of the chemical enters the liver via the portal circulation before reaching the general circulation. A compound may be completely extracted and biotransformed by the liver with subsequent excretion into bile without gaining access to the systemic circulation. A chemical with a high first-pass effect that is toxic in an organ other than the liver and GI tract is likely to be less toxic when administered intraperitoneally than when administered by other routes (intravenously, intramuscularly, or subcutaneously) because the intraperitoneal route favors extraction in the liver to reduce what is available systemically. Therefore, preliminary information on the contribution of biotransformation and excretion of xenobiotics to toxic outcome can be derived by comparing toxic responses after administration by different

routes.

DISTRIBUTION

After entering the bloodstream, regardless of route of exposure, a toxicant distributes to tissues throughout the body. The rate of distribution to organs or tissues is determined primarily by blood flow and the rate of diffusion out of the capillary bed into the cells of an organ or a tissue, and usually occurs rapidly. The final distribution depends largely on the affinity of a xenobiotic for various tissues. In general, the initial phase of distribution is dominated by blood flow, whereas the eventual distribution is determined largely by affinity.

Volume of Distribution

A key concept in understanding the disposition of a toxicant is its volume of distribution (Vd), a primary determinant of the concentration of a toxicant in blood that is used to quantify distribution throughout the body. It is defined as the volume in which the amount of drug would need to be uniformly dissolved in order to produce the observed blood concentration. Total body water is derived from that which is either extracellular or intracellular and represents three distinct compartments: plasma water and interstitial water comprise the extracellular compartment and are distinguished from intracellular water (Box 5–3). If a chemical distributes only to the plasma compartment (no tissue distribution), it has a high plasma concentration and hence a low Vd. In contrast, if a chemical distributes throughout the body (total body water), the effective plasma concentration is low and hence a high Vd. However, the distribution of toxicants is complex and under most circumstances cannot be simply equated with distribution into one of the water compartments of the body. Binding to and/or dissolution in various storage sites of the body, such as fat, liver, and bone, are important factors in determining the distribution of chemicals.

Some toxicants do not readily cross cell membranes and therefore have restricted distribution, whereas other toxicants rapidly pass through cell membranes and are distributed throughout the body. Some toxicants selectively accumulate in certain parts of the body as a result of protein binding, active transport, or high solubility in fat.

Storage of Toxicants in Tissues

Because only the free fraction of a chemical is in equilibrium throughout the body, binding to or dissolving in certain body constituents greatly alters the distribution of a xenobiotic. Some xenobiotics attain their highest concentrations at the site of toxic action. Other chemicals concentrate at sites other than the target organ. The compartment where a toxicant is concentrated but is not the major site of toxicity for that chemical is described as a storage depot. Toxicants in these depots are always in equilibrium with the free fraction in plasma, so that as a chemical is biotransformed or excreted from the body, more is released from the storage site. As a result, the biological half-life of stored compounds can be very long.

Plasma Proteins as Storage Depot— Several plasma proteins bind xenobiotics and some

endogenous constituents of the body (Fig. 5–6). Albumin is the major protein in plasma, and it binds many different drugs and xenobiotics. Glycoproteins, β-globulins, α- and β-lipoproteins, and plasma γ-globulins also bind chemicals.

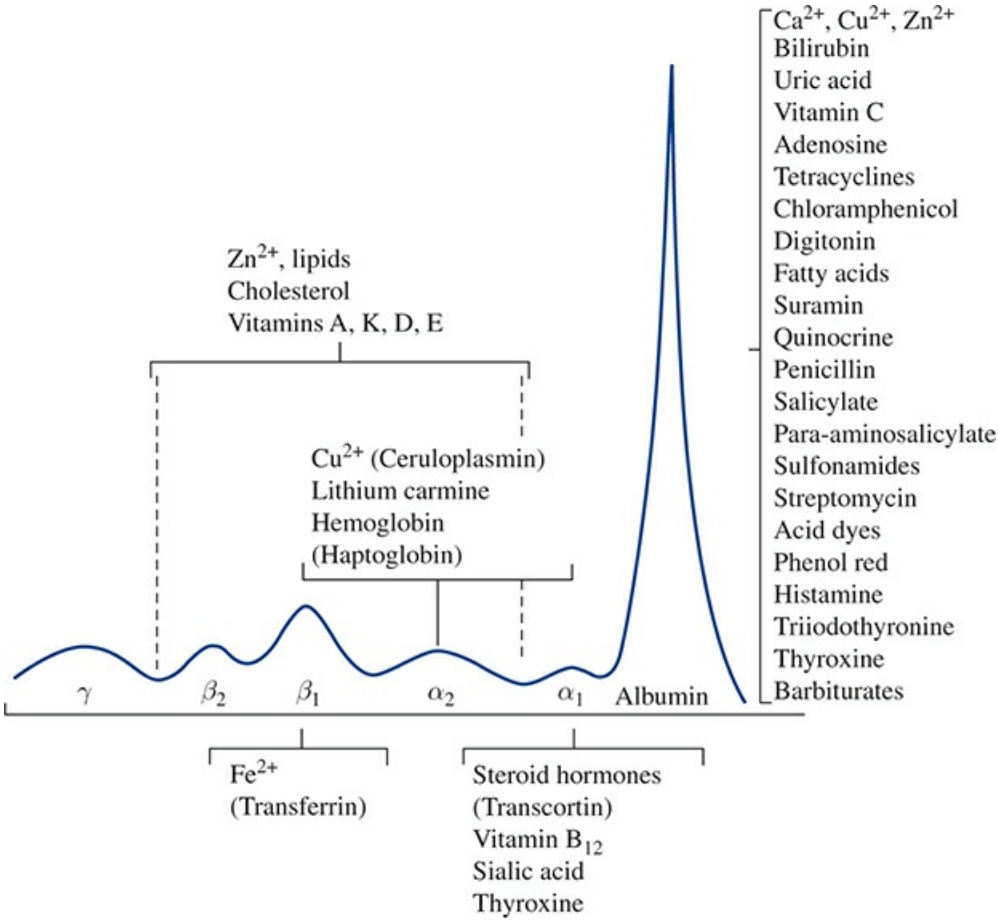


FIGURE 5–6 *Schematic representation of the electrophoretic separation of plasma proteins and xenobiotics that interact with these proteins.*

Protein–ligand interactions occur primarily as a result of hydrophobic forces, hydrogen bonding, and Van der Waals forces. Because of their high molecular weight, plasma proteins with bound toxicants cannot cross capillary walls. Consequently, the fraction of a toxicant bound to plasma proteins is not immediately available for distribution into the extravascular space or filtration by the kidneys. However, the interaction of a chemical with plasma proteins is a reversible process, and as unbound chemical diffuses out of capillaries, bound chemical dissociates from the protein until the free fraction reaches equilibrium between the vascular space and the extravascular space. In turn, diffusion in the extravascular space to sites more distant from the capillaries continues, and the resulting concentration gradient provides the thermodynamic force for continued dissociation of the bound fraction in plasma.

The binding of chemicals to plasma proteins is an important concept in toxicology. Toxicity is typically manifested by the amount of a xenobiotic that is unbound. Therefore, a compound with a high degree of plasma protein binding may not show toxicity when compared to one that is less extensively bound to plasma proteins. Severe toxic reactions can occur if a toxicant with a high

degree of protein binding is displaced from plasma proteins by another chemical, increasing the free fraction of the toxicant in plasma. Xenobiotics can also compete with and displace endogenous compounds that are bound to plasma proteins.

Plasma protein binding can also give rise to species differences in the disposition of xenobiotics. Plasma protein binding across species may be influenced by differences in albumin concentration, in binding affinity, and/or in competitive binding of endogenous substances.

BOX 5–3 Estimates of the Size of the Major Compartments That Contribute to Volume of Distribution

COMPARTMENT	PERCENTAGE OF TOTAL	LITER IN 70-kg HUMAN	PLASMA CONCENTRATION AFTER 1 g OF CHEMICAL (mg/L)
Plasma water	4.5	3	333
Total extracellular water	20	14	71
Total body water	55	38	26
Tissue binding	—	—	0–25

Liver and Kidney as Storage Depots—The liver and kidney have a high capacity for acting as a storage depot for many chemicals. These two organs probably concentrate more toxicants than do all the other organs combined, and active transport or binding to tissue components is likely to be involved.

Fat as Storage Depot—Many organic compounds are highly stable and lipophilic, leading to their distribution and concentration in body fat where they are retained for a long time. Storage lowers the concentration of the toxicant in the target organ such that toxicity is likely to be less severe in an obese person than in a lean individual. However, a sudden increase in the concentration of a chemical in blood and the target organ of toxicity may occur if rapid mobilization from fat occurs.

Bone as Storage Depot—Skeletal uptake of xenobiotics is essentially a surface chemistry phenomenon, with exchange taking place between the bone surface and the fluid in contact with it. Toxicants can be released from bone by ionic exchange at the crystal surface and dissolution of bone crystals through osteoclastic activity. Ultimately, deposition and storage of toxicants in bone may or may not be detrimental.

Blood–Brain Barrier

Access to the brain is restricted by the presence of two barriers: the blood–brain barrier (BBB) and the blood–cerebral spinal fluid barrier (BCSFB). Although neither represents an absolute barrier to the passage of toxic chemicals into the CNS, many toxicants do not enter the brain in appreciable quantities because of these barriers.

The BBB is formed primarily by the endothelial cells of blood capillaries in the brain. Each

endothelial cell forms a tight junction with adjacent cells, essentially forming a tight seal between the cells and preventing diffusion of polar compounds through paracellular pathways. Diffusion of more lipophilic compounds through endothelial cell membranes is counteracted by xenobiotic efflux transporters present in the endothelial cells. Glial cells, particularly astrocytes, contribute to the BBB by secreting chemical factors that modulate endothelial cell permeability, and astrocytes and perivascular microglial cells extend processes that support the integrity of the BBB. Active transport processes also play a pivotal role in determining the concentration of xenobiotics in the brain. ATP-dependent transporters have been identified in the BBB, comprising various members of the ABC and SLC families. Efflux transporters including MDR1 (PGP), BCRP, MRP1, MRP2, MRP4, and MRP5 are located on the apical (blood side) plasma membrane and function to move xenobiotics absorbed into the capillary endothelial cells out into the blood. Uptake transporters including OATP1A2 and OATP1C1 are found on both the basolateral and apical sides of the endothelium and can drive a concentrative efflux if coupled energetically to the electrical potential difference across the endothelial cell membrane. Uptake transporters on the apical membranes include OAT3 and CTN2. In combination, these transporters can efficiently efflux a wide range of anionic, cationic, uncharged, and drug conjugates from the brain.

The BCSFB is found between the circulating blood and the circulating cerebral spinal fluid in the brain. It comprises elements of the choroid plexus, the arachnoid membrane, and certain periventricular locations (including the area postrema). The choroid plexus has highly permeable endothelial cells on the blood side but is lined by epithelial cells on the cerebrospinal fluid (CSF) side that form the barrier. Xenobiotic transporters contribute to removing compounds that may enter the endothelial cells. In general, toxicants achieve concentrations in the CSF that are no higher than the concentration of the unbound toxicant in the plasma.

The entrance of toxicants into the brain follows the same principle that applies to transfer across other cells in the body. Only the free fraction of a toxicant (i.e., not bound to plasma proteins) equilibrates rapidly with the brain. Lipid solubility and the degree of ionization help determine the rate of entry of a compound into the CNS. A few xenobiotics may enter the brain by carrier-mediated processes. The BBB is not fully developed at birth, and this is one reason why some chemicals are more toxic in newborns than adults.

Passage of Toxicants Across the Placenta

The placenta is a multifunctional organ that also provides nutrition for the conceptus, exchanges maternal and fetal blood gases, disposes of fetal excretory material, and maintains pregnancy through complex hormonal regulation. Placental structure and function show more species differences than any other mammalian organ.

Most vital nutrients necessary for fetal development, including vitamins, amino acids, essential sugars, and ions such as calcium and iron, are transported by active transport systems from mother to fetus against a concentration gradient. Like nutrients, toxic chemicals can be transported across the placental barrier or pass through the placenta via passive permeability. A few xenobiotics are physiological substrates for active transport from the maternal to the fetal circulation. The placenta also has biotransformation capabilities that may prevent some toxic substances from reaching the fetus. Among the substances that cross the placenta by passive diffusion, more lipid-soluble substances rapidly attain a maternal–fetal equilibrium. Attributes including degree of ionization, lipophilicity, protein binding, and molecular weight along with

blood flow and the concentration gradient across the barrier influence chemical movement. Under steady-state conditions, concentrations of a toxic compound in the plasma of the mother and fetus are usually the same.

In addition to chemicals, viruses (e.g., rubella virus), cellular pathogens (e.g., syphilis spirochetes), and globulin antibodies can traverse the placenta. The human placenta includes the syncytiotrophoblast and cytotrophoblast layers. The apical membrane of the syncytiotrophoblast, which forms a continuous epithelial layer, is bathed in maternal blood and the basolateral surface is in contact with the discontinuous cytotrophoblast layer, the stromal tissue, or the fetal vasculature. To reach the fetus, toxins must traverse the apical and basolateral membranes of the syncytiotrophoblast as well as the endothelium of the fetal capillaries. Xenobiotic transporters are differentially expressed in these various cells and contribute to the barrier function that restricts distribution of toxicants to the fetus. The transporters are also critical to the movement of nutrients from the maternal circulation to the fetus along with the transfer of toxicants or waste products from the fetus back to the maternal circulation. Of the various cells in the placenta, the syncytiotrophoblasts appear to have the most extensive cohort of xenobiotics transporters.

Redistribution of Toxicants

The most critical factors that affect the distribution of xenobiotics are the organ blood flow and its affinity for a xenobiotic. The initial phase of distribution is determined primarily by blood flow to the various parts of the body. Therefore, a well-perfused organ such as the liver may attain high initial concentrations of a xenobiotic. However, chemicals may have a high affinity for a binding site (e.g., intracellular protein or bone matrix) or to a cellular constituent (e.g., fat), and, with time, will redistribute to these high-affinity sites.

EXCRETION

Toxicants are eliminated from the body by several routes. Biotransformation to more water-soluble products is usually a prerequisite to the excretion of xenobiotics through urine (see Chapter 6). Renal and fecal exretion are qualitatively very important. All body secretions appear to have the ability to excrete chemicals; toxicants have been found in sweat, saliva, tears, and milk.

Urinary Excretion

Toxic compounds are excreted in urine by the same mechanisms the kidney uses to remove the end products of intermediary metabolism from the body, including glomerular filtration, tubular excretion by passive diffusion, and active tubular secretion.

A toxicant filtered at the glomerulus may remain in the tubular lumen and be excreted in urine. Depending on the physicochemical properties of the compound, it may be reabsorbed across the tubular cells of the nephron back into the bloodstream. Toxicants with a high lipid/water partition coefficient are reabsorbed efficiently, whereas polar compounds and ions are excreted in urine. The pH of urine may vary but it is usually slightly acidic (approximately 6 to

6.5). Just as the Henderson–Hasselbach calculations determine the absorption of nonionized compounds from the GI tract, they also determine urinary excretion. In this case, urinary excretion of the ionized moiety is favored.

Xenobiotics can also be excreted into urine by active secretion. This process involves the uptake of toxicants from the blood into the cells of the renal proximal tubule, with subsequent efflux from the cell into the tubular fluid from which urine is formed. Figure 5–7 illustrates the families of transporters expressed in the human kidney that are directly involved in xenobiotic disposition. Other transporters such as specific glucose transporters that play a role predominantly in the flux of endogenous substances are not presented here. Transporters may be expressed on the apical cell membrane where efflux pumps contribute to tubular secretion and influx pumps are important for reabsorption. Transporters localized to the basolateral membranes serve to transport xenobiotics to and from the systemic circulation or the renal tubular cells contributing to reabsorptive and excretory processes.

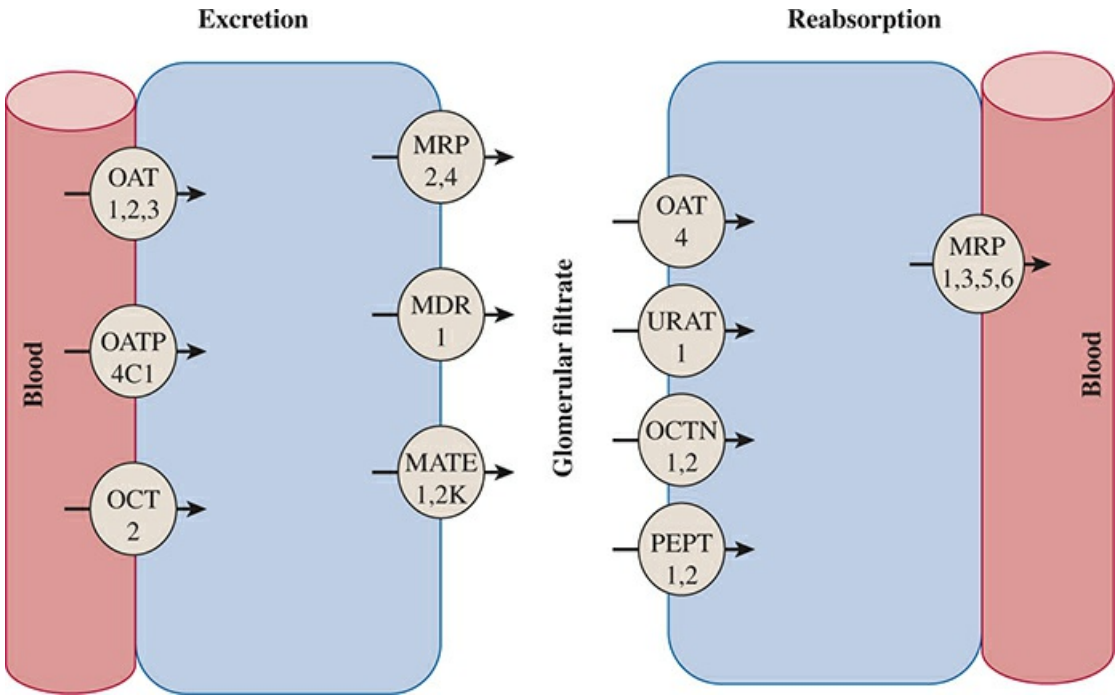


FIGURE 5–7 *Schematic model showing the transport systems in the human proximal tubule of the kidney.* The families of transporters are organic-anion transporters (OAT), organic-cation transporters (OCT), multidrug-resistant protein (MDR), multiresistant drug protein (MRP), peptide transporters (PEP), and urate transporter (URAT).

Many functions of the kidney are incompletely developed at birth, causing some xenobiotics to be eliminated more slowly in newborns than in adults. Species differences regarding the urinary excretion of weak organic acids and bases are observed frequently, as the pH of urine varies widely among species. Renal clearance can vary for compounds filtered at the glomeruli because of differences in plasma protein binding. Similarly, variations in xenobiotic transporter expression, regulation, and function as well as in biotransformaton can contribute to differences in the renal excretion of toxicants.

Fecal Excretion

Fecal excretion, the second major pathway for the elimination of xenobiotics, is a complex process. Excretion of toxicants via the feces can result from direct elimination of nonabsorbed compounds in the GI tract, from delivery to the GI tract via the bile, and from secretion into intestinal luminal contents from the enterocytes.

Nonabsorbed Ingesta—In addition to undigested material, varying proportions of nutrients and xenobiotics that are present in food or are ingested voluntarily (drugs) pass through the alimentary canal unabsorbed. Although most chemicals are lipophilic to some extent, it is rare for 100% of a compound to be absorbed. The nonabsorbed portion of xenobiotics contributes to the fecal excretion of most chemicals to some extent. Intestinal secretion, which likely occurs by passive diffusion out of enterocytes or via transporter-mediated processes, contributes to fecal excretion.

Biliary Excretion—The biliary route of elimination contributes significantly to the fecal excretion of xenobiotics and their metabolites. The liver can remove toxic chemicals from blood after absorption from the GI tract because blood from the GI tract passes via the portal circulation through the liver before reaching the general circulation, thereby preventing distribution to other parts of the body. The liver is also the main site for biotransformation of toxicants, and metabolites may be excreted directly into bile. In this manner, the liver can remove xenobiotics and their metabolites before entering the general circulation, which is referred to as the “first-pass effect.” Furthermore, xenobiotics and/or their metabolites excreted into bile enter the intestine and may be excreted with feces. However, if the physicochemical properties favor reabsorption, an enterohepatic circulation may ensue.

Toxic chemicals bound to plasma proteins are fully available for active biliary excretion. The factors that determine whether a chemical will be excreted into bile or into urine are not fully understood. Generally, low-molecular-weight compounds (<325) are poorly excreted into bile. Glutathione and glucuronide conjugates have a high predilection for excretion into bile, but there are marked species differences in the biliary excretion of foreign compounds with consequences for the biological half-life of a compound and its toxicity.

Biliary excretion is regulated predominantly by xenobiotic transporters present on the canalicular membrane, which include MRP2, BCRP, MDR1 (PGP), MATE1, and BSEP (Fig. 5–8). MRP2 is extremely important in biliary excretion because it is largely responsible for the transport of organic anions including glucuronide and glutathione conjugates of many xenobiotics. BCRP has particular affinity for sulfated conjugates of toxicants, whereas MDR1 (PGP) transports primarily organic bases into bile. MATE1 is specifically involved in biliary excretion of organic cations, and BSEP is critical for the secretion of bile acids and the regulation of bile flow.

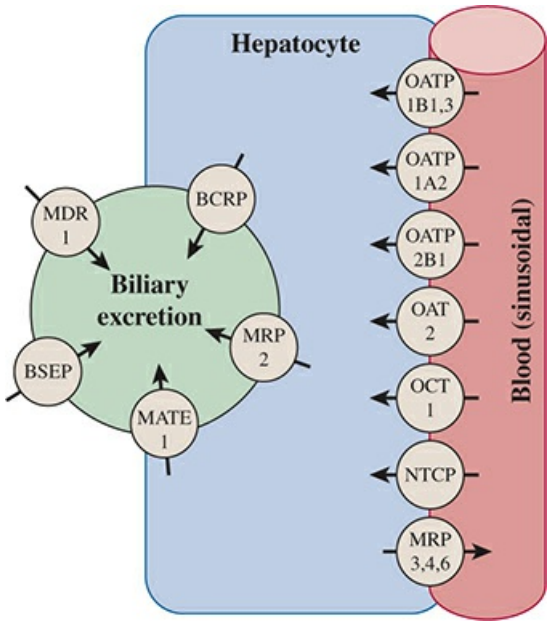


FIGURE 5–8 *Schematic model showing the xenobiotic transporting systems present in the human liver.* OATP = organic-anion transporting polypeptide, OCT = organic-cation transporter, BSEP = bile salt excretory protein, MDR = multidrug-resistant protein, MRP = multiresistant drug protein, BCRP = breast cancer resistance protein, and NTCP = sodium-dependent taurocholate peptide.

Other xenobiotic transporters localized to the sinusoidal membranes are also important in determining hepatic concentrations of toxicants and thereby contribute to hepatic disposition and biliary elimination. Transporters present on sinusoidal membranes include the ABC transport family members MRP3, MRP4, and MRP6, along with OATPs, OATs, and OCT. The Na^+/taurocholate cotransporting polypeptide is also found on the sinusoidal membrane where it functions specifically in the uptake of bile acids into the liver. Thus, the efficacy of hepatic extraction of drugs and xenobiotics from blood can impact extra-hepatic concentrations of drug and cause potential adverse drug toxicities.

An important concept relating to biliary excretion is the phenomenon of enterohepatic circulation. After a compound is excreted into bile, it enters the intestine where it can be reabsorbed or eliminated with feces. Many organic compounds are conjugated with UDP-glucuronic acid, sulfate, or glutathione before excretion into bile, and these polar metabolites are not sufficiently lipid soluble to be reabsorbed. However, enzymes found in the intestinal microflora may hydrolyze glucuronide and sulfate conjugates, liberating a more lipophilic moiety and increasing the likelihood of reabsorption. Intestinal reabsorption of the liberated xenobiotic completes a cycle in which the compound can return to the liver, where it can again be metabolized and excreted back into bile. Repeated enterohepatic cycling can lead to very long half-lives of xenobiotics in the body. Therefore, it is often desirable to interrupt this cycle to hasten the elimination of a toxicant from the body.

An increase in hepatic excretory function also has been observed after pretreatment with some drugs causing induction of biotransformation enzymes and xenobiotic transporters. Induction of these processes increases the capacity for a xenobiotic to be (1) taken up into the liver; (2) metabolized to conjugates that are likely to be excreted into bile; and (3) excreted into bile and

removed from the general circulation.

Exhalation

Substances that exist predominantly in the gas phase at body temperature are eliminated mainly by the lungs because volatile liquids are in equilibrium with their gas phase in the alveoli. The amount of a liquid eliminated via the lungs is proportional to its vapor pressure. A practical application of this principle is seen in the breath analyzer test for determining the amount of ethanol in the body.

No specialized transport systems have been described for the excretion of toxic substances by the lungs. Some xenobiotic transporters, including MRP1 and MDR1, have been identified in the lung, but overall, compounds excreted via exhalation in the lung are most likely to be eliminated by simple diffusion. Elimination of gases is roughly inversely proportional to the rate of their absorption. The rate of elimination of a gas with low solubility in blood is perfusion-limited, whereas that of a gas with high solubility in blood is ventilation-limited.

Other Routes of Elimination

Cerebrospinal Fluid—A specialized route of removal of toxic chemicals from a specific organ is represented by the cerebrospinal fluid (CSF). All compounds can leave the CNS with the bulk flow of CSF through the arachnoid villi. In addition, lipid-soluble toxicants also can exit at the site of the BBB. Toxicants also can be removed from the CSF by active transport, using the transport systems present in the BCSFB.

Milk—Secretion of toxic compounds into milk is extremely important because (1) toxic material may be passed with milk from the mother to the nursing offspring and (2) compounds can be passed from cows to people in dairy products. Toxic agents are excreted into milk by simple diffusion. Because milk has an acidic pH (about 6.5), basic compounds may be concentrated in milk, whereas acidic compounds may attain lower concentrations in milk than in plasma. About 3% to 4% of milk consists of lipids, and lipid-soluble xenobiotics diffuse along with fats from plasma into the mammary glands and are excreted during lactation.

Sweat and Saliva—The excretion of toxic agents in sweat and saliva is quantitatively of minor importance. Again, excretion depends on the diffusion of the nonionized, lipid-soluble form of an agent. Toxic compounds excreted into sweat may produce dermatitis. Substances excreted in saliva enter the mouth, where they are usually swallowed to become available for GI absorption.

COMPUTATIONAL AND EXPERIMENTAL APPROACHES TO ASSESS XENOBIOTIC DISPOSITION

Absorption—Various models are used to estimate log *P* because the lipid solubility is an

important feature of any toxicant. One model predicts poor absorption as a function of the calculated log *P* (*C* log *P*), the molecular weight, and the presence of hydrogen bond donors and acceptors. The concept based on these four elements is referred to as the “rule of 5” because the determinants are based on multiples of 5 and include (1) molecular weight greater than 500; (2) *C* log *P* greater than 5; (3) more than 5 H-bond donors; and (4) 10 H-bond acceptors.

The human colon adenocarcinoma cell line, Caco-2, is widely used to evaluate xenobiotic permeability. These cells form a confluent epithelial monolayer with well-defined tight junctions and typical microvilli on the apical surface. A variety of xenobiotic transporters are expressed on Caco-2 cells. The uptake transporters OATP2B1, PEPT1, and OCTN2 are expressed on the apical membrane along with efflux transporters MDR1, MRP2, and BCRP. On the basolateral membrane, efflux transporters MRP3, MRP4, and MRP6 have been identified. Caco-2 cells are specifically used to estimate the fraction of a xenobiotic that will be absorbed in the GI tract. Another model used to predict absorption utilizes artificial membranes such as the parallel artificial membrane permeability assay (PAMPA). This system lacks transporters or paracellular pathways and is most useful for assessing the non-energy-dependent diffusion of toxicants.

Dermal exposure can be evaluated in several in vitro systems, either with skin biopsy samples or with models developed to represent human skin. Mathematical models, with emphasis on the lipid matrix as the principal pathway of permeation, have also been developed and used with increasing frequency. Such methods emphasize log *P* and molecular size in model development.

Hepatobiliary Excretion—Xenobiotic transporter function can be evaluated with membrane vesicles isolated from specific organs or with expressed cell systems. The development of transporter-deficient models, particularly in mice, has also proved to be useful for assessing transporter contribution to toxicity. The latter tools along with more sophisticated primary hepatocyte culture models are typically utilized to aid in the prediction of transporter-mediated effects in absorption and excretion processes. The use of sandwich-cultured human hepatocytes (SCHH) has become a standard tool to aid in the prediction of biliary excretion and drug-induced liver injury. Briefly, when hepatocytes are cultured between two layers of gelled collagen (hence the sandwich configuration), they retain molecular and biochemical characteristics more consistent with their properties in the whole organ than monolayer cultures of cells. These features include the formation of canalicular networks necessary for biliary excretion. This system has been optimized to assess toxicant accumulation, estimate biliary excretion, and investigate the interplay between metabolism and transport, and aid in the evaluation of hepatobiliary disposition.

Classification Systems—Physiochemical properties can be utilized to predict solubility, disposition, and excretion for chemicals of environmental concern and predict the impact of active pharmaceutical ingredients excreted and present in sewage. The Biopharmaceutics Drug Disposition Classification System (BDDCS) was developed to aid in the prediction of drug disposition characteristics at early stages of drug discovery and development. The Extended Clearance Classification System (ECCS) uses known physicochemical properties and in vitro/in silico data readily available from early drug discovery to predict absorption trends, impact of intestinal and hepatic first-pass effect on new molecular entities oral profiles, drug clearance rate–determining step, and extent of metabolism.

CONCLUSION

Humans are in continuous contact with toxic chemicals in the food we eat, the water we drink, and the air we breathe. Depending on their physical and chemical properties, toxic chemicals may be absorbed by the GI tract, the lungs, and/or the skin. Fortunately, the body can biotransform and excrete these compounds. When the rate of absorption exceeds the rate of elimination, toxic compounds may accumulate, reach a critical concentration at a certain target site, and cause toxicity. Whether a chemical elicits toxicity depends not only on its inherent potency and site specificity but also on its absorbtion, distribution, and elimination. Many chemicals have low inherent toxicity but can be metabolically activated into toxic metabolites, and toxicity may be determined by the rate of formation of toxic metabolites. Alternatively, a potent toxicant may be detoxified rapidly by biotransformation. The fundamental and overarching concept is that adverse effects are related to the unbound concentration of the "toxic chemical" at the site of action (in the target organ), whether a chemical is administered or generated by biotransformation in the target tissue or at a distant site. Accordingly, the toxic response exerted by chemicals is critically influenced by the rates of absorption, distribution, biotransformation, and excretion.

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QUESTIONS

1. Biotransformation is vital in removing toxicants from the circulation. All of the following statements regarding biotransformation are true EXCEPT:
   - **a.** Many toxicants must be biotransformed into a more lipid-soluble form before they can be excreted from the body.
   - **b.** The liver is the most active organ in the biotransformation of toxicants.
   - **c.** Water solubility is required in order for many toxicants to be excreted by the kidney.
   - **d.** The kidney plays a major role in eliminating toxicants from the body.
   - **e.** The lungs play a minor role in ridding the body of certain types of toxicant.

2. Which of the following statements about active transport across cell membranes is FALSE?
   - **a.** Unlike simple or facilitated diffusion, active transport pumps chemicals against an electrochemical or concentration gradient.
   - **b.** Unlike simple diffusion, there is a rate at which active transport becomes saturated and cannot move chemicals any faster.
   - **c.** Active transport requires the expenditure of ATP in order to move chemicals against electrochemical or concentration gradients.

d. Active transport exhibits a high level of specificity for the compounds that are being moved.
e. Metabolic inhibitors do not affect the ability to perform active transport.

3. Which of the following might increase the toxicity of a toxicants administered orally?
   a. increased activity of the MDR transporter (p-glycoprotein).
   b. increased biotransformation of the toxicant by gastrointestinal cells.
   c. increased excretion of the toxin by the liver into bile.
   d. increased dilution of the toxin dose.
   e. increased intestinal motility.

4. Which of the following most correctly describes the first-pass effect?
   a. The body is most sensitive to a toxicant the first time that it passes through the circulation.
   b. Orally administered toxicants are partially removed by the GI tract before they reach the systemic circulation.
   c. It only results from increased absorption of toxicant by GI cells.
   d. It is often referred to as “postsystemic elimination.”
   e. A majority of the toxicant is excreted after the first time the blood is filtered by the kidneys.

5. Which of the following is an important mechanism of removing particulate matter from the alveoli?
   a. coughing.
   b. sneezing.
   c. blowing one’s nose.
   d. absorption into the bloodstream, followed by excretion via the kidneys.
   e. swallowing.

6. For a toxicant to be absorbed through the skin, it must pass through multiple layers in order to reach the systemic circulation. Which of the following layers is the most important in slowing the rate of toxicant absorption through the skin?
   a. stratum granulosum.
   b. stratum spinosum.
   c. stratum corneum.
   d. stratum basale.
   e. dermis.

7. A toxicant is selectively toxic to the lungs. Which of the following modes of toxicant delivery would most likely cause the LEAST damage to the lungs?
   a. intravenous.
   b. intramuscular.
   c. intraperitoneal.
   d. subcutaneous.

e. inhalation.

8. Which of the following is NOT an important site of toxicant storage in the body?
   a. adipose tissue.
   b. bone.
   c. plasma proteins.
   d. muscle.
   e. liver.

9. Which of the following regarding the blood–brain barrier is TRUE:
   a. The brains of adults and newborns are equally susceptible to harmful blood-borne chemicals.
   b. The degree of lipid solubility is a primary determinant in whether or not a substance can cross the blood–brain barrier.
   c. Astrocytes play a role in increasing the permeability of the blood–brain barrier.
   d. Active transport processes increase the concentration of xenobiotics in the brain.
   e. The capillary endothelial cells of the CNS possess large fenestrations in their basement membranes.

10. Which of the following will result in DECREASED excretion of toxic compounds by the kidneys?
   a. a toxic compound with a molecular weight of 25,000 Da.
   b. increased activity of the multidrug-resistance (MDR) protein.
   c. increased activity of the multiresistant drug protein (MRP).
   d. increased activity of the organic cation transporter.
   e. increased hydrophilicity of the toxic compound.

CHAPTER 6

Biotransformation of Xenobiotics

Andrew Parkinson, Brian W. Ogilvie, David B. Buckley, Faraz Kazmi, and Oliver Parkinson

KEY POINTS

- *Biotransformation* is the metabolic conversion of endogenous and xenobiotic chemicals to more water-soluble compounds.
- Xenobiotic biotransformation is accomplished by a limited number of enzymes with broad substrate specificities.
- Phase I reactions involve hydrolysis, reduction, and oxidation. These reactions expose or introduce a functional group (—OH, —NH_2, —SH, or —COOH), and usually result in only a small increase in hydrophilicity.
- Phase II biotransformation reactions include glucuronidation, sulfonation (more commonly called sulfation), acetylation, methylation, and conjugation with glutathione (mercapturic acid synthesis), which usually result in increased hydrophilicity and elimination.

INTRODUCTION

The overall fate or disposition of a xenobiotic, which encompasses its absorption, distribution, metabolism, and elimination or ADME, is determined by three major factors, namely, passive diffusion across biological membranes or between cells (i.e., transcellular and paracellular passive diffusion, respectively), facilitated transport by uptake and/or efflux transporters, and biotransformation (invariably called metabolism in the case of drugs) (Figs. 6–1 and 6–2). Biotransformation has been described as an enzymatic process of chemical modification that changes the physicochemical properties of a xenobiotic from those that favor absorption and distribution (i.e., high passive permeability associated with high lipophilicity) to those that favor elimination (i.e., low passive permeability associated with high hydrophilicity).

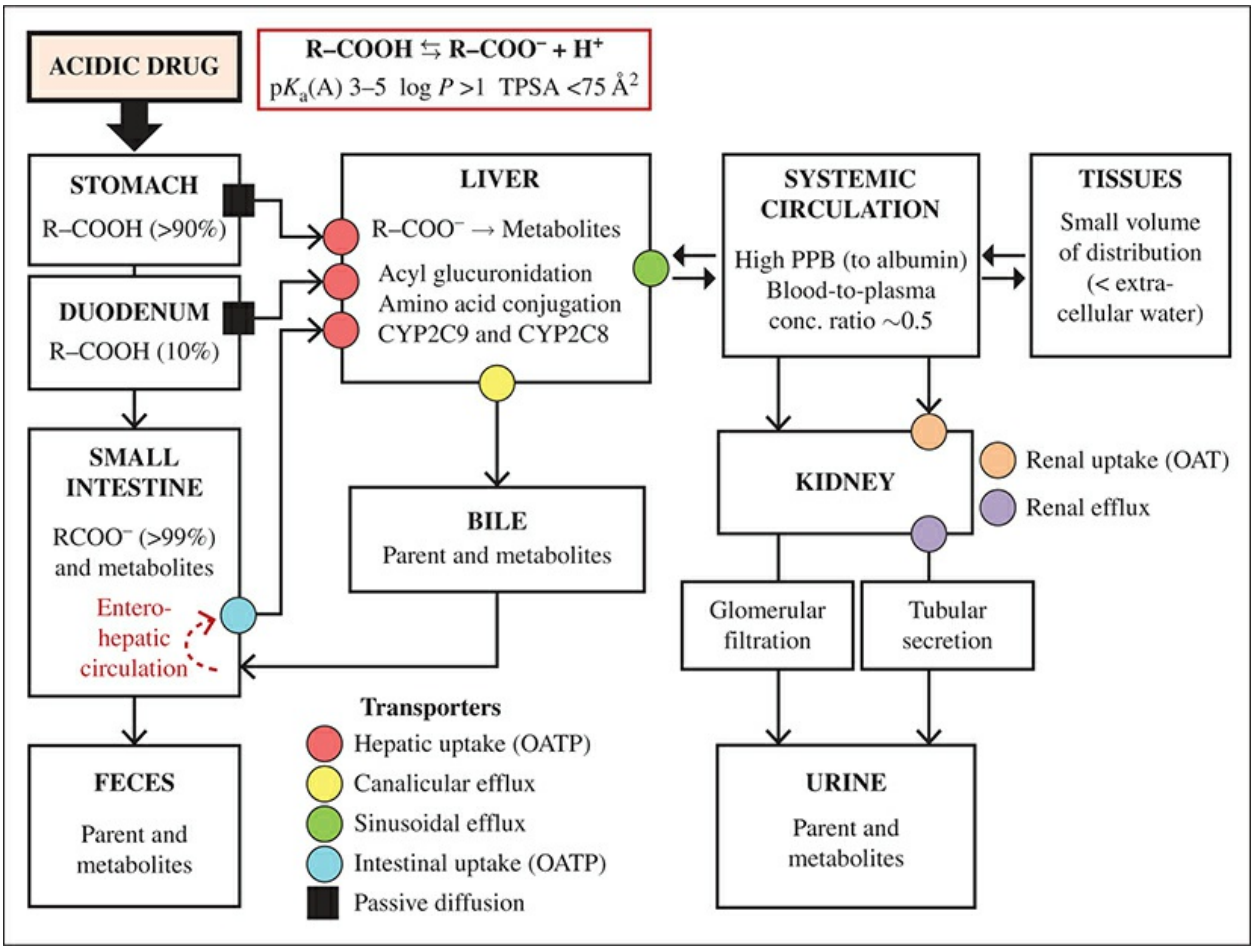


FIGURE 6–1 *Role of passive diffusion, transport, and biotransformation in the disposition of a lipophilic acidic xenobiotic.* OAT, organic anion transporter; OATP, organic anion transporting polypeptide; PPB, plasma protein binding; TPSA, topological polar surface area.

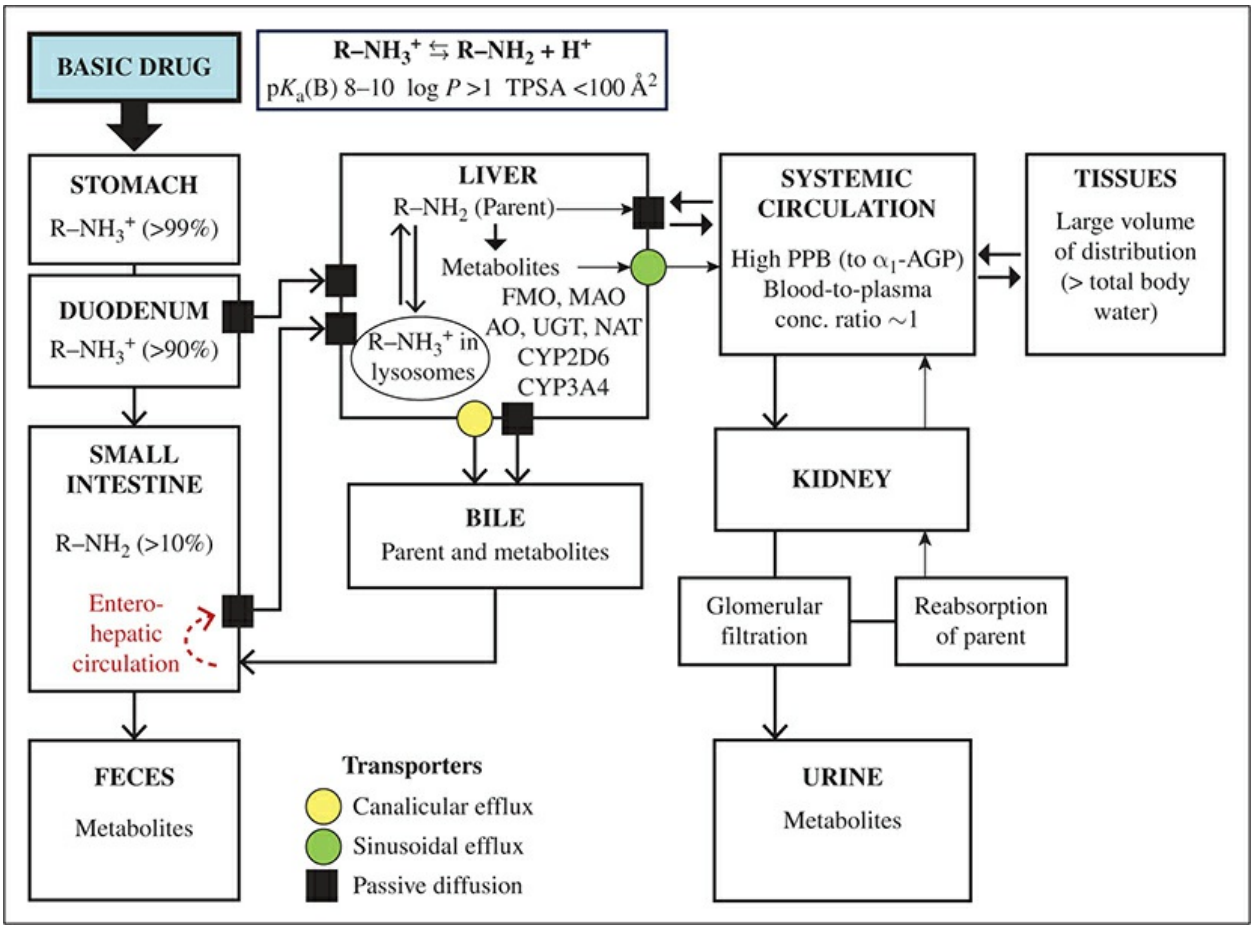


FIGURE 6–2 *Role of passive diffusion, transport, and biotransformation in the disposition of a lipophilic basic xenobiotic.* α1-AGP, α1-acid glycoprotein; AO, aldehyde oxidase; CYP, cytochrome P450; MAO, monoamine oxidase; NAT, *N*-acetyltransferase; PPB, plasma protein binding; TPSA, topological polar surface area; UGT, UDP-glucuronosyltransferase.

Biotransformation has traditionally been divided into two phases. Phase 1 biotransformation involves oxidation, reduction, and hydrolysis, which result in the introduction or exposure of functional groups such as –OH, –NH_2, –SH, or –COOH. Phase 2 biotransformation involves conjugation of the xenobiotic or its Phase 1 metabolite(s) with an acid (glucuronic acid or sulfonic acid) or zwitterion (glutathione or amino acids such as glycine, taurine, and glutamine). Phase 1 biotransformation facilitates the elimination of xenobiotics by increasing their topological surface area (TPSA) and lowering their log *P*, both of which decrease membrane permeation by passive diffusion and decrease the likelihood of the excreted metabolites being reabsorbed from the small intestine or kidney. Phase 1 biotransformation also produces metabolites that potentially undergo Phase 2 biotransformation, which facilitates the elimination of xenobiotics by adding an ionizable group (often an acidic moiety that is predominantly [>99%] negatively charged above pH 7) that is associated with relatively large increases in TPSA and decreases in log *P*.

This chapter describes some fundamental principles of xenobiotic biotransformation, and describes the major enzyme systems and their subcellular localization (see Table 6–1) involved in the biotransformation (or metabolism) of drugs and other xenobiotics.

TABLE 6–1 General Pathways of Xenobiotic Biotransformation and Their Major Subcellular Location

Reaction	Enzyme or Specific Reaction	Localization
Hydrolysis	Carboxylesterase	Microsomes, cytosol, lysosomes, blood
	Butyrylcholinesterase	Plasma, most tissues
	Acetylcholinesterase	Erythrocytes, most tissues
	Paraoxonases	Plasma, microsomes, inner mitochondrial membrane
	Alkaline phosphatase	Plasma membrane
	Peptidase	Blood, lysosomes
	β-Glucuronidase	Microsomes, lysosomes, microflora
	Epoxide hydrolase	Microsomes, plasma membrane, cytosol
Reduction	Azo- and nitro-reduction	Microflora
	Carbonyl (aldo-keto) reduction	Cytosol, microsomes, blood
	Disulfide reduction	Cytosol
	Sulfoxide reduction	Cytosol
	Quinone reduction	Cytosol, microsomes
	Dihydropyrimidine dehydrogenase	Cytosol
	Reductive dehalogenation	Microsomes
	Dehydroxylation (mARC)	Mitochondria
	Dehydroxylation (aldehyde oxidase)	Cytosol
Oxidation	Alcohol dehydrogenase	Cytosol
	Aldehyde dehydrogenase	Mitochondria, cytosol
	Aldehyde oxidase	Cytosol
	Xanthine oxidoreductase	Cytosol
	Class I Amine Oxidases	Outer mitochondrial membrane, platelets
	MAO-A and B	Cytosol, peroxisomes, plasma
	PAO	Cytosol, nucleus
	SMOX	Cytosolic, membrane-associated forms
	Class II Amine Oxidases (CuAOs)	Microsomes, extracellular matrix
	SSAOs (e.g., AOC3)	Extracellular matrix
	DAOs	Microsomes, lysosomes, saliva
	LOs	Microsomes
	Peroxidases	Microsomes, mitochondria
	Flavin-monooxygenases	
	Cytochrome P450	
Conjugation	UDP-glucuronosyltransferase	Microsomes
	Acyl-CoA synthetase	Mitochondria
	Sulfotransferase	Cytosol
	Glutathione transferase	Cytosol, microsomes, mitochondria
	Amino acid transferase	Mitochondria, microsomes
	N-acetyltransferase	Mitochondria, cytosol
	Methyltransferase	Cytosol, microsomes, blood

Abbreviation: mARC, mitochondrial amidoxime-reducing complex.

PRINCIPLES OF XENOBIOTIC BIOTRANSFORMATION

The following principles/rules about xenobiotic biotransformation apply in the majority of cases:

Point 1 Xenobiotic biotransformation or drug metabolism is the process of converting lipophilic (fat-soluble) chemicals, which are readily absorbed from the gastrointestinal tract and other sites, into hydrophilic (water-soluble) chemicals, which are readily excreted in urine or bile. Exceptions to this most basic rule include acetylation and methylation, which result in a nominal increase or a small decrease in the water solubility of xenobiotics.

Point 2 Xenobiotic biotransformation is catalyzed by enzyme systems that can be divided into four categories based on the reaction they catalyze: 1. Hydrolysis (e.g., carboxylesterase); 2. Reduction (e.g., carbonyl reductase); 3. Oxidation (e.g., cytochrome P450 [CYP]); and 4. Conjugation (e.g., UDP-glucuronosyltransferase [UGT]). The mammalian enzymes involved in the hydrolysis, reduction, oxidation, and conjugation of xenobiotics are listed in Table 6–1, together with their principal subcellular location. The conjugation reactions include glucuronidation, sulfonation, acetylation, methylation, conjugation with glutathione (GSH), and conjugation with amino acids (such as glycine, taurine, and glutamine).

Point 3 In general, individual xenobiotic-biotransforming enzymes are located in a single organelle. In Table 6–1, some enzymes are listed with two or more subcellular locations.

Point 4 Xenobiotic biotransformation is accomplished by a limited number of enzymes with broad substrate specificities. In humans, two CYP enzymes—namely, CYP2D6 and CYP3A4—metabolize over half of orally taken drugs and natural products/endobiotics. Many enzymes involved in biotransformation are arranged in families and subfamilies and named according to nomenclature systems based on primary amino acid sequence of the individual enzymes.

The amino acid sequence of a given xenobiotic-biotransforming enzyme may differ among individuals, which can give rise to differences in rates of drug metabolism. The broad substrate specificity of xenobiotic-biotransforming enzymes makes them catalytically versatile but slow compared with most other enzymes (with the exception of hydrolytic reactions). The sequential oxidation, conjugation, and transport of a xenobiotic tend to proceed quicker at each subsequent step, which prevents the accumulation of intracellular metabolites.

Point 5 Hydrolysis, reduction, and oxidation expose or introduce a functional group (such as −OH, $-NH_2$, −SH, or −COOH) that can be converted to a water-soluble conjugate. The functional group introduced or exposed by hydrolysis, reduction, or oxidation must be nucleophilic (in the case of glucuronidation, sulfonation, methylation, acetylation, and conjugation with glycine or taurine) or electrophilic (in the case of glutathionylation). All biotransformation reactions are capable of increasing the toxicity of xenobiotics.

Point 6 Oxidation, reduction, hydrolysis, methylation, and acetylation generally cause a modest increase (or a small decrease in the case of methylation) in the water solubility of a xenobiotic, whereas glucuronidation, sulfonation, glutathionylation, and amino acid conjugation generally cause a marked increase in hydrophilicity.

Point 7 Some biotransformation reactions are catalyzed by enzymes that participate in intermediary (endobiotic) metabolism, whereas others are catalyzed by enzymes in the gut microbiota (largely anaerobic bacteria in the colon).

Point 8 Just as some xenobiotics are biotransformed by the so-called endobiotic-metabolizing

enzymes (Point 7), certain endobiotics are biotransformed by the so-called xenobiotic-metabolizing enzymes. For example, the same CYP enzymes implicated in xenobiotic biotransformation also contribute to the hepatic catabolism of steroid hormones, and the same UGTs that conjugate xenobiotics also glucuronidate bilirubin, thyroid hormones, and steroid hormones. On a case-by-case basis, there is often no clear-cut distinction between endobiotic- and xenobiotic-biotransforming enzymes.

Point 9 Several xenobiotic-biotransforming enzymes are inducible, meaning their expression can be increased (upregulated) usually in response to exposure to high concentrations of xenobiotics. Induction is mediated by ligand-activated receptors (so-called xenosensors) that are activated by xenobiotics (ligands) to DNA-binding proteins that upregulate the transcription of various genes encoding xenobiotic-biotransforming enzymes, especially CYP enzymes. The major xenosensors are the aryl hydrocarbon receptor (AhR), which induces CYP1 enzymes, the constitutive androstane receptor (CAR), which induces CYP2B, CYP2C, and CYP3A enzymes, the pregnane X receptor (PXR), which also induces CYP2B, CYP2C, and CYP3A enzymes, and the peroxisome proliferator–activated receptor α (PPARα), which induces CYP4 enzymes.

Certain xenosensors are activated by endogenous ligands (e.g., bilirubin, bile acids, and fatty acids activate CAR, PXR, and PPARα, respectively), and certain nuclear receptors, such as the vitamin D receptor (VDR), can mimic PXR and induce CYP3A4, which inactivates the active metabolite of vitamin D. Induction is a reversible, adaptive response to xenobiotic exposure. When the induced enzymes (and transporters) accelerate the elimination of the xenobiotic that triggered the induction process, the xenobiotic is said to be an autoinducer (one that induces its own metabolism). Induction is a pleiotropic response: activation of AhR, CAR, PXR, PPARα, and Nrf2 all results in alterations in the expression of numerous genes.

Downregulation of drug-metabolizing enzymes is often associated with inflammatory diseases (such as arthritis), cancer, infectious diseases (both bacterial and viral), vaccination, and treatment with certain proinflammatory biologics (therapeutic proteins). These disease processes activate nuclear factor kappa-B (NF-κB) (and other nuclear receptors), which suppresses the expression and induction of CYP and other xenobiotic-metabolizing enzymes. Activated NF-κB suppresses all four xenosensors (AhR, CAR, PXR, and PPARα), as well as several other nuclear receptors. By reversing the disease process—such as lessening the inflammation associated with rheumatoid arthritis—some biologics (large drug molecules such as monoclonal antibodies and other types of therapeutic proteins) can reverse the suppression of drug-metabolizing enzymes and restore their activity to normal (predisease) levels.

Point 10 The ability of certain xenobiotic-biotransforming enzymes to metabolize hormones and other endobiotics (Point 8) and the ability of certain xenobiotics to induce xenobiotic-biotransforming enzymes (Point 9) can aid our understanding of how certain xenobiotics can alter homeostasis or cause toxicity.

Point 11 Xenobiotic biotransformation can alter the biological properties of a xenobiotic. It can make the xenobiotic less toxic (detoxication), but in some cases it can make it more toxic (activation). The biotransformation of drugs can result in (1) a loss of pharmacological activity, (2) no change in pharmacological activity, or (3) an increase in pharmacological activity.

Point 12 In many cases, the toxicity of a xenobiotic is due to the parent compound (the compound that was absorbed), and biotransformation serves as a detoxication mechanism.

CYP is particularly effective at converting proximate carcinogens to ultimate carcinogens by converting the former to electrophilic metabolites that bind to and mutate DNA, thereby leading to mutations and tumor initiation.

Point 13 The toxicity and potential carcinogenicity of electrophilic metabolites produced by CYP and other xenobiotic-biotransforming enzymes are reduced and often altogether eliminated by their conjugation with GSH.

Point 14 The biotransformation of some xenobiotics produces reactive oxygen species, which can cause cell toxicity through oxidative stress and lipid peroxidation. GSH, GSTs, and glutathione peroxidases (GPXs) limit the toxic effects of reactive oxygen species just as they limit the toxicity of reactive metabolites formed directly from xenobiotics. Oxidative stress and the formation of electrophilic metabolites reduce GSH levels and thus result in the concurrent oxidation of KEAP-1, which then releases Nrf2, which in turn upregulates the enzymes that detoxify electrophilic metabolites (e.g., epoxides) and those metabolites that generate reactive oxygen species.

Point 15 The balance between activation and detoxication by xenobiotic-biotransforming enzymes is often a key determinant of chemical toxicity, and is often the basis for organ or species differences in toxicity.

Point 16 The small intestine and liver are highly developed to limit systemic exposure to orally ingested xenobiotics, a process known as *first-pass elimination* (*or presystemic elimination*). The enterocytes at the tips of the small intestinal villi express various efflux transporters, such as P-gp, BCRP, and MRP2, which restrict intestinal absorption, thereby limiting systemic exposure to many xenobiotics. Enterocytes and hepatocytes express high levels of certain CYP and UGT enzymes, which biotransform a wide variety of xenobiotics.

Point 17 The same biotransformation mechanisms that protect the small intestine and liver from xenobiotic toxicity also protect certain organs such as the brain and reproductive organs.

Point 18 Although the small intestine and liver contain the highest concentrations, xenobiotic-biotransforming enzymes are widely distributed throughout the body.

Point 19 Species differences in xenobiotic-biotransforming enzymes are often the basis for species differences in the qualitative and quantitative aspects of xenobiotic biotransformation and toxicity.

Point 20 In sexually mature rats and, to a lesser extent, mice, there are marked gender differences in the expression of certain xenobiotic-biotransforming enzymes. In other species, including humans, gender differences either do not exist or generally represent less than a twofold difference.

Point 21 Idiosyncratic drug reactions (IDRs) are rare adverse events (generally <0.1%) that do not involve an exaggerated pharmacological response, do not occur in most patients at any dose, and typically occur after weeks or months of repeated administration.

Point 22 Large interindividual differences in pharmacokinetic parameters upon administration or exposure to the parent compound can reflect genetically determined differences in the activity of xenobiotic-biotransforming enzymes or transporters or in environmental factors, such as drug–drug interactions.

Point 23 Environmental factors can introduce as much variation in drug metabolism as can genetic factors, and this is especially true of drug–drug interactions.

Point 24 Although drug–drug interactions can cause an increase in the incidence of adverse events or, in the case of induction, a loss of therapeutic efficacy, not all drug–drug interactions are undesirable.

Point 25 Stereochemical aspects may be important in the interaction between a xenobiotic and its biotransforming enzyme, and xenobiotic-biotransforming enzymes can convert one

stereoisomer to another, a process known as *mutarotation* or *inversion of configuration*.

Point 26 Mass spectrometry is widely used to characterize the structure of metabolites, and many instruments now come equipped with software to assist in this process, based on the fact that certain xenobiotic reactions are associated with discrete changes in mass. For example, the loss of 2 mass/charge (m/z) units signifies dehydrogenation, whereas the loss of 14 m/z units usually signifies demethylation ($-CH_2$). Several reactions result in an increase in mass, including reduction (+2 m/z units = 2H), methylation (+14 m/z units = CH_2), oxidation (+16 m/z units = O), hydration (+18 m/z units = H_2O), acetylation (+42 m/z units = C_2H_2O), glucosidation (+162 m/z units = $C_6H_{10}O_5$), sulfonation (+80 m/z units = SO_3), glucuronidation (+176 m/z units = $C_6H_8O_6$), carbamoyl glucuronidation (+220 m/z units = $C_7H_8O_8$), and conjugation with GSH (+305 m/z units = $C_{10}H_{15}N_3O_6S$), glycine (+57 m/z units = C_2H_3NO), taurine (+107 m/z units = $C_2H_5NO_2S$), and glutamine (+107 m/z units = $C_5H_7NO_3$).

HYDROLYSIS, REDUCTION, AND OXIDATION

Hydrolysis

Carboxylesterases—Carboxylesterases are predominantly microsomal enzymes (about 60 kDa glycoproteins) that are present in liver, intestine, kidney, and other tissues. CES1 (the major liver form, which is also expressed in lung and other tissues) and CES2 (the major intestinal form, which is also expressed in liver, kidney, and brain) represent two of the six families of human carboxylesterases. The other enzymes are CES3 (expressed in brain, liver, and colon), CES4A (expressed in brain, lung, and kidney), CES5A (expressed in brain, lung, and testis), and CES6 (expressed in brain cerebellum). The mechanism of catalysis involves charge relay among a catalytic triad comprising an acidic amino acid residue (glutamate [Glu_{335}]), a basic residue (histidine [His_{448}]), and a nucleophilic residue (serine [Ser_{203}]).

Cholinesterases (AChE and BChE)—Humans have two cholinesterases, namely, acetylcholinesterase (AChE; gene name ACHE) and butyrylcholinesterase (BChE, also known as pseudocholinesterase; gene name BCHE), which are related enzymes (about 54% identical). They are present in most tissues. AChE is highly selective for acetylcholine and plays little or no significant role in the hydrolysis of xenobiotics, whereas BChE hydrolyzes numerous drugs and other xenobiotics.

Paraoxonases (Lactonases)—Paraoxonases catalyze the hydrolysis of a broad range of organophosphates, organophosphinites, aromatic carboxylic acid esters (such as phenylacetate), cyclic carbonates, lactones, and oxidized phospholipids. They are calcium-dependent enzymes containing a critical sulfhydryl (−SH) group. Humans express three paraoxonases designated PON1, PON2, and PON3. PON1 is present in liver microsomes and plasma, where it is associated exclusively with high-density lipoprotein (HDL). PON2 is expressed in the inner mitochondrial membrane of vascular cells and many tissues. PON3 is expressed in liver and kidney microsomes and plasma.

Prodrugs and Alkaline Phosphatase—Many prodrugs are designed to be hydrolyzed by hydrolytic enzymes to become active agents. Hydrolytic enzymes such as carboxylesterases, cholinesterases, and alkaline phosphatase are commonly invoked for this purpose. Potent anticancer agents exist that are highly selective for certain sites and only release active drug in the vicinity of tumor cells.

Peptidases—Peptidases are proteolytic enzymes that hydrolyze the amide (peptide) bond connecting adjacent amino acids in peptides and proteins (R_1–NH–CO–R_2 + H_2O → R_1–NH_2 + R_2–COOH). Numerous human peptides and several recombinant peptide hormones, growth factors, cytokines, soluble receptors, and monoclonal antibodies are used therapeutically. These peptides are hydrolyzed in the blood and tissues by a variety of peptidases, which cleave the amide linkage between adjacent amino acids.

β-Glucuronidase—β-Glucuronidase that is present in liver lysosomes and microsomes and in gut microbiota hydrolyzes xenobiotic glucuronides (if the glucuronide is in the β-configuration). When a drug is glucuronidated directly and excreted in bile, hydrolysis by β-glucuronidase in the gut can release the aglycone (the parent drug) and result in its enterohepatic circulation.

Epoxide Hydrolases—Epoxide hydrolases catalyze the *trans*-addition of water to alkene epoxides and arene oxides (oxiranes), which can form during the CYP-dependent oxidation of aliphatic alkenes and aromatic hydrocarbons, respectively. Epoxide hydrolases are important in detoxifying electrophilic epoxides that might otherwise bind to proteins and nucleic acids and cause cellular toxicity and genetic mutations. There are four distinct forms of epoxide hydrolase in mammals: microsomal epoxide hydrolase (mEH), soluble epoxide hydrolase (sEH), cholesterol-5,6-epoxide hydrolase (ChEH), and leukotriene A_4 hydrolase (LTA_4 hydrolase).

Many epoxides and oxides are intermediary metabolites formed during the CYP-dependent oxidation of unsaturated aliphatic and aromatic xenobiotics. These electrophilic metabolites might otherwise bind to proteins and nucleic acids and cause cellular toxicity and genetic mutations. In general, sEH and mEH are found in the same tissues and cell types that contain CYP enzymes. The colocalization of epoxide hydrolase and CYP presumably ensures the rapid detoxication of alkene epoxides and arene oxides generated during the oxidative metabolism of unsaturated xenobiotics.

Electrophilic epoxides and arene oxides are constantly produced during the CYP-dependent oxidation of unsaturated aliphatic and aromatic xenobiotics, and are potentially reactive to cellular macromolecules such as DNA and protein. Epoxide hydrolase can rapidly convert these potentially toxic metabolites to the corresponding dihydrodiols, which are less reactive and easier to excrete. Thus, epoxide hydrolases are widely considered as detoxication enzymes.

Reduction

Certain metals (e.g., pentavalent arsenic) and xenobiotics containing an aldehyde, ketone, alkene, disulfide, sulfoxide, quinone, *N*-oxide, azo, or nitro group are often reduced in vivo. The reaction may proceed enzymatically or nonenzymatically by interaction with reducing agents (such as the reduced forms of glutathione, FAD, FMN, and NAD[P]). Enzymes, such as alcohol dehydrogenase (ADH), aldehyde oxidase (AO), and CYP, can catalyze both reductive and oxidative reactions depending on the substrate or conditions (e.g., aerobic vs. anaerobic).

Azo- and Nitro-Reduction—Azo- and nitro-reduction reactions are catalyzed by intestinal microbiota. However, under conditions such as low oxygen tension, the reactions can be catalyzed by liver microsomal CYP and NAD(P)H-quinone oxidoreductase (NQO1, also known as DT-diaphorase) and, in the case of nitroaromatics, by cytosolic AO. The anaerobic environment of the lower gastrointestinal tract is well suited for azo- and nitro-reduction.

Carbonyl Reduction: AKRs, SDRs, and Others—The reduction of aldehydes to primary alcohols and of ketones to secondary alcohols is generally catalyzed in mammals by NAD(P)H-dependent reductases belonging to one of several superfamilies, the aldo–keto reductases (AKRs), the short-chain dehydrogenases/reductases (SDRs), the medium-chain dehydrogenases/reductases (MDR), aldehyde dehydrogenases (ALDH), and NAD(P)H-quinone oxidoreductases (NQO).

The 15 human AKRs are members of a superfamily of cytosolic, generally monomeric, enzymes that reduce both xenobiotic and endobiotic compounds. Humans contain at least 80 SDR members, three of which, namely, cytosolic carbonyl reductases (CBR1 and CBR3) and a microsomal carbonyl reductase (HSD11B1), catalyze the reduction of a wide variety of carbonyl-containing xenobiotics (other species express more than two carbonyl reductases).

Disulfide Reduction—Disulfide reduction by glutathione is a three-step process, the last of which is catalyzed by glutathione reductase. The first step can be catalyzed by glutathione transferase, or it can occur nonenzymatically.

Sulfoxide and N-Oxide Reduction—Thioredoxin-dependent enzymes in liver and kidney cytosol have been reported to reduce sulfoxides, which themselves may be formed by CYP or flavin monooxygenases. Under reduced oxygen tension, the NADPH-dependent reduction of *N*-oxides in liver microsomes appears to be catalyzed by CYP or in some cases NADPH-cytochrome P450 reductase.

Quinone Reduction—Quinones can be reduced to hydroquinones by two closely related, cytosolic flavoproteins, namely, NQO1 and NQO2. NAD(P)H-quinone oxidoreductase (DT-diaphorase) and NAD(P)H-quinone oxidoreductase-2 have different substrate specificities. The two-electron reduction of quinones is a nontoxic reaction that is not associated with semiquinone formation and oxidative stress—provided the resultant hydroquinone is sufficiently stable to undergo glucuronidation or sulfonation.

Dehalogenation—Three major mechanisms remove halogens (F, Cl, Br, and I) from aliphatic xenobiotics. The first, known as *reductive dehalogenation*, involves replacement of a halogen with hydrogen. In the second mechanism, known as *oxidative dehalogenation*, a halogen and hydrogen on the same carbon atom are replaced with oxygen. A third mechanism of *double dehalogenation* involves the elimination of two halogens on adjacent carbon atoms to form a carbon–carbon double bond. A variation on this third mechanism is *dehydrohalogenation*, in which a halogen and hydrogen on adjacent carbon atoms are eliminated to form a carbon–carbon double bond.

Oxidation

Alcohol Dehydrogenase (ADH)—Alcohol dehydrogenases are zinc-containing, cytosolic

enzymes present in several tissues including liver (which has the highest levels), kidney, lung, and gastric mucosa. Class I comprises three hepatically expressed genes: ADH1A, ADH1B, and ADH1C, which were formerly known as ADH1, ADH2, and ADH3, respectively. The class I isozymes consist of homo- and hetero-dimeric forms of the three subunits (e.g., αα, αβ, ββ, βγ, and γγ). Class II contains ADH4, which is composed of two pi subunits (ππ). Class III contains ADH5, which is composed of two chi subunits (χχ). Class IV contains ADH7, which is composed of two sigma subunits (σσ). Class V ADH6 has no subunit designation. Action on ethanol is shown in Fig. 6–3.

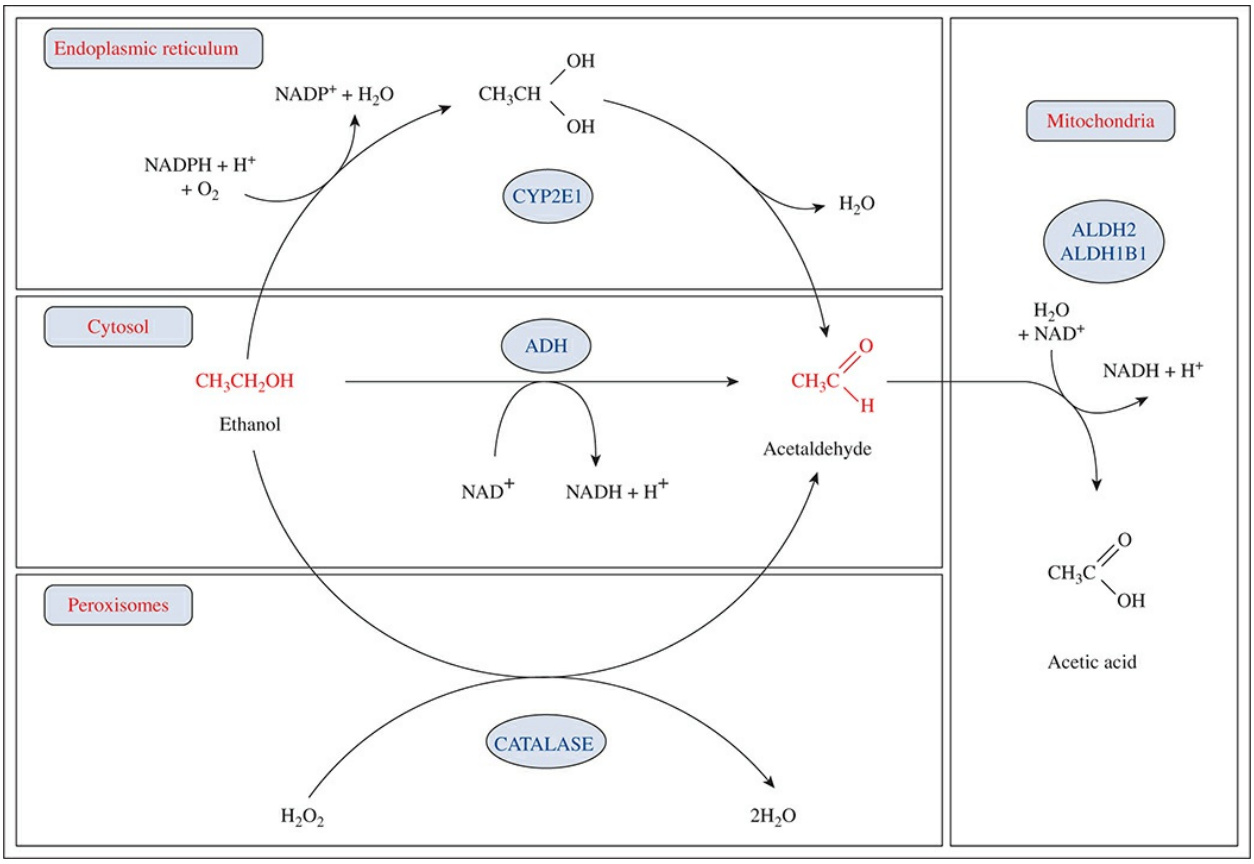


FIGURE 6–3 *Oxidation of alcohol to acetaldehyde by alcohol dehydrogenase (ADH), cytochrome P450 (CYP2E1), and catalase.* Note that the oxidation of alcohol to acetic acid involves multiple organelles.

Aldehyde Dehydrogenase—Aldehyde dehydrogenase (ALDH) oxidizes aldehydes to carboxylic acids with NAD+ as the cofactor. The enzymes also have esterase activity. The 19 identified ALDHs differ in their primary amino acid sequences and in the quaternary structure. In contrast to ALDH1A1 and ALDH2, which specifically reduce NAD+, ALDH3A1 reduces both NAD+ and NADP+. ALDH2 is a mitochondrial enzyme that, by virtue of its high affinity, is primarily responsible for oxidizing simple aldehydes, such as acetaldehyde.

Dimeric Dihydrodiol Dehydrogenase—Several members of the aldo–keto reductase (AKR) superfamily (particularly AKR1A1, 1C1, 1C2, 1C3, and 1C4) are dihydrodiol dehydrogenases that oxidize the *trans*-dihydrodiols of various polycyclic aromatic hydrocarbons to the

corresponding *ortho*-quinones, The overall reaction catalyzed by dimeric dihydrodiol dehydrogenase is a two-electron oxidation of one of the hydroxyl groups of a *trans*-dihydrodiol to an intermediate ketol, which rapidly enolizes to the catechol.

Molybdenum Hydroxylases—Two major molybdenum hydroxylases or molybdozymes participate in the biotransformation of xenobiotics: aldehyde oxidase and xanthine oxidoreductase (also known as xanthine oxidase [XO]). Sulfite oxidase, a third molybdozyme, oxidizes sulfite, an irritating air pollutant, to sulfate, which is relatively innocuous. All three molybdozymes are flavoprotein enzymes. During substrate oxidation, aldehyde oxidase and XO are reduced and then reoxidized by molecular oxygen. The oxygen incorporated into the xenobiotic is derived from water rather than oxygen, which distinguishes the oxidases from oxygenases. Xenobiotics that are good substrates for molybdozymes tend to be poor substrates for cytochrome P450, and vice versa.

Xanthine Oxidoreductase—Xanthine dehydrogenase (XD) and xanthine oxidase (XO) are two forms of the same cytosolic enzyme (xanthine oxidoreductase or XOR) that differ in the electron acceptor used in the final step of catalysis. In the case of XD, the final electron acceptor is NAD^+ (dehydrogenase activity), whereas in the case of XO, the final electron acceptor is oxygen. The highest xanthine oxidoreductase expression in humans is found in the proximal intestine, liver, and lactating mammary glands. The conversion of XD to XO in vivo may be important in ischemia–reperfusion injury, lipopolysaccharide-mediated tissue injury, and alcohol-induced hepatotoxicity. XO contributes to oxidative stress and lipid peroxidation because the oxidase activity of XO involves reduction of molecular oxygen, which can lead to the formation of ROS.

Aldehyde Oxidase—Aldehyde oxidase is a molybdoenzyme that is found in the liver and adrenal gland, with somewhat less expression in the small and large intestine. Aldehyde oxidase transfers electrons to molecular oxygen, which can generate reactive oxygen species and lead to oxidative stress and lipid peroxidation.

Amine Oxidases—Human amine oxidases can be divided into two classes: (1) FAD-containing mitochondrial monoamine oxidases, MAO-A and MAO-B and polyamine oxidase (PAOX) and spermine oxidase (SMOX); and (2) the copper-containing amine oxidases (CuAOs) that contain a tightly bound Cu^{II} and a quinone residue (typically 2,4,5-trihydroxyphenylalanine quinone [TPQ]) as the redox cofactor. Monoamine oxidase, spermine oxidase (SMOX), and polyamine oxidase (PAO) are involved in the oxidative deamination of primary, secondary, and tertiary amines including serotonin (5-hydroxytryptamine), monoacetylated derivatives of spermine and spermidine, and a number of xenobiotics. Oxidative deamination of a primary amine produces ammonia and an aldehyde, whereas oxidative deamination of a secondary amine produces a primary amine and an aldehyde. The aldehydes formed by MAO are usually oxidized further by other enzymes to the corresponding carboxylic acids. Monoamine oxidase is located throughout the brain, liver, kidney, intestine, heart, and platelets.

The substrate is oxidized by the enzyme, which itself is reduced ($FAD \rightarrow FADH_2$). The oxygen incorporated into the substrate is derived from water, not molecular oxygen; hence, the enzyme functions as a true oxidase. The catalytic cycle is completed by reoxidation of the reduced enzyme ($FADH_2 \rightarrow FAD$) by oxygen, which generates hydrogen peroxide.

Semicarbazide-sensitive amine oxidase (SSAO) is a copper containing enzyme catalyzes

fundamentally the same reaction as MAOs. It can be distinguished from MAO by its sensitivity to inhibitors and presence in plasma and on various cell surfaces, whereas MAO is found in mitochondria.

Peroxidase-Dependent Cooxidation—The oxidative biotransformation of xenobiotics couples the reduction of hydrogen peroxide (or a lipid hydroperoxide) to the one-electron oxidation of other substrates via a process known as cooxidation. An important peroxidase is prostaglandin H synthetase (PHS), which possesses two catalytic activities: a cyclooxygenase that converts arachidonic acid to prostaglandins and a peroxidase that converts the hydroperoxide to the corresponding alcohol PGH2. PHS has two forms (PHS1 and PHS2) that are better known as two forms of cyclooxygenase, namely, COX1 and COX2. PHS peroxidases are important in the activation of xenobiotics to toxic or tumorigenic metabolites, particularly in extrahepatic tissues that contain low levels of cytochrome P450. Oxidation of xenobiotics by peroxidases involves direct transfer of the peroxide oxygen to the xenobiotic.

Xenobiotics that serve as electron donors, such as amines and phenols, can also be oxidized to free radicals during the reduction of a hydroperoxide by peroxidases. In this case, the hydroperoxide is still converted to the corresponding alcohol, but the peroxide oxygen is reduced to water instead of being incorporated into the xenobiotic. For each molecule of hydroperoxide reduced (which is a two-electron process), two molecules of xenobiotic can be oxidized (each by a one-electron process). Many of the metabolites produced are reactive electrophiles that can cause tissue damage.

PHS is unique among peroxidases because it can both generate hydroperoxides and catalyze peroxidase-dependent reactions, as shown in Fig. 6–4. Xenobiotic biotransformation by PHS is controlled by the availability of arachidonic acid, whereas conversion by other peroxidases is controlled by the availability of hydroperoxide substrates.

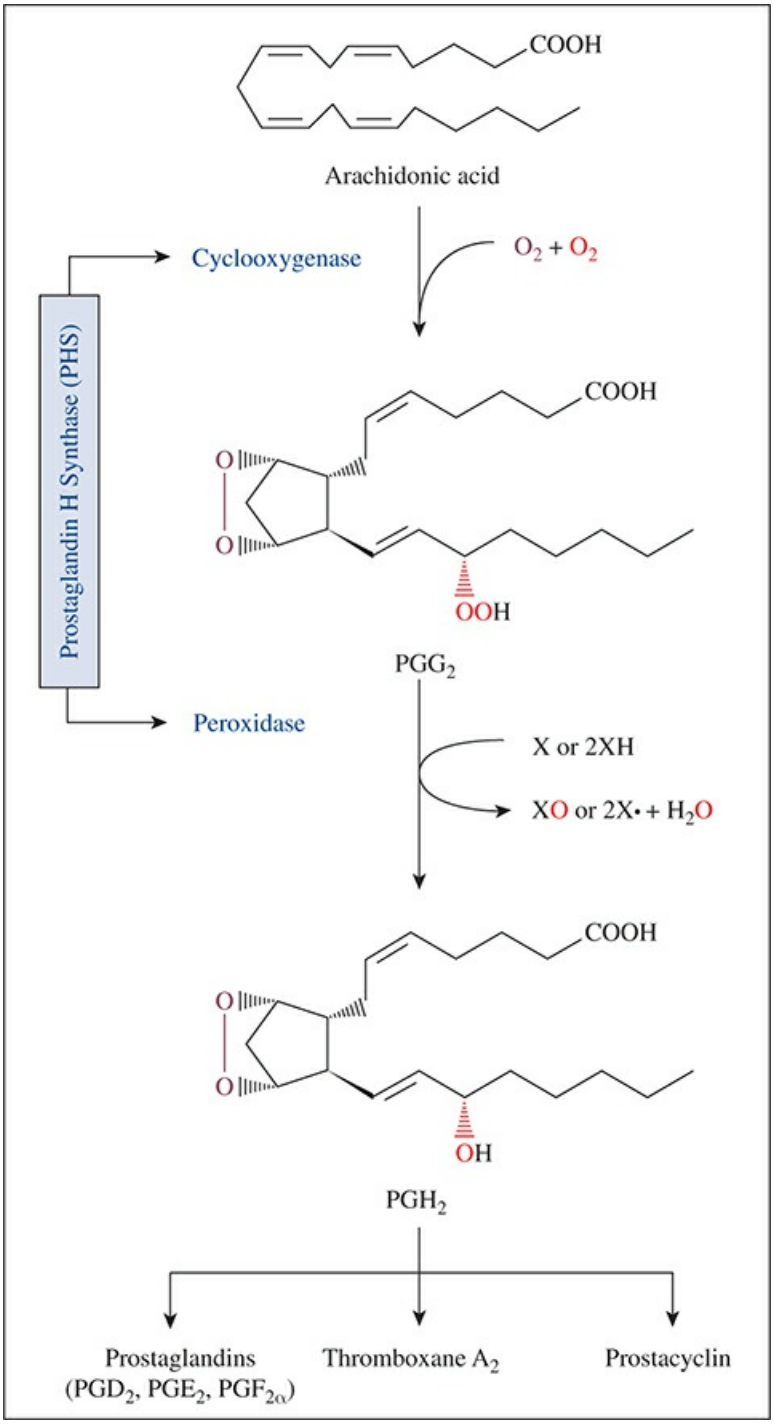


FIGURE 6–4 *Cooxidation of xenobiotics (X) during the conversion of arachidonic acid to PGH2 by prostaglandin H synthase.*

Flavin Monooxygenases—Humans and other mammals express five different flavin monooxygenases (FMO1, FMO2, FMO3, FMO4, and FMO5) in a species- and tissue-specific manner. The liver, kidney, intestine, brain, and lung (among other tissues) contain one or more FAD-containing monooxygenases (FMO) that oxidize soft nucleophilic nitrogen, sulfur, selenium, and phosphorus atoms of xenobiotics. The FMOs are microsomal enzymes that require

O_2 and NADPH (or NADH in some cases) and that catalyze a single two-electron oxidation. FMO catalyzes the oxidation of nucleophilic tertiary amines to *N*-oxides, secondary amines to hydroxylamines and nitrones, and primary amines to hydroxylamines and oximes.

The mechanism of catalysis by FMO is depicted in Fig. 6–5. After the FAD moiety is reduced to $FADH_2$ by NADPH, the oxidized cofactor, $NADP^+$, remains bound to the enzyme. $FADH_2$ then binds oxygen to produce a peroxide. During the oxygenation of xenobiotics (depicted as X → XO in Fig. 6–5), the nucleophilic heteroatom (N or S) attacks the terminal oxygen of the C(4a)-hydroperoxyflavin resulting in oxygen transfer to the xenobiotic (to form an *N*-oxide or sulfoxide) and formation of C(4a)-hydroxyflavin. The metabolites produced by FMO are generally the products of a chemical reaction between a xenobiotic and a peroxide or peracid. The final step in the catalytic cycle involves dehydration of C(4a)-hydroxyflavin (which restores FAD to the ground state) and release of $NADP^+$.

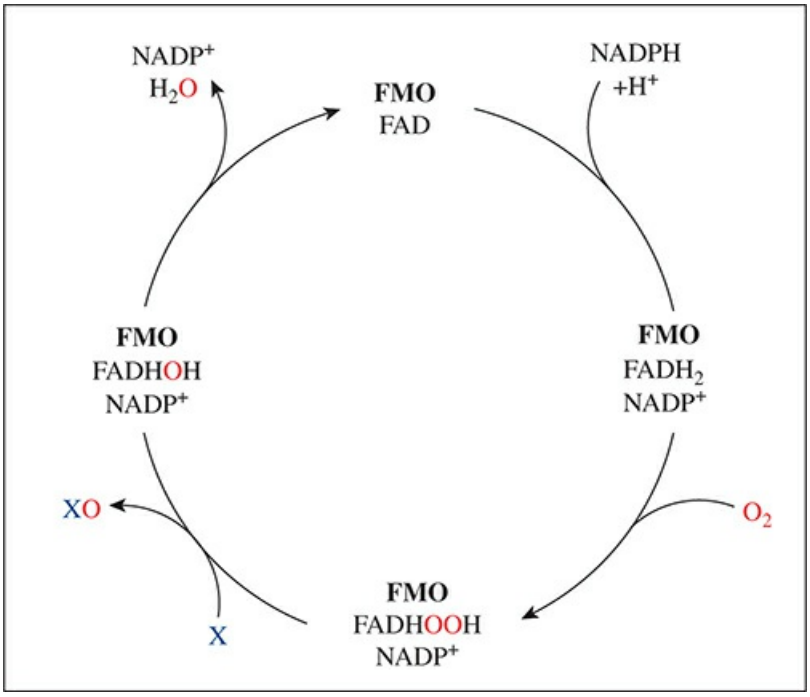


FIGURE 6–5 *Catalytic cycle of flavin monooxygenase (FMO). X and XO are the xenobiotic substrate and oxygenated product, respectively.* The C(4a)-hydroperoxyflavin and C(4a)-hydroxyflavin of FAD are depicted as FADHOOH and FADHOH, respectively.

Cytochrome P450

***General Features, Reactions, and Catalytic Cycle*—**The CYP enzyme system ranks first in terms of catalytic versatility and the sheer number of xenobiotics it detoxifies or activates to reactive intermediates. The highest levels of CYP enzymes involved in xenobiotic biotransformation are found in liver endoplasmic reticulum (microsomes), but CYP enzymes are present in virtually all tissues. The 55 human CYP enzymes can be broadly categorized on the basis of their role in (1) xenobiotic biotransformation, (2) fatty acid/eicosanoid hydroxylation/epoxidation, (3) steroidogenesis, (4) bile acid synthesis, vitamin D activation/inactivation, (5) retinoic acid metabolism, and (6) unknown function (a diminishing group of so-called orphan enzymes). Many CYP enzymes actively metabolize both endobiotic

and xenobiotics.

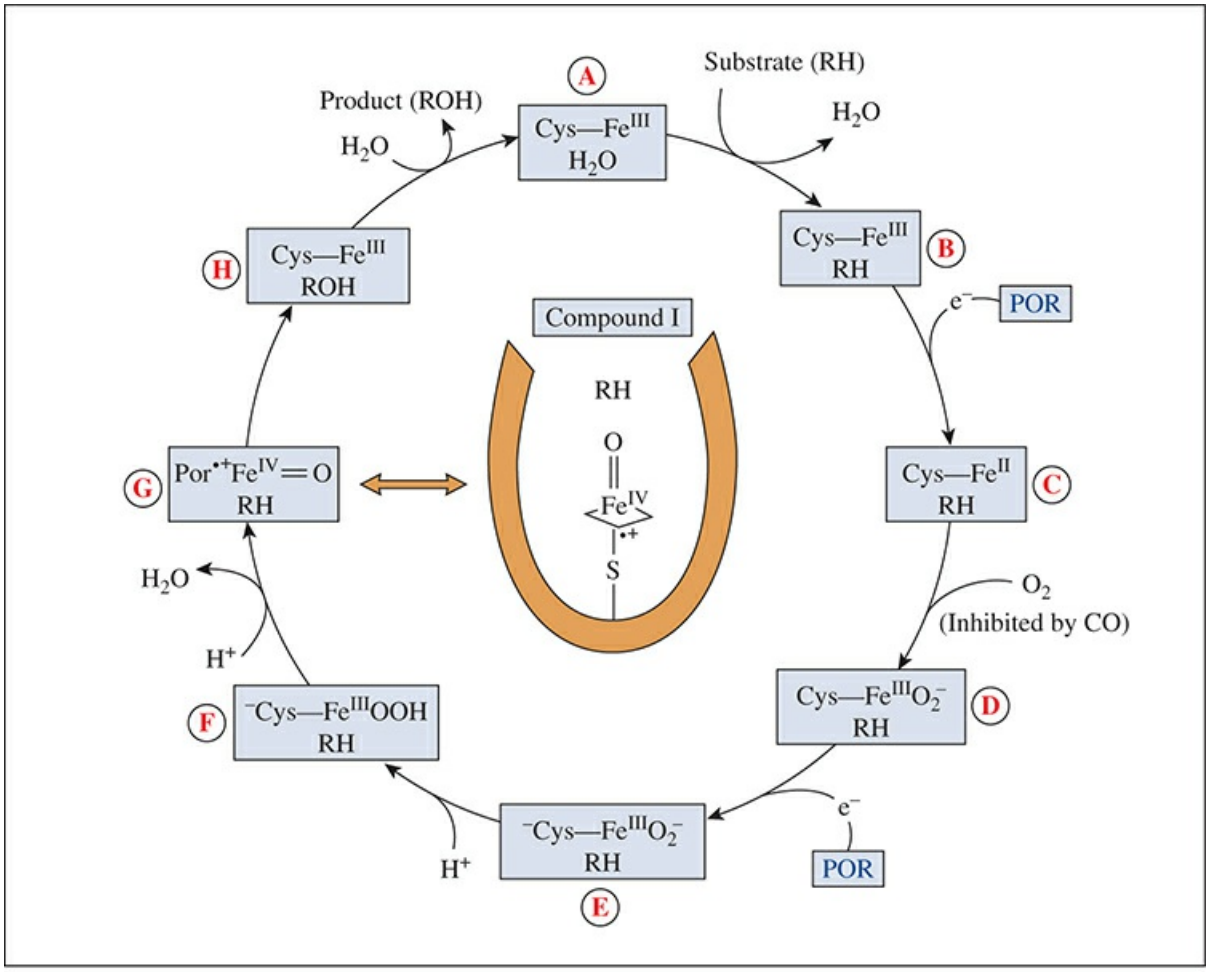
All CYP enzymes are heme-containing proteins. The basic reaction catalyzed by CYP enzymes is monooxygenation in which one atom of oxygen from O_2 is incorporated into a substrate, designated RH, and the other is reduced to water with reducing equivalents derived from NADPH, as follows:

$$\text{Substrate(RH)} + O_2 + \text{NADPH} + H^+ \rightarrow \text{Product(ROH)} + H_2O + \text{NADP}^+$$

During catalysis, CYP binds directly to the substrate and molecular oxygen, but it does not interact directly with NADPH or NADH. The mechanism by which CYP receives electrons from NAD(P)H depends on the subcellular localization of CYP. In the endoplasmic reticulum where most of the CYP enzymes involved in xenobiotic biotransformation are localized, electrons are relayed from NADPH to CYP via a flavoprotein called NADPH–cytochrome P450 reductase or POR.

The catalytic cycle of CYP involves eight steps (A → H), as shown in Fig. 6–6, for the oxidation of a substrate (RH) to its hydroxylated metabolite (ROH). In this scheme, iron is shown bound to its fifth ligand, a heme thiolate provided by a highly conserved cysteine (Cys) residue. The first steps of the cycle (A → G) involve the activation of oxygen to compound I, and the final steps involve substrate oxidation by compound I (G → H) followed by release of the metabolite (ROH) to restore the enzyme to its resting (ferric) state (H → A). Following the binding of substrate (RH) to CYP (A → B), the heme iron is reduced from the ferric (Fe^{III}) to the ferrous (Fe^{II}) state by the introduction of a single electron from POR (B → C). It is at stage C, when the iron is in the ferrous state, that CYP can bind oxygen and CO. In the third step (C → D), oxygen binds to the ferrous iron, which transfers an electron to oxygen to form ferric-bound superoxide anion, designated $Cys{-}Fe^{III}O^-_2$. At this stage, the cycle can be interrupted (uncoupled) to release superoxide anion and the enzyme can be restored to its resting (ferric) state (see "Other reactions" in Fig. 6–6). In the fourth step (D → E), a second electron is introduced from POR, which is delocalized over the thiolate bond to form the supernucleophilic ferriperoxo intermediate $Cys{-}Fe^{III}O^-_2$. Uncoupling of the cycle at this stage releases hydrogen peroxide and restores the enzyme to its resting (ferric) state. In the fifth step (E → F), addition of a proton (H^+) converts the supernucleophilic ferriperoxo anion intermediate to its corresponding hydroperoxide, the ferrihydroperoxy $^-Cys{-}Fe^{III}OOH$. In the sixth step (F → G), addition of a second proton followed by release of water converts the ferrihydroperoxy intermediate to compound I, an $iron^{IV}$-oxo porphyrin radical cation species. The formation of compound I ($por^{\bullet +}Fe^{IV}{=}O$) by protonation of the ferrihydroperoxy intermediate involves the heterolytic cleavage of oxygen with the two-electron oxygen atom going to water, a reaction facilitated by the strong electron-donating effects of the heme thiolate anion. Heterolytic (two-electron) cleavage of oxygen to produce compound I places the iron in the perferryl (Fe^V) oxidation state, which is a considerably stronger oxidant than that formed by homolytic cleavage of oxygen, which produces the less reactive $Por\ Fe^{IV}{-}OH$ with iron in oxidation state IV. (*Note*: It is somewhat confusing that compound I is written as $por^{\bullet +}Fe^{IV}{=}O$ because, without taking the porphyrin ring into account, the formula gives the erroneous impression that the iron is in the Fe^{IV} [ferryl] state, whereas it is actually in the Fe^V [perferryl] state.) In the seventh step (G → H), the highly electrophilic oxygen from compound I is transferred to the substrate (RH) to produce metabolite

(ROH). In the final step (H → A), the metabolite is released, which restores the enzyme to its initial resting (ferric) state.



Other reactions

One-electron reduction	**C** (Cys—Fe^{II} RH)	→	**A** (Cys—Fe^{III} + $RH^{\bullet}$)
Superoxide anion production	**D** (Cys—$Fe^{III}O_2^-$ RH)	→	**B** (Cys—Fe^{III} RH) + $O_2^{\bullet-}$
Hydrogen peroxide production	**E** ($^-$Cys—$Fe^{III}O_2^-$ RH) + $2H^+$	→	**B** (Cys—Fe^{III} RH) + H_2O_2
Hydrogen peroxide shunt	**B** (Cys—Fe^{III} RH) + H_2O_2	→	**F** ($^-$Cys—Fe^{III}OOH RH) + H^+
Peroxide shunt to form Compound I	**B** (Cys—Fe^{III} RH) + XOOH	→	**G** ($Por^{\bullet+}Fe^{IV}$=O RH) + XOH

FIGURE 6–6 *Catalytic cycle of cytochrome P450.* Cytochrome P450 is represented as Cys-FeIII, where Cys represents the fifth ligand (a cysteine thiolate) to the ferric heme iron. RH and ROH represent the substrate and product (hydroxylated metabolite), respectively. The intermediates in the catalytic cycle are as follows: A, ferric resting state; B, substrate bound; C, ferrous intermediate; D, ferrisuperoxo anion intermediate; E, ferriperoxo intermediate with

an electron delocalized over the Cys thiolate bond; F, ferrihydroperoxy intermediate (with a negative charge on the Cys thiolate bond); G, compound I, an ironIV-oxo porphyrin cation, which is responsible for most substrate oxidation reactions; H, enzyme in its resting state prior to the release of product formed by hydrogen abstraction followed by oxygen rebound (see text for details). FeII, FeIII, FeIV, and FeV refer to iron in the ferrous, ferric, ferryl, and perferryl state, respectively. It should be noted that although it is written as por•+FeIV=O, compound I is in the highly oxidized perferryl (FeV) state when the oxidation state of the porphyrin ring is also taken into account.

CYP catalyzes several types of oxidation reactions, including the following:

1. Hydroxylation of an aliphatic or aromatic carbon
2. Epoxidation of a double bond
3. Heteroatom (*S*-, *N*-, and *I*-) oxygenation and *N*-hydroxylation
4. Heteroatom (*O*-, *S*-, and *N*-) dealkylation
5. Oxidative group transfer
6. Cleavage of esters and carbamates
7. Dehydrogenation

Cytochrome P450 Nomenclature

Liver microsomes from all mammalian species contain numerous CYP enzymes, each with the potential to catalyze the various types of reactions shown in Figs. 6–7 to 6–14. Hepatic microsomal CYP enzymes are categorized into families and subfamilies and named individually on the basis of their amino acid sequence. The CYP enzymes involved in xenobiotic biotransformation belong mainly to the CYP1, CYP2, and CYP3 gene families. The CYP enzymes involved in endobiotic metabolism generally have the same name in all mammalian species.

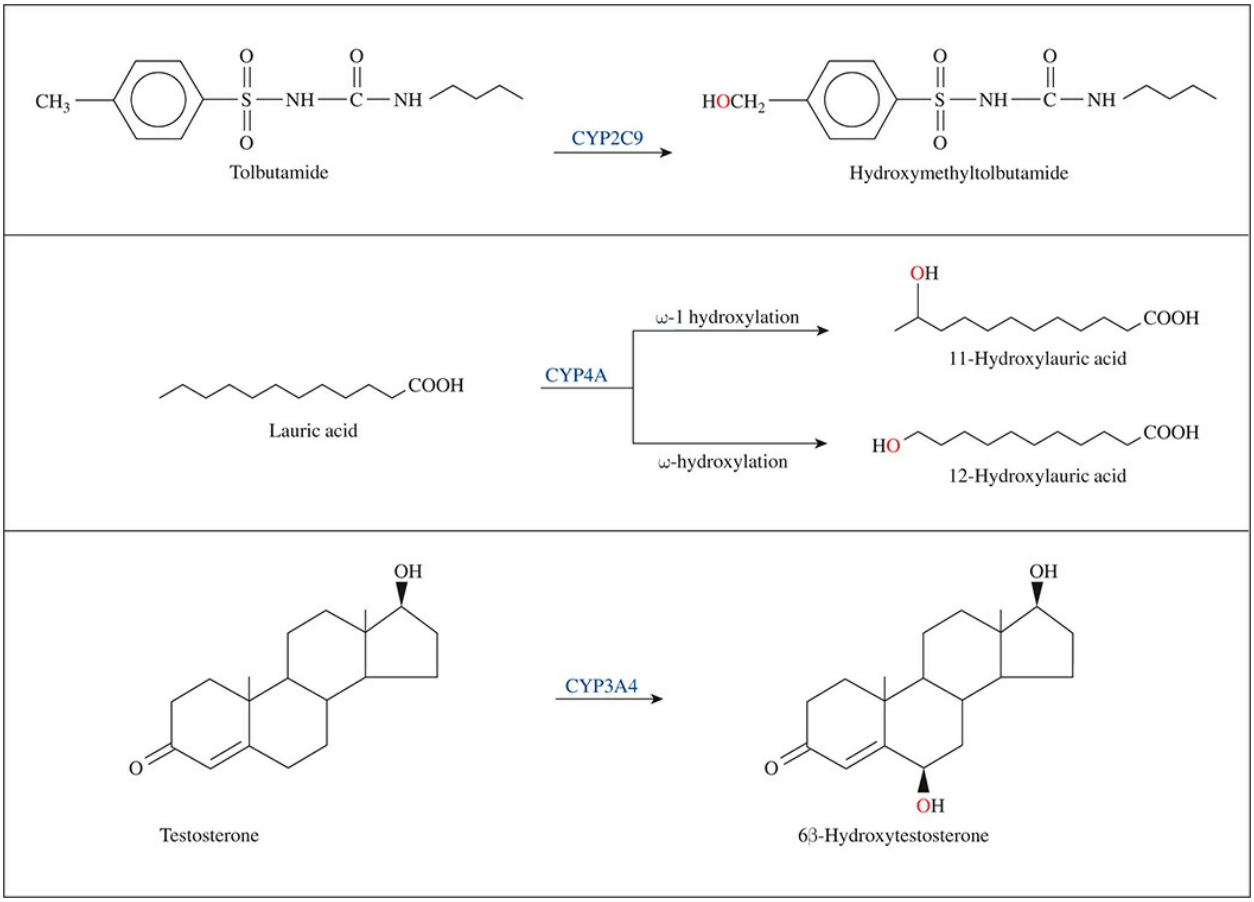


FIGURE 6–7 *Examples of reactions catalyzed by cytochrome P450: hydroxylation of aliphatic carbon.*

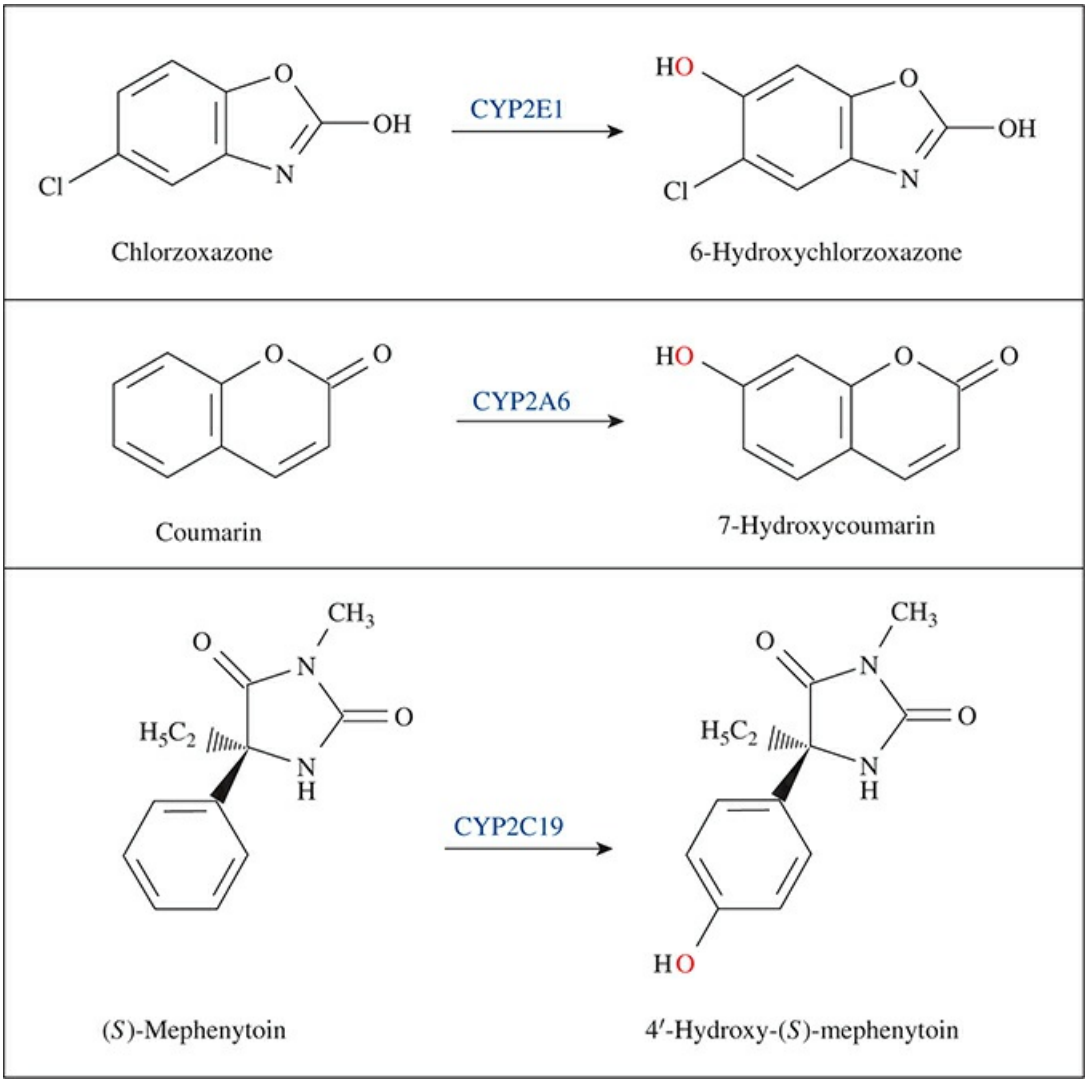


FIGURE 6–8 *Examples of reactions catalyzed by cytochrome P450: hydroxylation of aromatic carbon.*

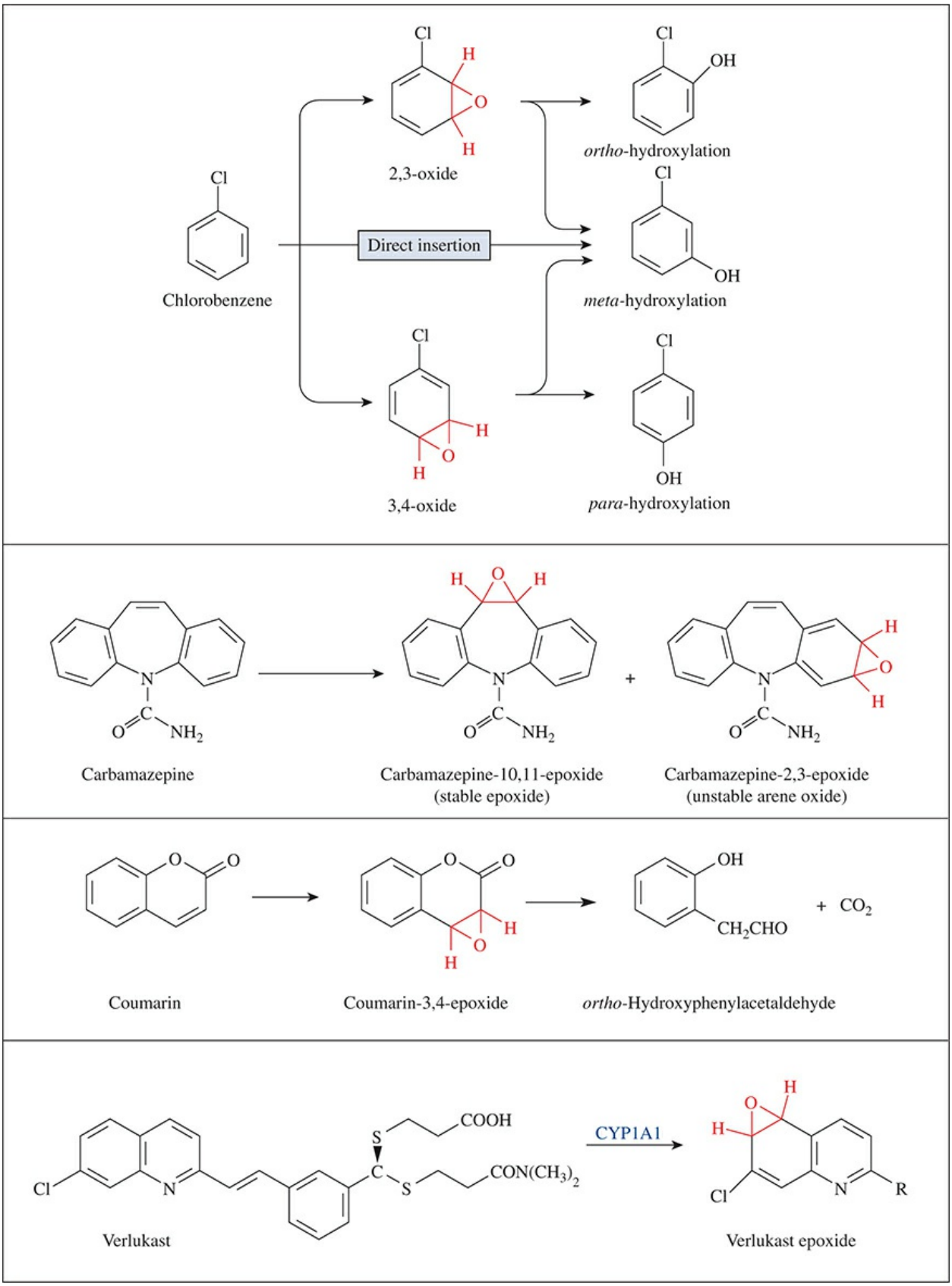


FIGURE 6–9 *Examples of reactions catalyzed by cytochrome P450: epoxidation.*

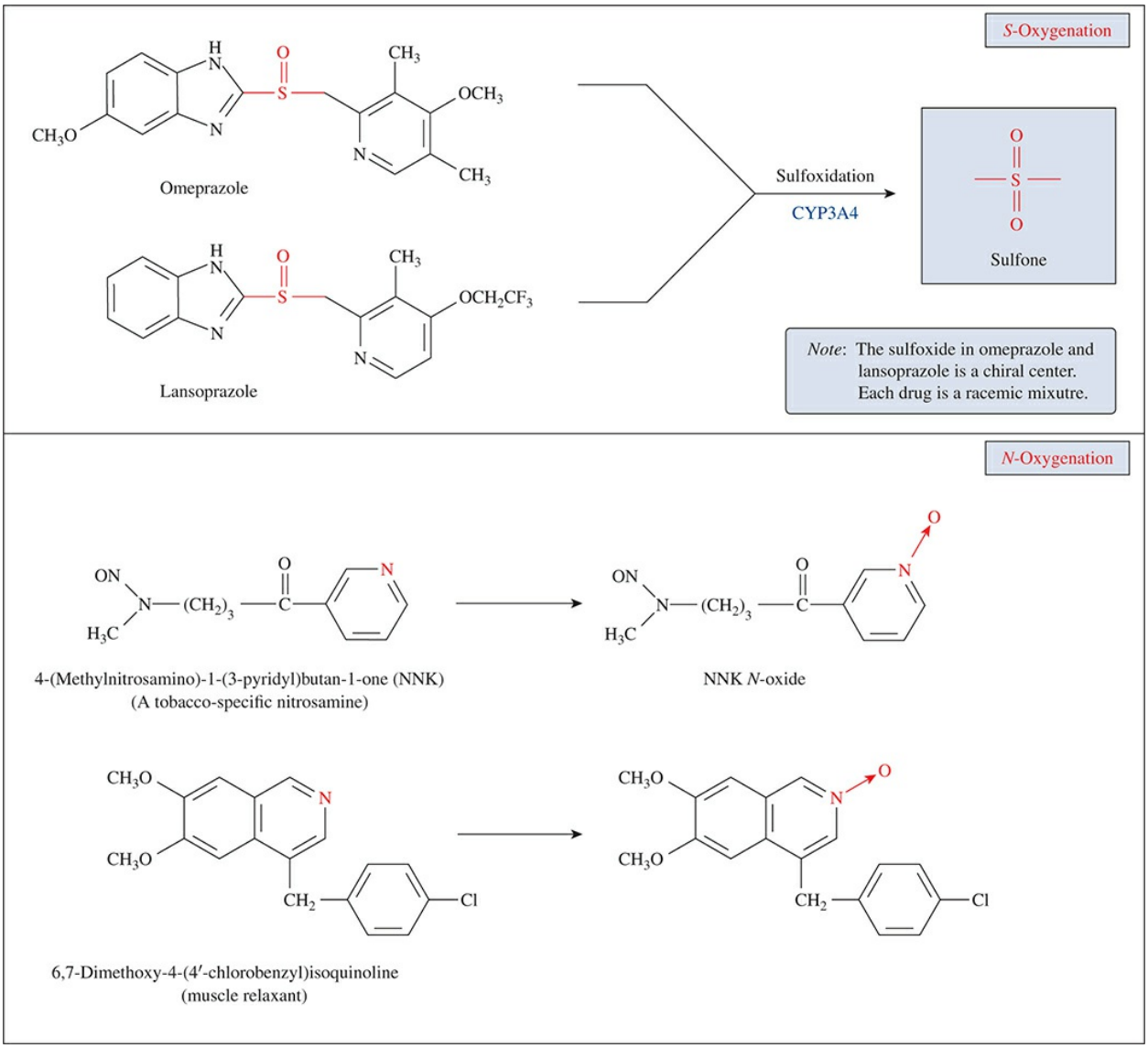


FIGURE 6–10 *Examples of reactions catalyzed by cytochrome P450: heteroatom oxygenation.*

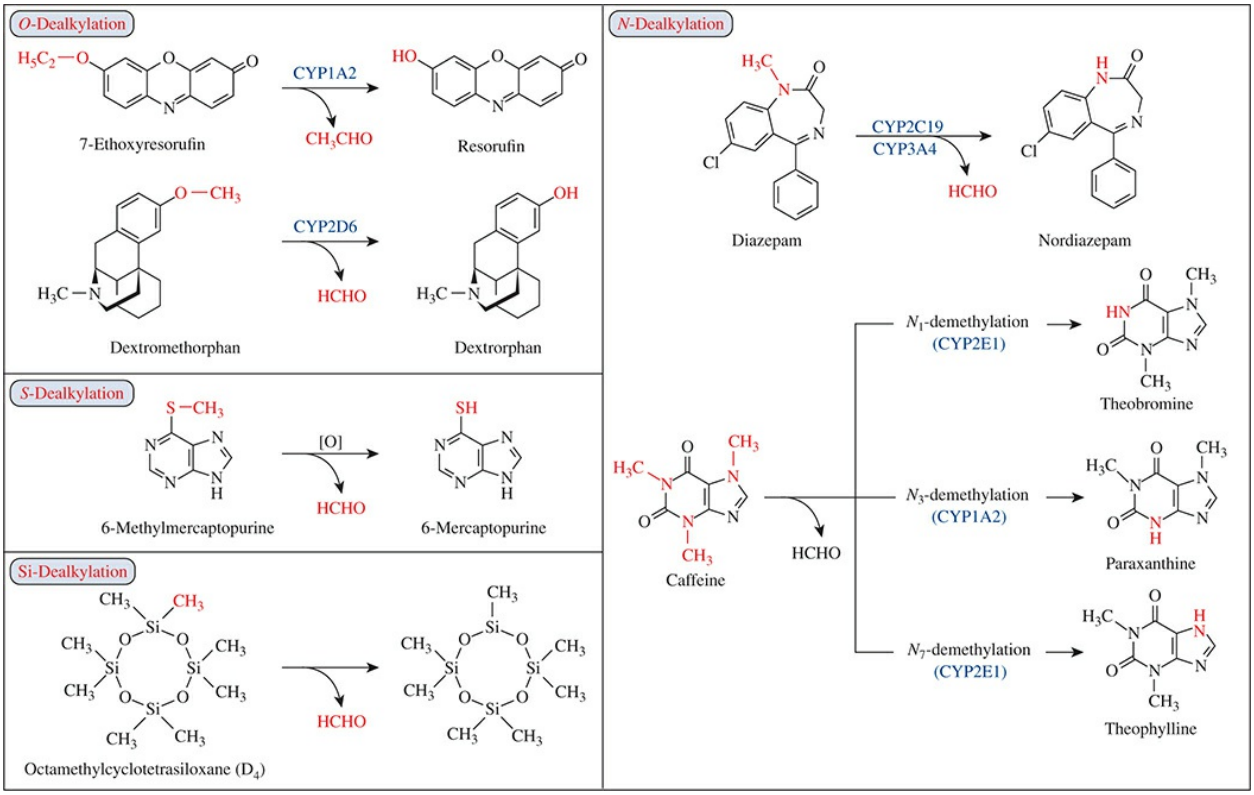


FIGURE 6–11 *Examples of reactions catalyzed by cytochrome P450: heteroatom dealkylation.*

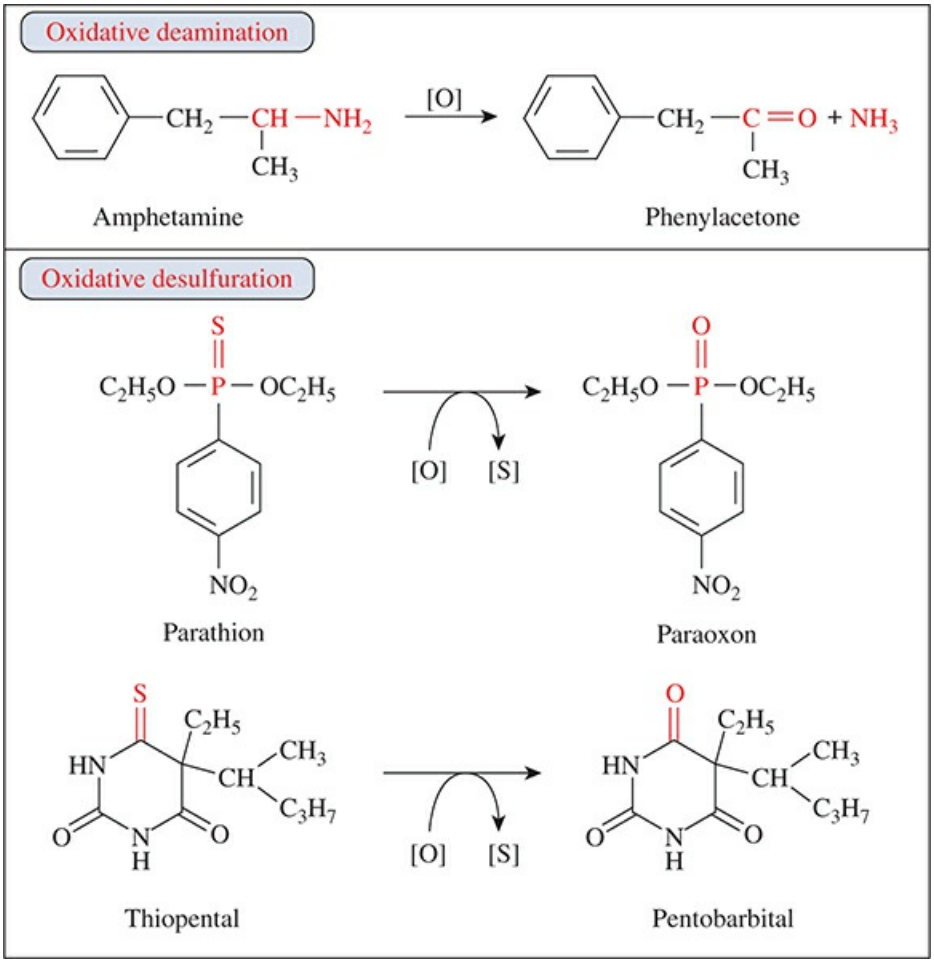


FIGURE 6–12 *Examples of reactions catalyzed by cytochrome P450: oxidative group transfer.*

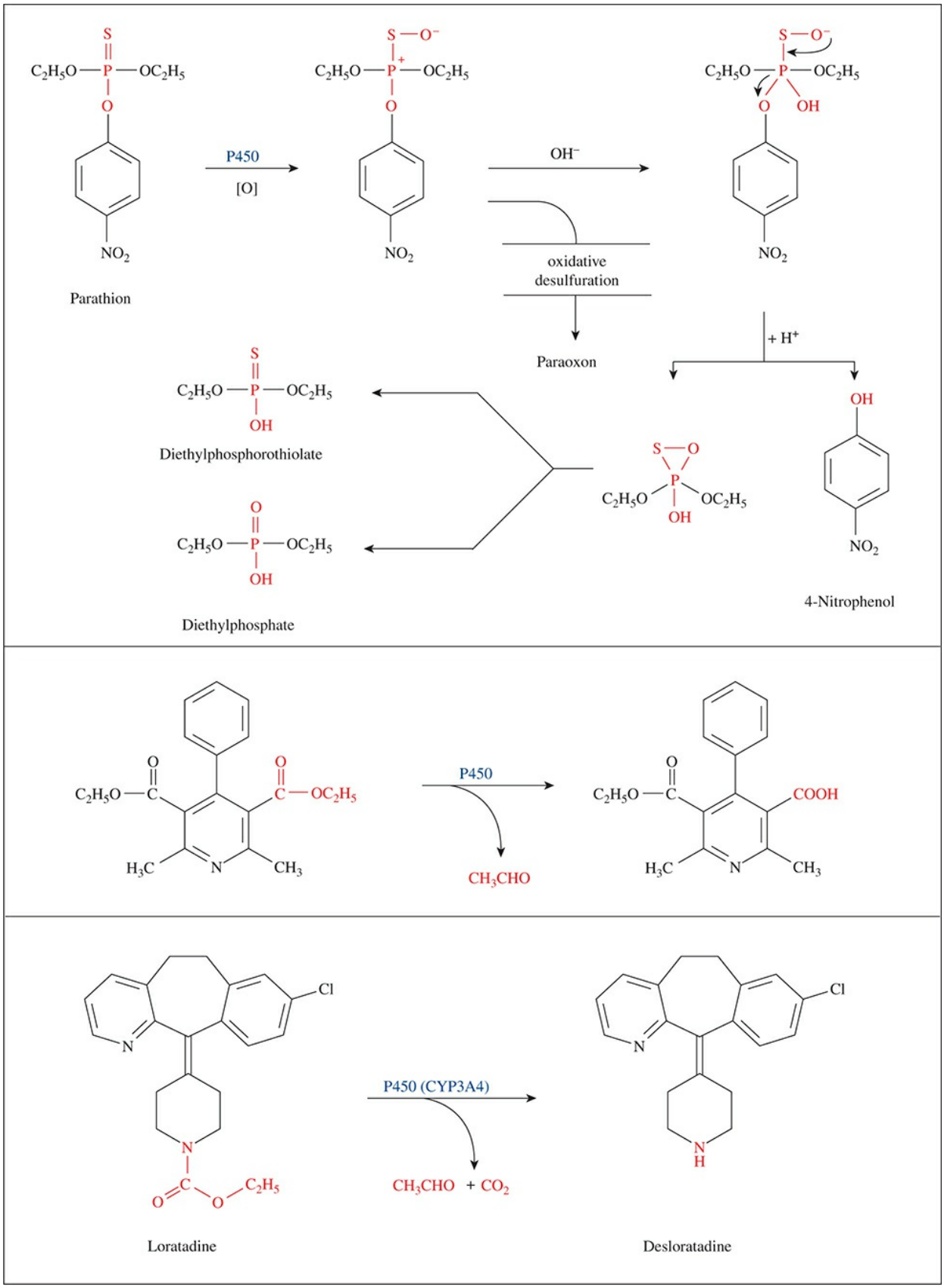


FIGURE 6–13 *Examples of reactions catalyzed by cytochrome P450 that resemble hydrolytic reactions: cleavage of a thiophosphate (parathion), a carboxylic acid ester (2,6-dimethyl-4-phenyl-3,5-pyridinecarboxylic acid diethyl ester), and a carbamate (loratadine).*

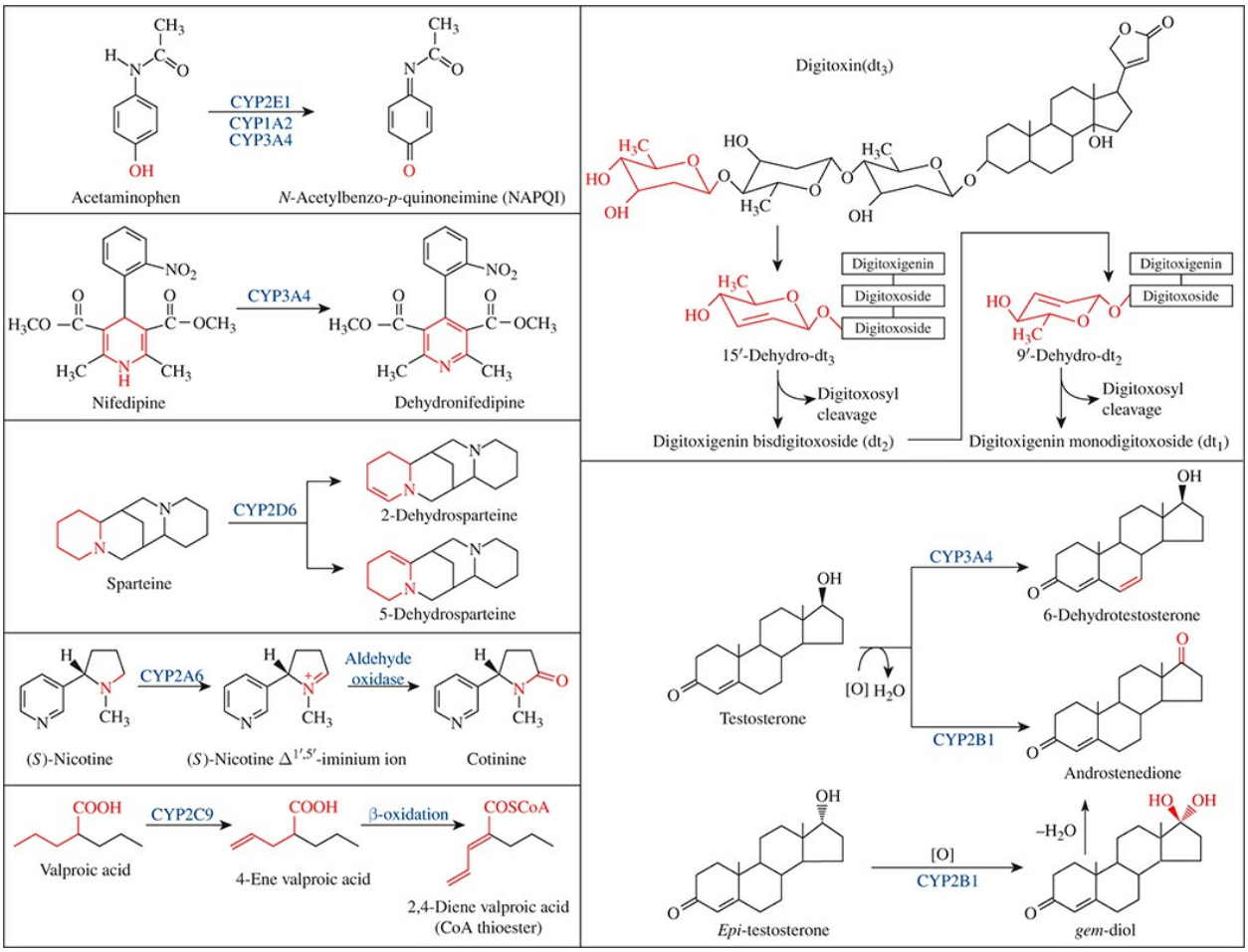


FIGURE 6–14 *Examples of reactions catalyzed by cytochrome P450: dehydrogenation.*

CYP1B1, CYP2E1, CYP2R1, CYP2S1, CYP2U1, and CYP2W1 are highly conserved homologs that can be given the same name across mammalian species. The levels and activity of each CYP enzyme have been shown to vary from one individual to the next, due to extrinsic (e.g., environmental) and/or intrinsic (e.g., genetic) factors. Decreased CYP enzymatic activity can result from (1) a genetic mutation that either blocks the synthesis of a CYP enzyme or leads to the synthesis of a catalytically compromised, inactive, or unstable enzyme, which gives rise to the PM and IM genotypes; (2) exposure to an environmental factor (such as an infectious disease or an inflammatory process) that suppresses CYP enzyme expression; or (3) exposure to a xenobiotic that inhibits or inactivates a preexisting CYP enzyme. By inhibiting CYP, one drug can impair the biotransformation of another, which may lead to an exaggerated pharmacological or toxicological response to the second drug. Increased CYP enzymatic activity can result from (1) gene duplication leading to overexpression of a CYP enzyme, which gives rise to the UM genotype; (2) gene mutations in the coding or promoter region that increase expression, activity, or stability of CYP; (3) exposure to drugs and other xenobiotics that induces the synthesis or

retard the degradation of CYP; or (4) exposure to drugs and other xenobiotics that stimulates the activity of a preexisting enzyme.

Induction of cytochrome P450 by xenobiotics increases CYP enzyme activity. By inducing cytochrome P450, one drug can stimulate the metabolism of a second drug and thereby decrease or ameliorate its therapeutic effect. Allelic variants, which arise by point mutations in the wild-type gene, are another source of interindividual variation in CYP activity. Environmental factors known to affect CYP levels include medications, foods, social habits (e.g., alcohol consumption and cigarette smoking), and disease status (diabetes, inflammation, viral and bacterial infection, hyperthyroidism, and hypothyroidism). When environmental factors influence CYP enzyme levels, considerable variation may be observed during repeated measures of xenobiotic biotransformation (e.g., drug metabolism) in the same individual. Due to their broad substrate specificity, it is possible that two or more CYP enzymes can contribute to the metabolism of a single compound.

The pharmacologic or toxic effects of certain drugs are exaggerated in a significant percentage of the population due to a heritable deficiency in a CYP enzyme. Inasmuch as the biotransformation of a xenobiotic in humans is frequently dominated by a single CYP enzyme, the considerable effort in identifying which CYP enzyme or enzymes are involved in eliminating the drug is known as reaction phenotyping or enzyme mapping. Four approaches to reaction phenotyping are as follows:

1. Correlation analysis involves measuring the rate of xenobiotic metabolism by several samples of human liver microsomes and correlating reaction rates with the variation in the level or activity of the individual P450 enzymes in the same microsomal samples.
2. Chemical inhibition evaluates the effects of known CYP enzyme inhibitors on the metabolism of a xenobiotic by human liver microsomes. Inhibitors of cytochrome CYP must be used cautiously because most of them can inhibit more than one CYP enzyme.
3. Antibody inhibition determines the effects of inhibitory antibodies against selected CYP enzymes on the biotransformation of a xenobiotic by human liver microsomes. This method alone can potentially establish which human CYP enzyme is responsible for biotransforming a xenobiotic.
4. Biotransformation by purified or recombinant human CYP enzymes establishes whether a particular CYP enzyme can or cannot biotransform a xenobiotic, but it does not address whether that CYP enzyme contributes substantially to reactions catalyzed by human liver microsomes.

Examples of substrates, inhibitors, and inducers for each CYP enzyme in human liver microsomes are given in Table 6–2. Because reaction phenotyping in vitro is not always performed with toxicologically relevant substrate concentrations, the CYP enzyme that appears responsible for biotransforming the drug in vitro may not be the CYP enzyme responsible for biotransforming the drug in vivo.

TABLE 6–2 Examples of Clinically Relevant Substrates, Inhibitors, and Inducers of the Major Human Liver Microsomal P450 Enzymes Involved in Xenobiotic Biotransformation

	CYP1A2	CYP2A6	CYP2B6	CYP2C8	CYP2C9	CYP2C19	CYP2E1
SUBSTRATES	Alosetron	Coumarin	Bupropion	Amodiaquine	Celecoxib	Clobazam	Aniline
	Caffeine	Nicotine	Efavirenz	Cerivastatin	Diclofenac	Diazepam	Acetaminophen
	Duloxetine	Efavirenz	Propofol	Dasabuvir	Fluoxetine	Esomeprazole	Chlorzoxazone
	7-Ethoxyresorufin	Tegafur	*(S)*-Mephenytoin	GSK1278863	Flurbiprofen	Fluoxetine	Lauric acid
	Melatonin		Cyclophosphamide	Montelukast	Meloxicam	*(R)*-Hexobarbital	4-Nitrophenol
	Phenacetin		Ketamine	Paclitaxel	Peroxicam	*(S)*-Mephenytoin	
	Ramelteon		Meperidine	Rosiglitazone	Phenytoin	Lansoprazole	
	Selegiline		Nevirapine	Repaglinide	Tolbutamide	Moclobemide	
	Tacrine		Ifosfamide		*(S)*-Warfarin	Omeprazole	
	Tizanidine		Pethidine			Proguanil	
	Theophylline		Methadone			Tilidine	
INHIBITORS	Acyclovir	Methoxsalen	Boceprevir	Clopidogrel glucuronide	Amiodarone	Allicin	Clomethiazole
	Allopurinol	Pilocarpine	Clopidogrel	Deferasirox	Azapropazone	Armodafinil	Diallyldisulfide
	Caffeine	Selegiline	Clorgyline	Fluvoxamine	Benzbromarone	Carbamazepine	Diethyldithiocarbamate
	Cimetidine	Tranylcypromine	3-Isopropenyl-3-methyl diamantane	Gemfibrozil glucuronide	Capecitabine	Cimetidine	Disulfiram
	Ciprofloxacin	Tryptamine	2-Isopropenyl-2-methyladamantane	Ketoconazole	Cotrimoxazole	Esomeprazole	Isoniazid
	Disulfiram		Pargyline	Montelukast	Etravirine	Efavirenz	
	Echinacea		Phencyclidine	Pioglitazone	Fluconazole	Etravirine	
	Famotidine		Phenelzine	Quercetin	Fluoxetine	Felbamate	
	Fluvoxamine		Phenylethylpiperidine	Rosiglitazone	Fluvastatin	Fluconazole	
	Furafylline		Prasugrel	Rosuvastatin	Fluvoxamine	Fluoxetine	
	Mexilitene		Rolapitant	Teriflunomide	Metronidazole	Fluvoxamine	
	Norfloxacin		Selegiline	Trimethoprim	Miconazole	Human growth hormone (rhGH)	
	Oltipraz		Sertraline		Oxandrolone	Ketoconazole	
	Oral contraceptives		Tenofovir		Phenylbutazone	Moclobemide	
	Propafenone		Thio-TEPA		Sulfaphenazole	Nootkatone	
	Propranolol		Ticlopidine		Sulfinpyrazone	Omeprazole	
	Terbinafine		Voriconazole		Tienilic acid	Oral contraceptives	
	Ticlopidine				Tigecycline	Ticlopidine	
	Verapamil				Voriconazole	Voriconazole	
	Zafirlukast				Zafirlukast		

INDUCERS	Cigarette smoking Lansoprazol Omeprazole 3-Methylcholanthrene Montelukast Moricizine β-Naphthoflavone Phenobarbital Phenytoin Rifampin Ritonivir TCDD Teriflunomide	Dexamethasone Pyrazole	Carbamazepine Efavirenz Nevirapine Phenobarbital Phenytoin Rifampin Ritonavir St. John's wort	Carbamazepine Oral contraceptives Phenobarbital Rifampin	Aprepitant Bosentan Carbamazepine Enzalutamide Phenobarbital Rifampin Ritonavir St. John's wort	Artemisinin Carbamazepine Efavirenz Enzalutamide Ginkgo Phenobarbital Rifampin St. John's wort	Cigarette smoking Ethanol Isoniazid
	CYP2D6		**CYP3A4**				
SUBSTRATES	Atomoxetine Amitriptyline Aripiprazole Brofaromine (±)-Bufuralol (*S*)-Chlorpheniramine Chlorpromazine Clomipramine Codeine Debrisoquine Desipramine Dextromethorphan, Dolasetron Doxepin Duloxetine Eliglustat	Methylphenidate Metoprolol Mexiletine Morphine Nebivolol Nicergoline Nortriptyline Ondansetron Paroxetine Perhexilene Perphenazine Pimozide Propafenone (+)-Propranolol Risperidone Sparteine	Alfentanil Alfuzosin Alisporivir Almorexant Alprazolam Amlodipine Amprenavir Aplaviroc Aprepitant Artemether Astemiziole Atazanavir Atorvastatin Avanafil Azithromycin Barnidipine	Cyclosporine Danoprevir Darifenacin Darunavir Dasatinib Depsipeptide Dexamethasone Dextromethorphan Diergotamine α-Dihydroergocriptine Dihydroergotamine Disopyramide Docetaxel Domperidone Dronedarone Dutasteride	Etoricoxib Felodipine Fentanyl Fluticasone Gallopamil Gefitinib Gepirone Gestodene Granisetron Grazoprevir Halofantrine Ibrutinib Imatinib Indinavir Isavuconazole Isradipine	Methylprednisolone Mexazolam Midazolam Midostaurin Mifepristone Mosapride Naloxegol Neratinib Nicardipine Nifedipine Nimoldipine Nisoldipine Nitrendipine Norethindrone Oxatomide Oxybutynin	Saquinavir Sildenafil Sibutramine Simeprevir Simvastatin Sirolimus Sunitinib Tacrolimus Tadalafil Telithromycin Terfenadine Testosteron† Tiagabine Ticagrelor Tilidine Tipranavir

	Encainide	Tamoxifen	Bexarotene	Ebastine	Itraconazole	Paritaprevir	Tirilazad
	Fentanyl	Thioridazine	Bortezomib	Eletriptan	Ivabradine	Perospirone	Tofisopam
	(S)-Fluoxetine	Timolol	Bosutinib	Elvitegravir	Ivacaftor	Pimozide	Tolvaptan
	Haloperidol (reduced)	Tolperisone	Brecanavir	Eplerenone	Karenitecin	Pranidipine	Triazolam
	Ibogaine	Tolterodine	Brotizolam	Ergotamine	Ketamine	Praziquantel	Trimetrexate
	Imipramine	Tramadol	Budesonide	Erlotinib	Laquinimod	Quetiapine	Ulipristal
	Loperamide	Traxoprodil	Buspirone	Erythromycin	Levomethadyl	Quinidine	Vardenafil
	(R)-Metoprolol	*(R)*-Venlafaxine	Capravirine	Eplerenone	Lomitapide	Quinine	Vicriviroc
		Vernakalant	Carbamazepine	Ethosuximide	Lonafarnib	Reboxetine	Vinblastine
			Casopitant	Etoperidone	Loperamide	Ridaforolimus	Vincristine
			Cibenzoline	Everolimus	Lopinavir	Rifabutin	Vinorelbine
			Cilastazol	Ethinyl estradiol	Lovastatin	Ritonavir	Voclosporin
			Cisapride		Lumefantrine	Rosuvastatin	Ziprasidone
			Clarithromycin		Lurasidone	Ruboxistaurin	Zonisamide
			Clindamycin		Maraviroc	Salmeterol	
			Clopidogrel		Medroxyprogesterone		
			Cobimetinib				
			Conivaptan				
INHIBITORS	Amiodarone	Hydralazine	Alprazolam	Cimetidine	Fluvoxamine	Lopinavir	Saquinavir
	Buproprion	Hydroxychloroquine	Amiodarone	Ciprofloxacin	Fosamprenavir	Mibefradil	St. John's wort
	Celecoxib	Imatinib	Amlodipine	Clarithromycin	Gestodene	Nefazodone	Telaprevir
	Chlorpheniramine	Methadone	Amprenavir	Cobicistat	Ginkgo	Nelfinavir	Telithromycin
	Cimetidine	Mibefradil	Aprepitant	Conivaptan	Goldenseal	Nilotinib	Ticagrelor
	Cinacalet	Oral contraceptives	Atazanavir	Crizotinib	Grapefruit Juice	Oral contraceptives	Tipranavir
	Clobazam	Paroxetine	Atorvastatin	Cyclosporine	Idelalisib	Pazopanib	Troleandomycin
	Clomipramine	Pazopanib	Azamulin	Darunavir	Imatinib	Posaconazole	Verapamil
	Dacomitinib	Propafenone	Bicalutamide	Diltiazem	Indinavir	Ranitidine	Voriconazole
	Desvenlafaxine	Quinidine	Bosentan	Erythromycin	Isoniazid	Ranolazine	Zileuton
	Diltiazem	Ranitidine	Boceprevir	Felbamate	Itraconazole	Ritonavir	
	Diphenhydramine		Cilostazol	Fluconazole	Ketoconazole	Roxithromycin	
				Fluoxetine	Lapatinib		

	CYP2D6		CYP3A4				
	Duloxetine	Ritonavir					
	Echinacea	Sertraline					
	Ecstasy	Telithromycin					
	Escitalopram	Terbinafine					
	Febuxostat	Verapamil					
	Fluoxetine	Vemurafenib					
	Gefitinib						
	Haloperidol						
INDUCERS	Carbamazepine		Amprenavir	Dabrafenib	Mifepristone	Pioglitazone	Spironolactone
	Corticosteroids (pregnancy)		Aprepitant	Dexamethasone	Mitotane	Prednisone	Sulfinpyrazole
	Phenobarbital		Armodafinil	Echinacea	Nafcillin	Rifabutin	Talviraline
	Rifampin		Avasimibe	Efavirenz	Nelfinavir	Rifampin	Telotristat ethyl
			Bosentan	Enzalutamide	Nifedipine	Rifapentine	Thioridazine
			Carbamazepine	Etravirine	Omeprazole	Ritonavir	Topotecan
			Clobazam	Etoposide	Paclitaxel	Rufinamide	Troglitazone
			Echinacea	Genistein	PCBs	Semagacestat	Troleandomycin
			Clotrimazole	Guggulsterone	Phenobarbital	St. John's wort	Vemurafenib
			Cyproterone acetate	Hyperforin	Phenytoin	Simvastatin	
				Lersivirine			
				Lopinavir			
				Lovastatin			
				Modafinil			

Endogenous Biomarkers of Cytochrome P450—The ability of many CYP enzymes (and other drug-metabolizing enzymes) to biotransform endogenous substrates (in addition to xenobiotics)

opens the possibility that their activity in vivo can be monitored with biomarkers (i.e., metabolites of endogenous compounds formed by a specific CYP or other drug-metabolizing enzyme). In principle, biomarkers could be used to assess the impact of a new investigational drug, herbal medication, or any other xenobiotic to which humans are exposed on one or more enzymes involved in xenobiotic biotransformation. In the case of investigational drugs, this assessment could be conducted early in the clinical phase of drug development to assess the potential of a new drug candidate to cause clinically relevant inhibition or induction of CYP enzymes.

Activation of Xenobiotics by Cytochrome P450—Various CYP-dependent reactions are involved in the activation of the chemicals listed in Table 6–3. Many of the chemicals listed in Table 6–3 are also detoxified by CYP by biotransformation to less toxic metabolites. In some cases, the same CYP enzyme catalyzes both activation and detoxication reactions. Complex factors determine the balance between xenobiotic activation and detoxication.

TABLE 6–3 Examples of Xenobiotics Activated by Human P450

CYP1A2	CYP2E1
Acetaminophen	Acetaminophen
2-Acetylaminofluorene	Acrylonitrile
4-Aminobiphenyl	Benzene
2-Aminofluorene	Carbon tetrachloride
2-Naphthylamine	Chloroform
NNK	Dichloromethane
Amino acid pyrolysis products	1,2-Dichloropropane
(DiMeQx, MeIQ, MeIQx, Glu P-2, IQ, PhIP, Trp P-1, Trp P-2)	Ethylene dibromide
	Ethylene dichloride
Tacrine	Ethyl carbamate
CYP2A6 AND 2A13	Halothane
NNK and bulky nitrosamines	*N*-Nitrosodimethylamine
N-Nitrosodiethylamine	Styrene
Aflatoxin B_1	Trichloroethylene
CYP2B6	Vinyl chloride
6-Aminochrysene	**CYP3A4**
Cyclophosphamide	Acetaminophen
Ifosfamide	Aflatoxin B_1 and G_1
CYP2C8, 9, 18, 19	6-Aminochrysene
Tienilic acid	Benzo[*a*]pyrene 7,8-dihydrodiol
Phenytoin	Cyclophosphamide
Valproic acid	Ifosfamide
CYP2D6	1-Nitropyrene
NNK	Sterigmatocystin
CYP2F1	Senecionine
3-Methylindole	*Tris*(2,3-dibromopropyl)phosphate
Acetaminophen	**CYP4B1 (IN ANIMALS BUT NOT HUMANS)***
Valproic acid	Ipomeanol
CYP1A1 AND 1B1	3-Methylindole
Benzo[*a*]pyrene and other polycyclic aromatic hydrocarbons	2-Aminofluorene

Abbreviation: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, a tobacco-specific nitrosamine.
* Due to lack of heme incorporation human CYP4B1 is probably inactive.
Adapted from Guengerich FP, Shimada T. Oxidation of toxic and carcinogenic chemicals by human cytochrome P-450 enzymes. *Chem Res Toxicol.* 1991;4(4):391-407.

Inhibition of Cytochrome P450—Inhibition of CYP is a major cause of drug–drug interactions (and occasionally the withdrawal of regulatory approval). The magnitude of the drug–drug interaction depends on the degree of CYP inhibition by the perpetrator drug (those xenobiotics that inhibit or induce the enzyme that is responsible for clearing a victim drug) and the fractional metabolism of the victim drug by the affected enzyme.

Inhibitory drug interactions generally fall into two categories, namely, reversible or irreversible. Reversible (direct) inhibitors can be competitive, noncompetitive, or uncompetitive (or mixed) inhibitors. Irreversible inhibitors are metabolism-dependent inhibitors (MDIs), which can be completely irreversible or quasi-irreversible (reversible under certain conditions). Direct inhibition can be subdivided into two types: 1) competition between two drugs that are metabolized by the same CYP enzyme, and 2) inhibition is when the inhibitor is not a substrate for the affected CYP enzyme. Metabolism dependent inhibition occurs when CYP converts a xenobiotic to a metabolite that is a more potent inhibitor than the parent compound.

Induction of Cytochrome P450: Xenosensors—The induction (upregulation) of xenobiotic-biotransforming enzymes and transporters is a receptor-mediated, adaptive process that augments xenobiotic elimination during periods of high xenobiotic exposure. It is not a toxicological or pathological response, but enzyme induction is often associated with liver enlargement (due to both hepatocellular hypertrophy and hyperplasia), and it may be associated with toxicological and pharmacological consequences, especially for the safety evaluation of drug candidates in laboratory animals and for clinical practice in humans. In animals and humans, enzyme induction may be associated with pharmacokinetic tolerance, whereby the xenobiotic induces its own elimination.

CYP induction is mediated by four ligand-activated receptors, namely, AhR, CAR, PXR, and PPARα, as summarized in Table 6–4. These so-called xenosensors resemble other nuclear receptors, such as steroid and thyroid hormone receptors. thyroid hormone receptors, which has consequences for receptor interactions (cross talk among xenosensors and between xenosensors and other nuclear receptors). Some xenosensors respond to endobiotics to regulate their metabolism, or to play a role in inducing or suppressing the expression of xenobiotic-biotransforming enzymes and membrane transporters. Xenosensors have a ligand-binding domain (LBD) and a highly conserved cysteine-rich DNA-binding domain (DBD).

TABLE 6–4 Receptors Mediating the Induction (or Suppression) of Cytochrome P450 Enzymes and Other Xenobiotic-Biotransforming Enzymes and Transporters

Nuclear Receptor	Response Element(s)	Receptor Activators	Regulated Genes*
AhR	XRE	PAHs, TCDD (other PHAHs), β-naphthoflavone, indigoids, tryptophan metabolites, omeprazole, lansoprazole, laquinimod, roquinimex	CYP1A1, 1A2, 1B1, 2S1, UGT1A1, UGT1A6, AKR1A1, AKR1C1–4
CAR	DR-3 DR-4 ER-6	Phenobarbital, phenytoin, carbamazepine, CITCO (human), TCPOBOP (mouse), clotrimazole, Yin Zhi Wuang (many PXR agonists are also CAR agonists, and vice versa)	CYP2A6, 2B6, 2C8, 2C9, 2C19, 3A4, UGT1A1, SULT1A1, AKR1D1, ALAS, MRP2, MRP3, MRP4
PXR	DR-3 DR-4 ER-6 ER-8	Amprenavir, avasimibe, bosentan, bile acids, carbamazepine, clindamycin, clotrimazole, cortisol, cyproterone acetate, dicloxacillin, efavirenz, etoposide, dexamethasone, griseofulvin, guggulsterone, hyperforin (SJW), indinavir, lovastatin, mifepristone, nafcillin, nelfinavir, nifedipine, omeprazole, paclitaxel, PCBs, phenobarbital, phthalate monoesters, 5β-pregnane-3,20-dione, rifabutin, rifampin, ritonavir, saquinavir, simvastatin, spironolactone, sulfinpyrazole, TAO, tetracycline, topotecan, transnanoclor, troglitazone, verapamil, vitamin E, vitamin K_2	CYP2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4, 3A7, 4F12, 7A1↓, CES2, SULT2A1, UGT1A1, 1A3, 1A4, 1A6, GSTA1, AKR1D1, PAPSS2, ALAS, MDR1, MRP2, AhR
PPARα	DR-1	Fibrates, WY-14643, perfluorodecanoic acid	CYP4A, UGT1A9, UGT2B4 in rodents CYP1A1, 2C8, and 3A4 in humans
Nrf2	ARE	β-Naphthoflavone, oltipraz, phenolic antioxidants (e.g., BHA and BHT), phenylisothiocyanate, acetaminophen, and various glutathione depletors (diethyl maleate, phorone)	NQO1, mEH, AKR7A, UGTs, GSTA1, γ-GCL, MRP1
GR	GRE	Glucocorticoids (e.g., dexamethasone)	CYP2C9, 2B6, 2D6, 3A4, 3A5, CAR, PXR
FXR	IR-1	Bile acids, GW4064, AGN29, AGN31	BSEP, I-BABP, MDR3, UGT2B4, SULT2A1, OATP8, OSTα/β, PPARα, SHP
LXRα	DR-4	GW3965, T0901317, paxiline, F_3methylAA,† acetylpodocarpic dimer (APD)	LRH1, SHP, CYP7A, LXRα, CYP3A4↓↓, 2B6↓
VDR	DR-3 ER-6 IR-0	1α,25-Dihydroxyvitamin D_3, lithocholate	CYP2B6, 2C9, 3A4, SULT2A1
HNF-1α	Not named‡		OATP-C, OATP8, CYP7A1, UGT1A6, 1A8, 1A9, 1A10, HNF4α, PXR, kidney specific expression of OAT1, OAT3, URAT1
HNF-4α	DR		CYP2A6, 2B6, 2C9, 2D6, 3A4, DD4, MDR1, PXR, CAR, FXR, PPARα, HNF1α
LRH-1	DR-4		CYP7A, ASBT
SHP	None		Targets of PPARα↓, AhR↓, PXR↓, CAR↓, LRH-1↓, HNF4α↓, LXRα↓, GR↓

* A downward arrow indicates downregulation (suppression). All others are upregulated (induced).
† [3-Chloro-4-(3-(7-propyl-3-trifluoromethyl-6-(4,5)-isoxazolyl)propylthio)-phenylacetic acid].
‡ The HNF-1α consensus sequence is GTTTAATNATTAAC.

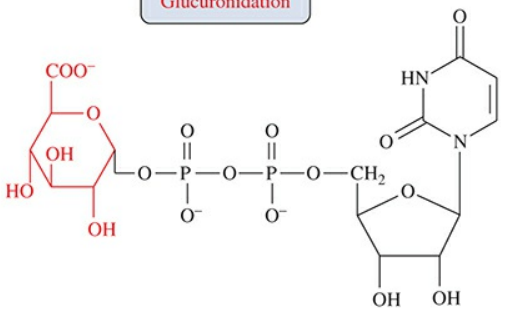
In general, CYP induction involves the following steps (with steps 2 and 3 reversed in the case of AhR): (1) binding of ligand (xenobiotic) to the receptor, which triggers conformational changes that promote its dissociation from accessory proteins (such as corepressors, chaperones, and cytoplasm retention proteins) and promote its association with coactivators; (2) dimerization of the ligand-bound receptor with a partner protein to form a DNA-binding heterodimer (which is analogous to the two halves of a clothespin coming together to form a functional unit); (3) translocation of the functional receptor heterodimer from the cytoplasm to the nucleus; (4)

binding of the functional receptor heterodimer to discrete regions of DNA (response elements) that are typically located in the 5′-promoter region of the gene (which is analogous to a clothespin being fastened to a clothes line); (5) recruitment of other transcription factors and coactivators (such as histone and RNA methyltransferases, histone and chromatin deacetylases, and histone remodeling helicases) and RNA polymerase to form a transcription complex; and (6) gene transcription, which leads to increased levels of CYP mRNA and protein (as well as other xenobiotic-biotransforming enzymes and transporters). As with all nuclear receptors, the details of the process of activating a xenosensor to its transcriptionally active form are complex and multifaceted. Over 200 coactivators/coregulators of nuclear receptor function have been identified.

CONJUGATION

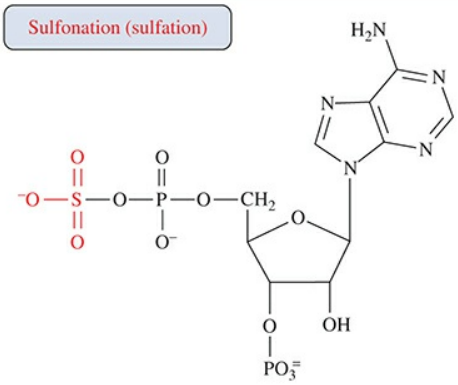
Conjugation reactions include glucuronidation, sulfonation (often called sulfation), acetylation, methylation, conjugation with glutathione (GSH; mercapturic acid synthesis), and conjugation with coenzyme A (CoA) followed by conjugation with amino acids such as glycine, taurine, and glutamine. The cofactors (or cosubstrates) for these reactions, which are shown in Fig. 6–15, react with functional groups that are either present on the xenobiotic or introduced/exposed during oxidation, reduction, or hydrolysis. With the exception of methylation and acetylation, conjugation reactions result in a large increase in xenobiotic hydrophilicity and total polar surface area, such that they greatly promote the excretion of foreign chemicals. Glucuronidation, sulfonation, acetylation, and methylation involve reactions with activated or “high-energy” cofactors, whereas conjugation with amino acids or GSH involves reactions with activated xenobiotics. Most conjugation enzymes are located in the cytosol, except for the UDP-glucuronosyltransferases (UGTs), which are found in the microsomes.

Glucuronidation



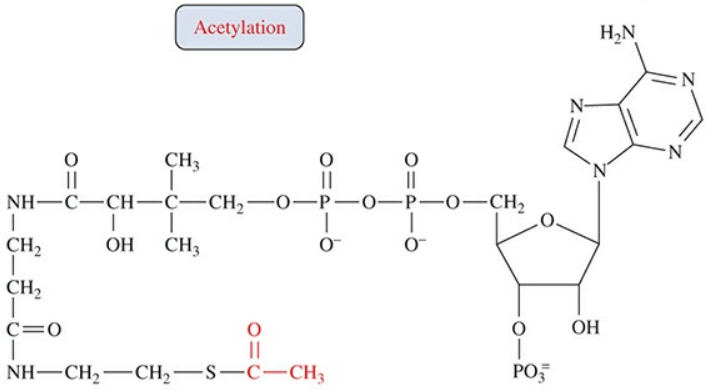
Uridine-5′-diphospho-α-D-glucuronic acid (UDPGA)

Sulfonation (sulfation)



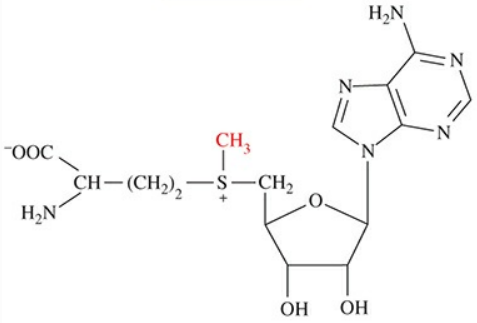
3′-Phosphoadenosine-5′-phosphosulfate (PAPS)

Acetylation



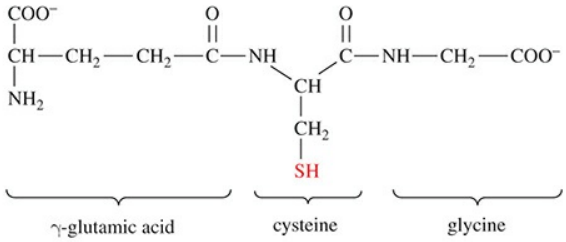
Acetyl-coenzyme A

Methylation



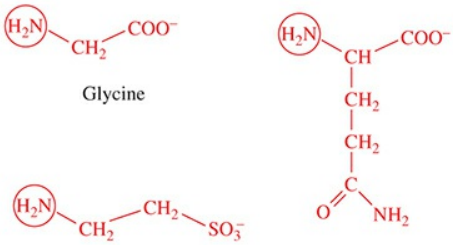
S-Adenosylmethionine (SAM)

Glutathione conjugation



Glutathione

Amino acid conjugation

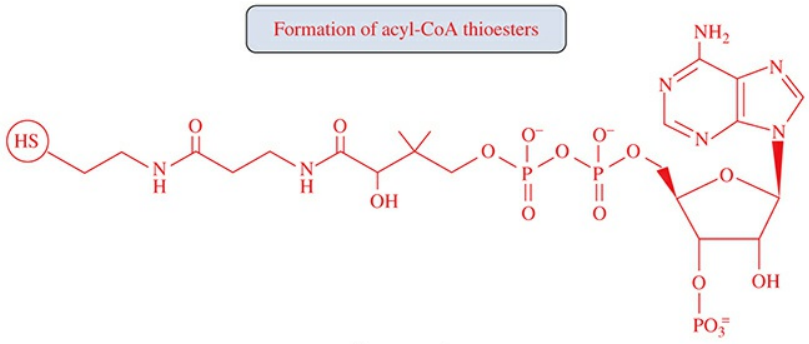


Glycine

Taurine

Glutamine

Formation of acyl-CoA thioesters



Coenzyme A

FIGURE 6–15 *Structures of cofactors for conjugation reactions. The functional group that reacts with or is transferred to the xenobiotic is shown in red.*

Glucuronidation and Formation of Acyl-CoA Thioesters

Glucuronidation is a major pathway of xenobiotic biotransformation in mammalian species except for members of the cat family. The primary cofactor is UDP-glucuronic acid (UDPGA), and UGTs are located predominantly in the endoplasmic reticulum of the liver, kidney, gastrointestinal tract, lungs, prostate, mammary glands, skin, brain, spleen, and nasal mucosa. Examples of xenobiotics that are glucuronidated are shown in Fig. 6–16. The site of glucuronidation is generally an electron-rich (nucleophilic) O, N, or S heteroatom of typically small lipophilic compounds such as aliphatic alcohols and phenols (which form *O*-glucuronide acetals), carboxylic acids (which form acyl glucuronides), primary and secondary aromatic and aliphatic amines (which form *N*-glucuronides), and free sulfhydryl groups (which form *S*-glucuronides).

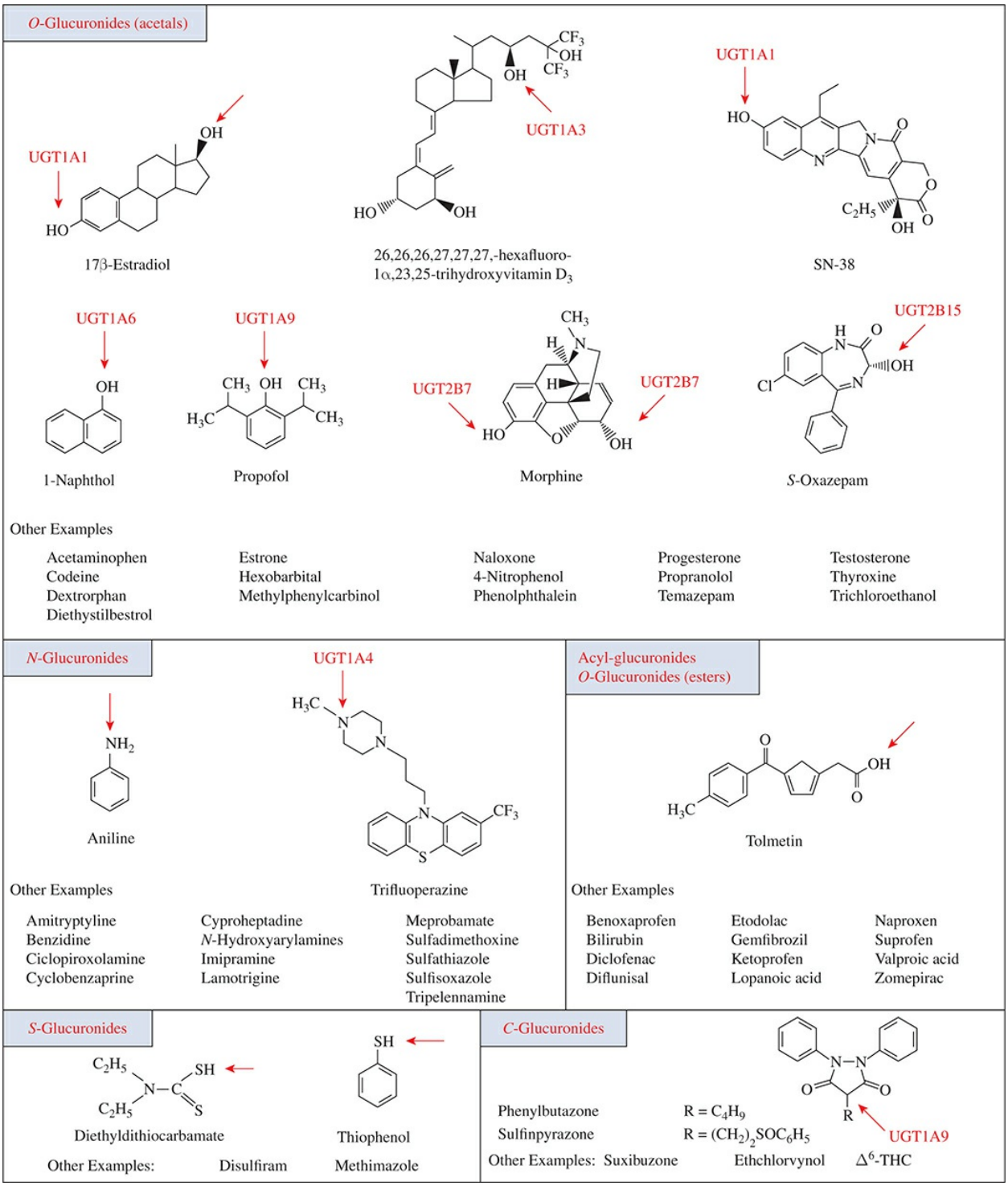


FIGURE 6–16 *Examples of xenobiotics and endogenous substrates that are glucuronidated.* The arrow indicates the site of glucuronidation, with the UGT enzyme if selective.

UGTs can form unusual conjugates: (1) bisglucuronides (e.g., bilirubin and morphine), where two different functional groups on the same molecule are glucuronidated; (2) diglucuronides (e.g., 5α-dihydroxytestosterone; DHT), where two glucuronides are attached in tandem to a

single site; (3) *N*-carbamoyl glucuronides (e.g., sertraline and varenicline), where carbonate is incorporated in the glucuronide; and (4) glycosidation with UDP-sugars other than UDPGA (e.g., glucosidation of barbiturates). Endogenous substrates for glucuronidation include bilirubin, steroid hormones, and thyroid hormones.

Glucuronide conjugates of xenobiotics and endogenous compounds are polar (typically anionic), with a pK_a(A) of 3 to 3.5, water-soluble metabolites that are eliminated from the body in urine or bile. Whether glucuronides are excreted from the body in bile (following their canalicular transport by MRP2) or urine (following their sinusoidal transport into blood by MRP3) depends, in part, on the size of the parent compound or its unconjugated metabolite. The carboxylic acid moiety of glucuronic acid (pK_a(A) 3–3.5), which is ionized at physiological pH, promotes excretion because (1) it increases the aqueous solubility of the xenobiotic and (2) it is recognized by the biliary and renal organic anion transport systems, enabling active secretion of the glucuronides into urine and bile. However, the elimination of drug conjugates in bile may not result in the elimination of the drug in feces because the conjugates may be hydrolyzed in the gut by bacterial β-glucuronidase and the parent drug may be reabsorbed or eliminated in feces.

Although human UGTs typically are highly selective in the use of UDPGA as a sugar donor, they can accommodate the use of other sugar donors such as UDP-glucose, UDP-galactose, and UDP-xylose in an aglycone-dependent manner. Cofactor availability can limit the rate of glucuronidation of drugs that are administered in high doses and are conjugated extensively.

Sulfonation

Many xenobiotics and endogenous substrates undergo *O*-sulfonation to a highly water-soluble sulfuric acid ester (see Table 6–5). The reaction is catalyzed by sulfotransferases (SULTs), a large multigene family of enzymes widely distributed throughout the body. In mammals, there are two major classes of SULTs: (1) membrane-bound SULTs in the Golgi apparatus and (2) soluble SULTs in the cytoplasm. The cytosolic SULTs catalyze the sulfonation of various drugs, mutagens, flavonoids, and other xenobiotics, as well as endogenous substrates such as bile acids, thyroid hormones, catecholamine neurotransmitters, and steroids. The cofactor for the sulfonation reaction is 3′-phosphoadenosine-5′-phosphosulfate (PAPS), the structure of which is shown in Fig. 6–15.

TABLE 6–5 Properties of the Human Cytosolic Sulfotransferases (SULTs)

Human Sult	Polymorphic?	Tissue Distribution	Major Substrates[†,‡]
SULT1A1	Yes*** *1–*4	Liver, platelets, placenta, adrenal gland, endometrium, colon, small intestine, leukocytes, brain	**4-Nitrophenol**, acetaminophen, apomorphine, 4-cresol, dopamine, epinephrine, 17β-estradiol (and other phenolic steroids), 4-ethylphenol, genistein, *N*-hydroxy-PhIP, minoxidil, morphine, naloxone, 2-naphthol (and other phenols), 2-nitropropane, numerous aromatic amines, hydroxylamines, hydroxamic acids, and benzylic alcohols, T2, T3, troglitazone
SULT1A2	Yes *1–*6	Liver, kidney, brain, gastrointestinal tract, leukocytes, bladder tumors	4-Nitrophenol, *N*-hydroxy-2-acetylaminofluorene, minoxidil, morphine, naloxone, 2-naphthol, various aromatic hydroxylamines and hydroxamic acids
SULT1A3	Yes *1–*4	Small intestine and colon, liver, platelets, placenta, brain, leukocytes, fetal liver	**Dopamine**, albuterol, dobutamine, 1-hydroxymethylpyrene, minoxidil, morphine, 4-nitrophenol, norepinephrine, salbutamol, vanillin
SULT1A4		Liver, pancreas, colon, brain	Likely similar to SULT1A3
SULT1B1		Gastrointestinal tract, liver, leukocytes, skeletal muscle	Benzylic alcohols, dopamine, 4-nitrophenol, T2, T3, r-T3, T4
SULT1C2	Yes *1–*5	Kidney, gastrointestinal tract, thyroid gland, heart, fetal lung, and kidney	Aromatic hydroxylamines, *N*-hydroxy-2- acetylaminofluorene, 4-nitrophenol, thyroid hormones
SULT1C3		Appendix, lymph nodes, thymus, adrenal gland, fetal liver	Unknown
SULT1C4		Kidney, ovary, spinal cord, fetal kidney, and lung	Bisphenol-A, diethylstilbestrol, estrone, 1-hydroxymethylpyrene, *N*-hydroxy-2- acetylaminofluorene, 4-nitrophenol, nonylphenol, 4-octylphenol
SULT1D1		Not characterized	
SULT1E1		Liver, small intestine, endometrium, heart, adrenal gland, mammary epithelial cells, and fetal liver, lung, and kidney	**17β-Estradiol**, dehydroepiandrosterone, diethylstilbestrol, equilenin, estrone, ethinyl estradiol, 2- and 4-hydroxyestrone, 2- and 4-hydroxyestradiol, 4-hydroxylonazolac, 1-naphthol, naringenin, pregnenolone, raloxifene, tamoxifen, thyroid hormones
SULT2A1	Yes *1–*3	Liver, adrenal gland, ovaries, prostate, small intestine, kidney, brain	**Dehydroepiandrosterone (DHEA)**, afitoxifene, bile acids, budesonide, buprenorphine, estradiol (and other hydroxysteroids), hycanthone, 1-hydroxymethylpyrene, 6-hydroxymethylbenzo[*a*]pyrene, 4-hydroxytamoxifen, pregnenolone, raloxifene, testosterone (and other androgens)
SULT2B1		Tongue, placenta, liver, prostate, trachea, skin, skeletal muscle, spleen, thymus, kidney, ovary, adrenal gland, gastrointestinal tract	**Cholesterol**, dehydroepiandrosterone, pregnenolone and other oxysterols and hydroxysteroids
SULT4A1		Brain	Estrone, 2-naphthol, 2-naphthylamine, 4-nitrophenol, T3, T4
SULT6B1		Testis, kidney	Unknown

[†]Substrates in bold are reported to be selective probe substrates for the SULT listed.
[‡]T4 is thyroxine. T2 and T3 are di- and tri-iodothyronine. r-T3 is reverse tri-iodothyronine.

Sulfonation involves the transfer of sulfonate, not sulfate (i.e., SO_3^-, not SO_4^-) from PAPS to the xenobiotic. PAPS is synthesized from inorganic sulfate (SO_4^{2-}) and ATP in a two-step reaction on a single enzyme called PAPS synthetase. The major source of sulfate required for the synthesis of PAPS is derived from cysteine through a complex oxidation sequence. The low cellular concentrations of PAPS (4 to 80 μM; versus UDPGA 200 to 350 μM and GSH 5 to 10 mM) limits the capacity for xenobiotic sulfonation.

Multiple SULTs have been identified in all mammalian species examined and they are arranged into gene families that share at least 45% amino acid sequence identity, which are subdivided into subfamilies that are at least 60% identical. The SULTs expressed in mammals

belong to six families (SULT1 to SULT6). Each family appears to work on a specific functional group (phenols, alcohols, and amines).

Table 6–5 lists some examples of xenobiotics and endogenous compounds that are sulfonated without prior biotransformation by oxidative enzymes. An even greater number of xenobiotics are sulfonated after a hydroxyl group is exposed or introduced during hydrolytic or oxidative biotransformation. Figure 6–17 highlights the sulfonation of three substrates: 2-acetylaminofluorene, safrole, and 7,12-dimethylbenz[a]anthracene.

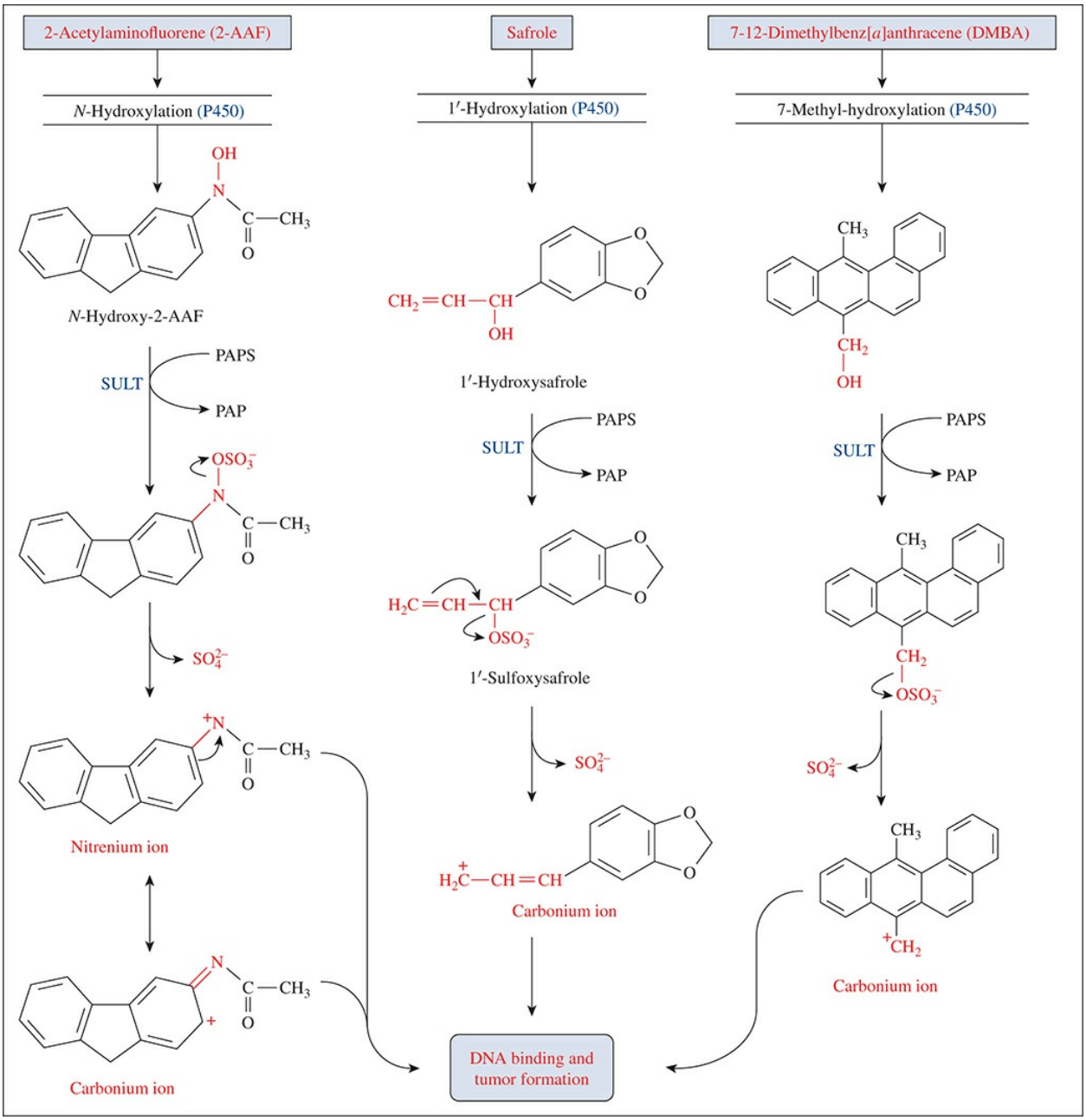


FIGURE 6–17 *Role of sulfonation in the generation of tumorigenic metabolites (nitrenium or carbonium ions) of 2-acetylaminofluorene, safrole, and 7,12-dimethylbenz[a]anthracene (DMBA).*

Sulfonate conjugates of xenobiotics are excreted mainly in urine. Those excreted in bile may be hydrolyzed by aryl sulfatases present in gut microbiota, which contributes to the enterohepatic circulation of certain xenobiotics. Sulfatases are also present in the endoplasmic reticulum and lysosomes, where they primarily hydrolyze sulfonates of endogenous compounds.

Methylation

Methylation, a minor pathway of xenobiotic biotransformation, generally decreases the aqueous solubility of xenobiotics and masks functional groups that might be otherwise conjugated by other enzymes. The cofactor for methylation is *S*-adenosylmethionine (SAM) as shown in Fig. 6–15. The methyl group bound to the sulfonium ion in SAM is transferred to xenobiotics and endogenous substrates by nucleophilic attack from an electron-rich heteroatom (*O*, *N*, or *S*). Examples of xenobiotics and endogenous substrates that undergo O-, N-, or S-methylation are shown in Fig. 6–18.

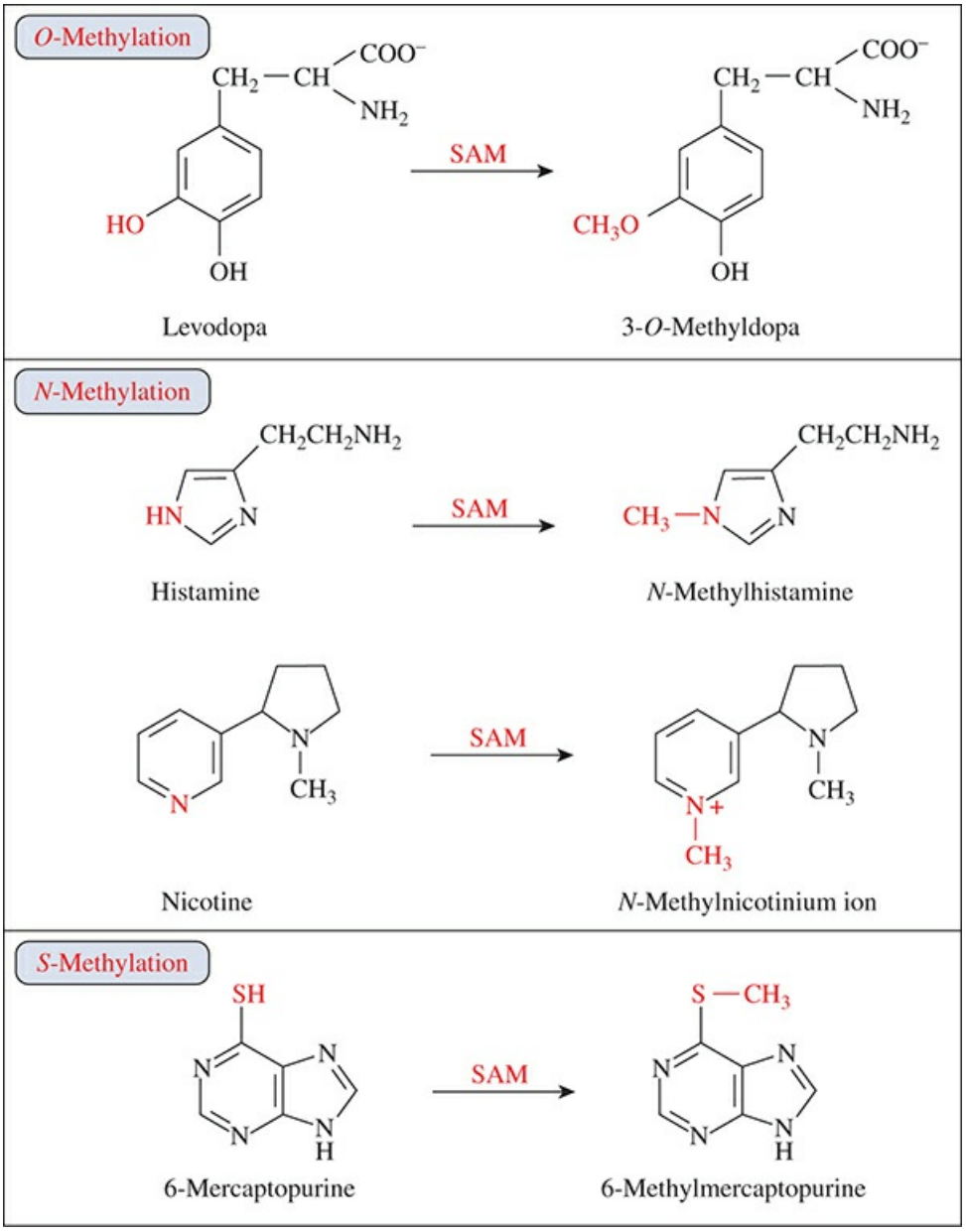


FIGURE 6–18 *Examples of compounds that undergo O-, N-, or S-methylation.*

The O-methylation of phenols and catechols is catalyzed by two different enzymes known as phenol O-methyltransferase (POMT) in microsomes and catechol-O-methyltransferase (COMT) in cytosol and microsomes. Substrates for COMT include several catecholamine neurotransmitters and catechol drugs, such as L-DOPA and methyldopa.

Several N-methyltransferases have been described in humans and other mammals. Phenylethanolamine N-methyltransferase catalyzes the N-methylation of the neurotransmitter norepinephrine to form epinephrine in the adrenal medulla and in certain regions of the brain, and is of minimal significance in xenobiotic biotransformation. However, histamine and nicotine N-methyltransferases expressed in liver, intestine, and/or kidney do methylate xenobiotics. S-Methylation is an important pathway in the biotransformation of sulfhydryl-containing xenobiotics. In humans, S-methylation is catalyzed by thiopurine methyltransferase in cytosol

and thiol methyltransferase in microsomes.

Acetylation

N-Acetylation is a major route of biotransformation for xenobiotics containing an aromatic amine (phenyl-NH_2) or a hydrazine group (R–NH–NH_2), which are converted to aromatic amides (phenyl-NH–$COCH_3$) and hydrazides (R–NH–NH–$COCH_3$), respectively. *N*-Acetylation masks an amine with a nonionizable group, making many *N*-acetylated metabolites less water soluble than the parent compound. Like methylation, *N*-acetylation has a negligible effect on aqueous solubility and does little to promote the renal and biliary excretion of xenobiotics.

The *N*-acetylation of xenobiotics is catalyzed by *N*-acetyltransferases (NATs) and requires the cofactor acetyl coenzyme A (acetyl-CoA, Fig. 6–15). The reaction occurs in two sequential steps according to a *ping-pong Bi–Bi* mechanism. In the first step, the acetyl group from acetyl-CoA is transferred to a cysteine residue in the NAT active site with release of CoA (E–SH + CoA–$SCOCH_3$ → E–S–$COCH_3$ + CoA–SH). In the second step, the acetyl group is transferred from the acylated enzyme to the amino group of the substrate with regeneration of the enzyme. For "strongly" basic amines (pK_a(B) >8), the rate of *N*-acetylation is determined by the first step (acetylation of the enzyme), whereas the rate of *N*-acetylation of "weakly" basic amines (pK_a(B) <6) is determined by the second step (transfer of the acetyl group from the acylated enzyme to the acceptor amine). In certain cases, NATs can catalyze the *O*-acetylation of hydroxylamines. Examples are shown in Fig. 6–19.

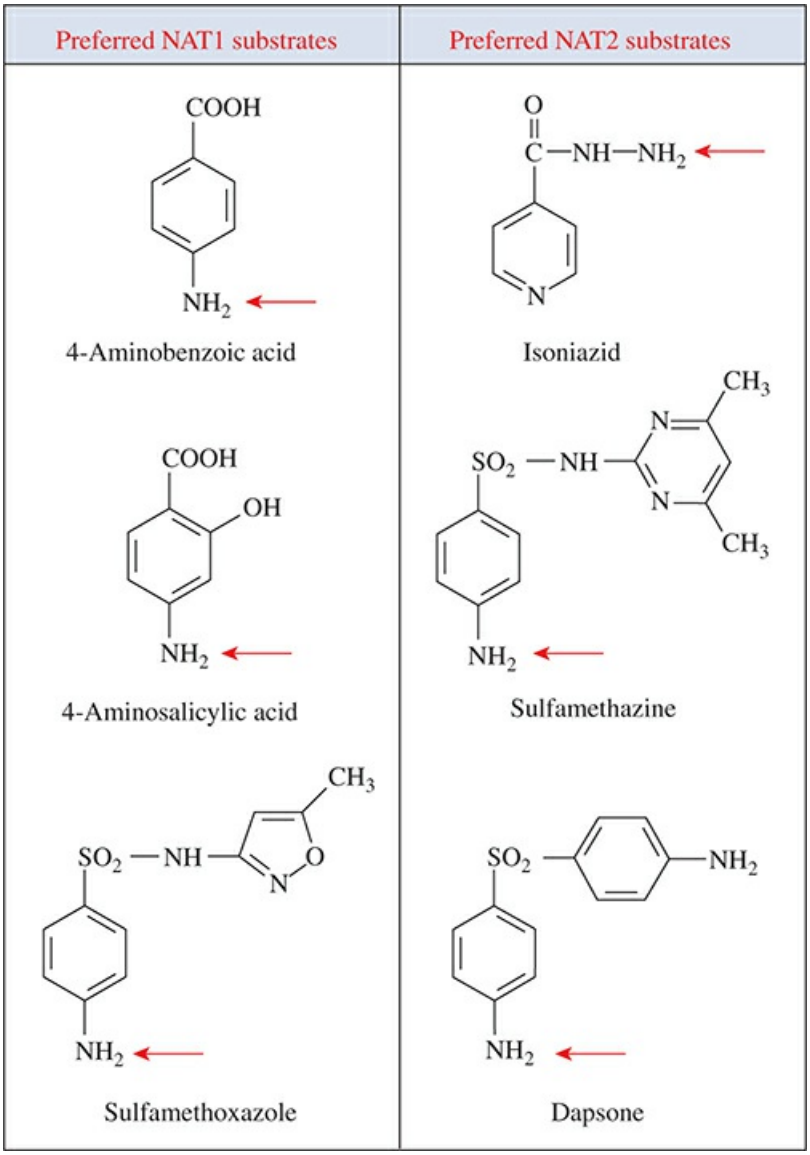


FIGURE 6–19 *Examples of substrates for the human N-acetyltransferases, NAT1, and the highly polymorphic NAT2.*

NATs are cytosolic enzymes found in the liver and many other tissues of most mammalian species, with the notable exception of the dog, fox, and musk shrew (*Suncus marinus*), which are unable to acetylate xenobiotics. The two xenobiotic-acetylating human enzymes are NAT1 and NAT2, which are encoded by two highly polymorphic genes located on chromosome 8. In each species examined, NAT1 and NAT2 are closely related proteins (79% to 95% identical in amino acid sequence) with an active-site cysteine residue (Cys_{68}) in the N-terminal region.

NAT1 and NAT2 have different but overlapping substrate specificities, although no substrate is exclusively *N*-acetylated by one enzyme or the other. Several drugs are *N*-acetylated following their biotransformation by hydrolysis, reduction, or oxidation.

NAT2 slow acetylator phenotype is caused by various single nucleotide polymorphisms in the NAT2 gene that decrease NAT2 activity, decrease enzyme stability, or target the enzyme for proteasomal degradation. At least 88 allelic variants of human NAT2 have been documented.

This is partly because different mutations in the NAT2 gene have different effects on NAT2 activity and/or enzyme stability, heterozygotes retain moderate NAT2 activity, and the *N*-acetylation of "NAT2-substrates" by NAT1 becomes significant in slow NAT2 acetylators.

Genetic polymorphisms in NAT2 have a number of pharmacological and toxicological consequences for drugs that are *N*-acetylated by this enzyme. The pharmacological effects of the antihypertensive drug hydralazine are more pronounced in slow NAT2 acetylators. Slow NAT2 acetylators are predisposed to several drug toxicities, including nerve damage (peripheral neuropathy) from isoniazid and dapsone, systemic lupus erythematosus from hydralazine and procainamide, and the toxic effects of coadministration of the anticonvulsant phenytoin with isoniazid.

Amino Acid Conjugation

Two principal pathways by which xenobiotics are conjugated with amino acids are illustrated in Fig. 6–20. The first involves conjugation of xenobiotics containing a carboxylic acid group with the *amino group* of amino acids such as glycine, glutamine, and taurine (see Fig. 6–15). After activation of the xenobiotic by conjugation with coenzyme A, the xenobiotic-CoA thioester reacts with the *amino group* of an amino acid to form an amide linkage. The second pathway involves conjugation of xenobiotics containing an aromatic hydroxylamine (*N*-hydroxy aromatic amine) with the *carboxylic acid group* of such amino acids as serine and proline. This pathway involves activation of an amino acid by aminoacyl-tRNA synthetase, which reacts with an aromatic hydroxylamine to form a reactive *N*-ester.

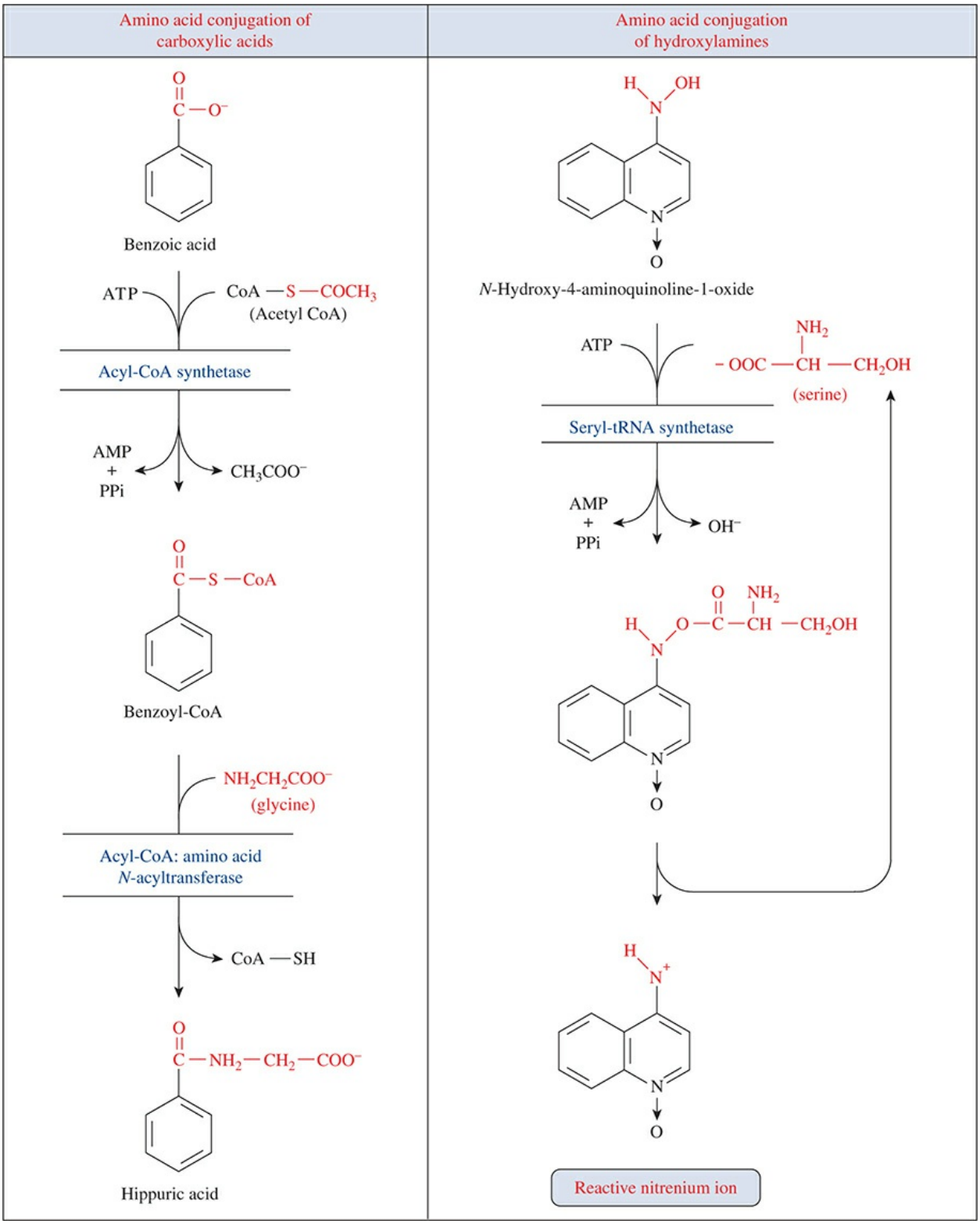


FIGURE 6–20 *Conjugation of xenobiotics with amino acids.*

Substrates for amino acid conjugation are restricted to certain aliphatic, aromatic, heteroaromatic, cinnamic, and arylacetic acids. The ability of xenobiotics to undergo amino acid

conjugation depends on steric hindrance around the carboxylic acid group, and by substituents on the aromatic ring or aliphatic side chain. Amino acid conjugation is apparently catalyzed by separate *N*-acyltransferases specific for each amino acid. The acceptor amino acid used for conjugation is both species- and xenobiotic-dependent.

Amino acid conjugates of bile acids are secreted into bile, whereas amino acid conjugates of xenobiotics are eliminated primarily in urine. The addition of an endogenous amino acid to xenobiotics may facilitate this elimination by increasing their ability to interact with the tubular organic anion transport system in the kidney.

Glutathione Conjugation

Conjugation of xenobiotics with the tripeptide glutathione (GSH) in a reaction also known as glutathionylation occurs with electrophilic xenobiotics or their electrophilic metabolites. GSH is composed of glycine, cysteine, and glutamic acid; the latter being linked to cysteine via the γ-carboxyl group, not the usual α-carboxyl group (Fig. 6–15). Although the abbreviation "GST" has survived to describe the enzyme family, the term "glutathione *S*-transferase" is technically incorrect because the glutathionyl group (GS-) is transferred rather than a single sulfur atom; hence, the correct term is glutathione transferase. GSTs function endogenously as part of a defense mechanism against reactive oxygen species; GSTs reduce the formation of hydroperoxides of fatty acids, phospholipids, and cholesterol and protect against the redox cycling of many quinone-containing compounds by conjugating them with GSH.

Substrates for glutathionylation include an enormous array of electrophilic xenobiotics, or xenobiotics that can be biotransformed to electrophiles.

The synthesis of GSH involves formation of the peptide bond between cysteine and glutamic acid, followed by peptide bond formation with glycine. The first and overall rate-limiting reaction is catalyzed by γ-glutamylcysteine synthetase (γ-GCL), the second by GSH synthetase. At each step, ATP is hydrolyzed to ADP and inorganic phosphate. Due to the sheer multiplicity of GSTs, they cannot be collectively inhibited or induced; therefore, the importance of glutathionylation in xenobiotic toxicity in vivo is often assessed by altering levels of GSH. This can be achieved by (1) inhibiting GSH synthesis, (2) depleting GSH, or (3) increasing GSH levels through Nrf2 activation.

The conjugation of xenobiotics with GSH is catalyzed by GSTs in cytosol, microsomes, and mitochondria. The only mitochondrial GST found to date has been referred to as Kappa GST, and it has a structure that is distinct from the cytosolic GSTs. The GSTs are present in most tissues, with high concentrations in the liver, intestine, kidney, testis, adrenal, and lung.

Substrates for GST share three common features: they are hydrophobic, they contain an electrophilic atom, and they react nonenzymatically with GSH at some measurable rate. The mechanism by which GST increases the rate of GSH conjugation involves deprotonation of GSH to GS^- by an active-site tyrosine or serine. The concentration of GSH in the liver is extremely high (5 to 10 mM) relative to plasma (0.5 to 10 μM); hence, the nonenzymatic conjugation of certain xenobiotics with GSH can be significant. However, some xenobiotics are conjugated with GSH stereoselectively, indicating that the reaction is largely catalyzed by GST. Like GSH, the GSTs are themselves abundant cellular components, accounting for up to 10% of the total cellular protein. These enzymes bind, store, and/or transport a number of compounds that are not substrates for GSH conjugation.

As shown in Fig. 6–21, substrates for GSH conjugation can be divided into two groups: those that are sufficiently electrophilic to be conjugated directly and those that must first be biotransformed to an electrophilic metabolite prior to conjugation. The second group of substrates for GSH conjugation includes reactive intermediates (often formed by CYP) such as oxiranes (arene oxides and alkene epoxides), nitrenium ions, carbonium ions, and free radicals. The conjugation reactions themselves can be divided into two types: *displacement reactions*, in which GSH displaces an electron-withdrawing group, and *addition reactions*, in which GSH is added to an activated double bond or strained ring system.

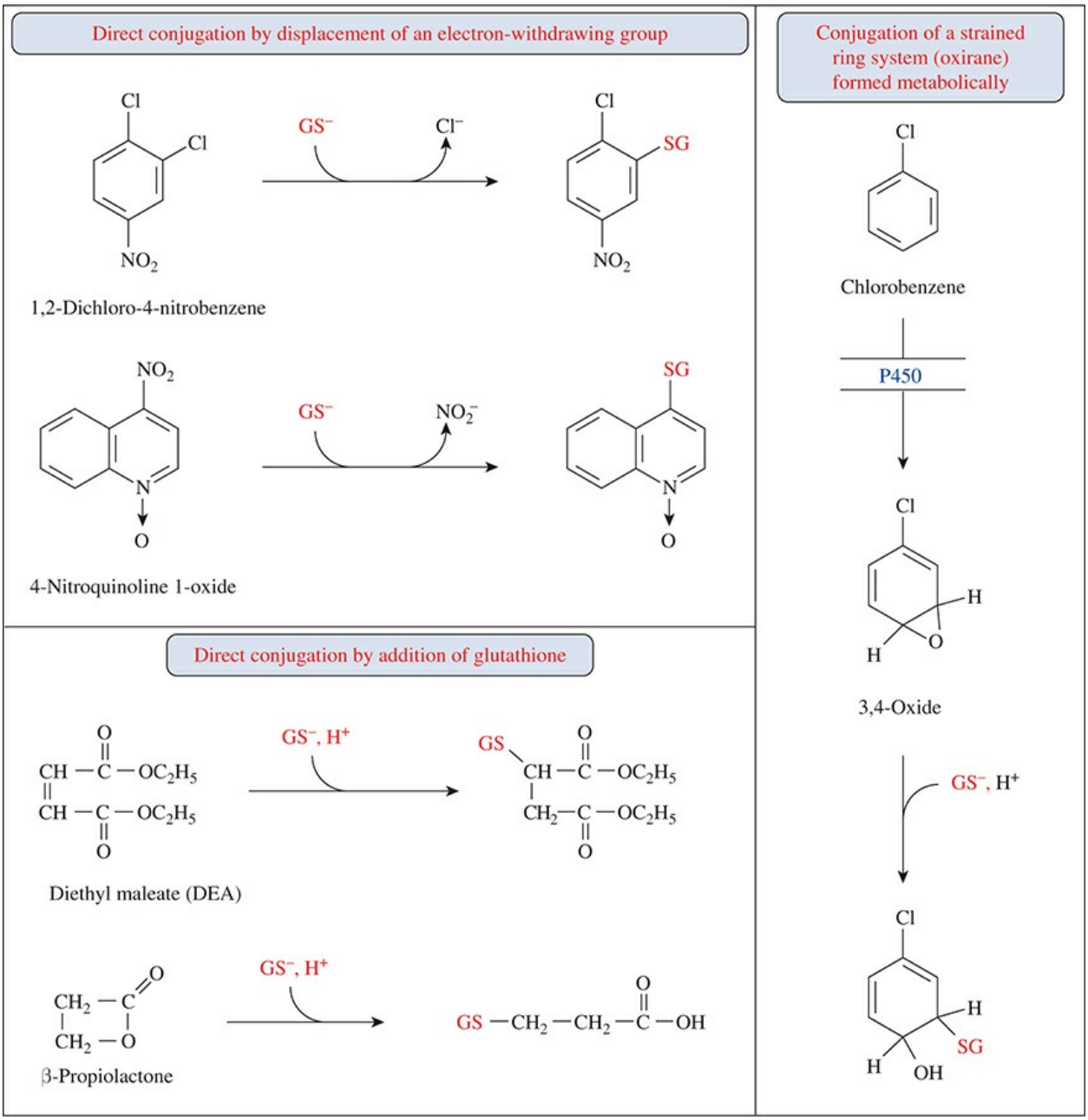


FIGURE 6–21 *Examples of glutathione conjugation of xenobiotics with an electrophilic carbon. GS− represents the anionic form of glutathione.*

The displacement of an electron-withdrawing group by GSH typically occurs when the substrate contains halide, sulfate, sulfonate, phosphate, or a nitro group (i.e., good *leaving groups*) attached to an allylic or benzylic carbon atom. Displacement of an electron-withdrawing group from aromatic xenobiotics is decreased by the presence of other substituents that donate electrons to the aromatic ring ($–NH_2$, –OH, –OR, and –R). Conversely, such displacement reactions are increased by the presence of other electron-withdrawing groups (–F, –Cl, –Br, –I, $–NO_2$, –CN, –CHO, and –COOR).

The addition of GSH to a carbon–carbon double bond is also facilitated by the presence of a nearby electron-withdrawing group; hence, substrates for this reaction typically contain a double bond attached to –CN, –CHO, –COOR, or –COR.

GSH can also conjugate xenobiotics with an electrophilic heteroatom (*O*, *N*, and *S*). In each of the examples shown in Fig. 6–22, the initial conjugate formed between GSH and the heteroatom is cleaved by a second molecule of GSH to form oxidized GSH, which is also known as glutathione disulfide (GSSG). The initial reactions are catalyzed by GST, whereas the second reaction (which leads to GSSG formation) generally occurs nonenzymatically.

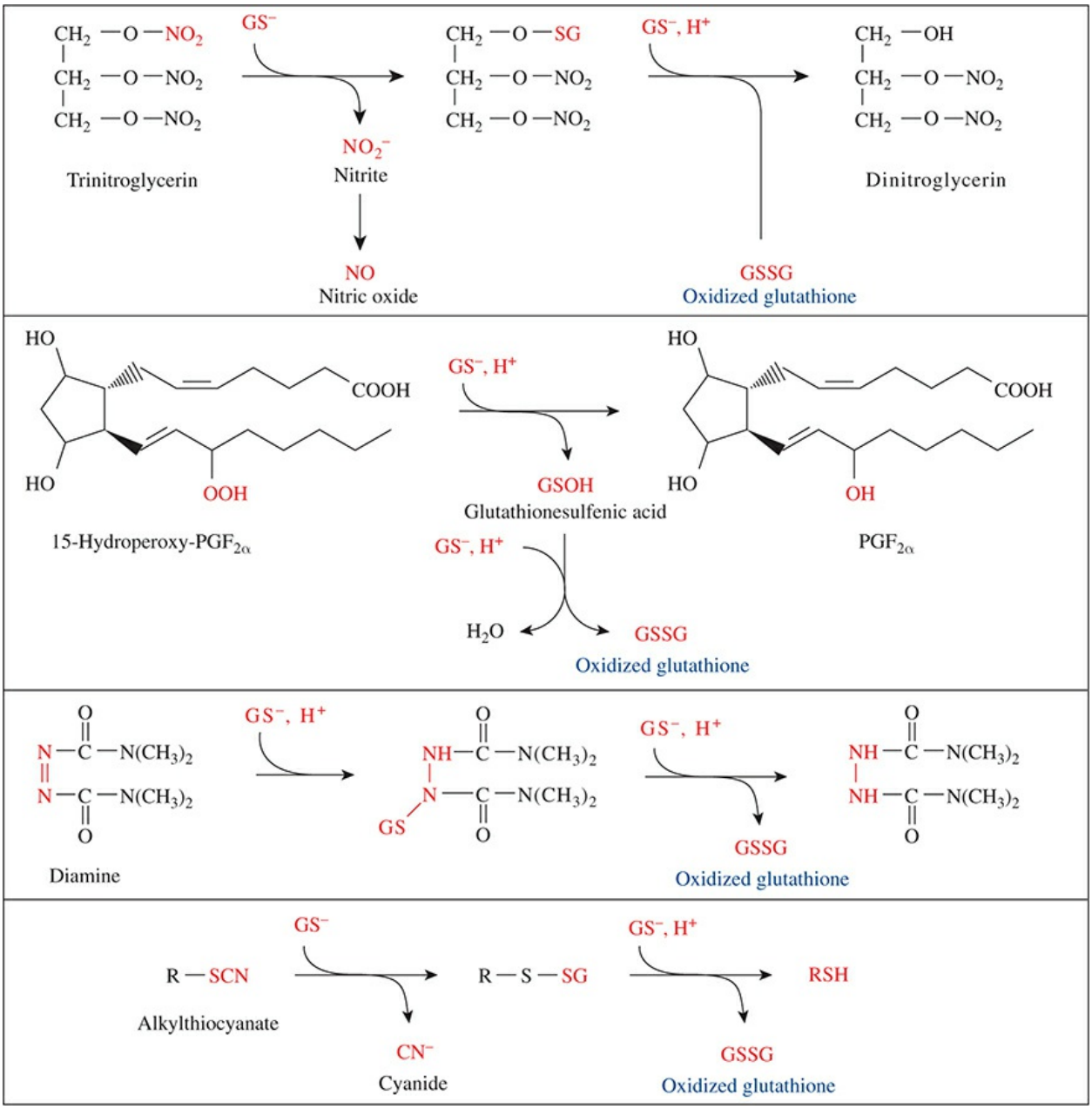


FIGURE 6–22 *Examples of glutathione conjugation of electrophilic heteroatoms.*

GSH conjugates formed in the liver can be effluxed into bile by MRP2 on the canalicular membrane, or they can be transported into blood by various transporters on the sinusoidal membrane such as MRP1, MRP3, MRP4, MRP5, and MRP6.

GSH conjugates can be converted to mercapturic acids (*N*-acetylcysteine conjugates) in the kidney and excreted in urine. As shown in Fig. 6–23, the conversion of GSH conjugates to mercapturic acids involves the sequential cleavage of glutamic acid and glycine from the GSH moiety, followed by *N*-acetylation of the resulting cysteine conjugate. The first two steps in mercapturic acid synthesis are catalyzed outside the cell by two membrane-bound enzymes on the luminal surface of the proximal tubule of the kidney (and the bile ducts of the liver), namely, γ-glutamyltransferase (GGT; aka γ-glutamyltranspeptidase), which cleaves the glutamic acid

residue, and alanyl aminopeptidase (ANPEP), which cleaves the glycine residue. After hydrolysis, the xenobiotic, now conjugated only to cysteine, is transported into the proximal tubule, where it is *N*-acetylated to a mercapturic acid by cytosolic NAT or deconjugated by mitochondrial β-lyase, as shown in Fig. 6–23. Mercapturic acids are transported back across the luminal surface of the proximal tubules for elimination in urine.

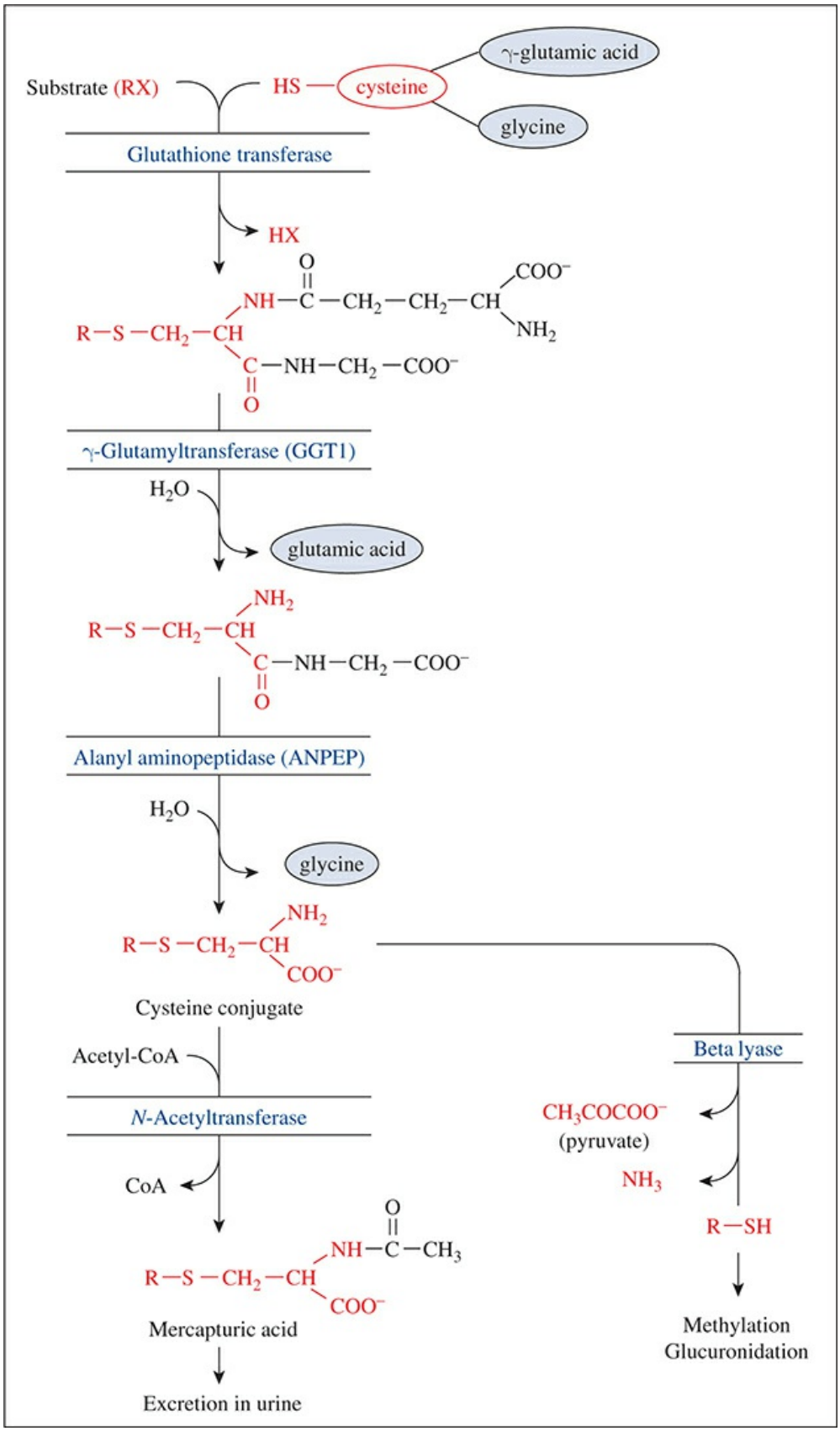


FIGURE 6–23 *Glutathione conjugation and mercapturic acid biosynthesis.*

Cytosolic GSTs are dimers, typically composed of identical subunits (M_r 23–29 kDa), although some forms are heterodimers. Each subunit contains 199 to 244 amino acids and one catalytic site. Numerous GST subunits have been cloned and sequenced, and differ in substrate specificity, tissue location, and cellular location. Glutathione is also a cofactor for glutathione peroxidase, which is important in protecting cells against lipid and hemoglobin peroxidation.

Conjugation with GSH represents an important detoxication reaction because electrophiles are potentially toxic species that can bind to critical nucleophiles, such as proteins and nucleic acids, and cause cellular damage and genetic mutations. All the enzymes involved in xenobiotic biotransformation have the potential to generate reactive intermediates, most of which are detoxified to some extent by conjugation with GSH. GSH is also a cofactor for GPXs, which play an important role in protecting cells against lipid and hemoglobin peroxidation. Resistance to toxic compounds is often associated with an overexpression of GST.

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QUESTIONS

1. Xenobiotic biotransformation is performed by multiple enzymes in multiple subcellular locations. Where would one of these enzymes most likely NOT be located?
   - **a.** cytosol.
   - **b.** Golgi apparatus.
   - **c.** lysosome.
   - **d.** mitochondria.
   - **e.** microsome.

2. All of the following statements regarding hydrolysis, reduction, and oxidation biotransformations are true EXCEPT:
   - **a.** The xenobiotic can be hydrolyzed.
   - **b.** The xenobiotic can be reduced.
   - **c.** There is a large increase in hydrophilicity.
   - **d.** The reactions introduce a functional group to the molecule.
   - **e.** The xenobiotic can be oxidized.

3. Which of the following is often conjugated to xenobiotics during phase II

biotransformations?

a. alcohol group.
b. sulfhydryl group.
c. sulfate group.
d. aldehyde group.
e. carbonyl group.

4. Which of the following is a true statement about the bio-transformation of ethanol?

a. Alcohol dehydrogenase is only present in the liver.
b. Ethanol is reduced to acetaldehyde by alcohol dehydrogenase.
c. Ethanol and hydrogen peroxide combine to form acetaldehyde with the aid of catalase.
d. In spite of its catalytic versatility, cytochrome P450 does not aid in ethanol oxidation.
e. Acetaldehyde is oxidized to acetic acid in the mitochondria by aldehyde dehydrogenase.

5. Which of the following enzymes is responsible for the bio-transformation and elimination of serotonin?

a. cytochrome P450.
b. monoamine oxidase.
c. flavin monooxygenase.
d. xanthine oxidase.
e. paraoxonase.

6. Which of the following reactions would likely NOT be catalyzed by cytochrome P450?

a. dehydrogenation.
b. oxidative group transfer.
c. epoxidation.
d. reductive dehalogenation.
e. ester cleavage.

7. All of the following statements regarding cytochrome P450 are true EXCEPT:

a. Poor metabolism or biotransformation of xenobiotics is often due to a genetic deficiency in cytochrome P450.
b. Cytochrome P450 can be inhibited by both competitive and noncompetitive inhibitors.
c. Certain cytochrome P450 enzymes can be induced by one's diet.
d. Increased activity of cytochrome P450 always slows the rate of xenobiotic activation.
e. Induction of cytochrome P450 can lead to increased drug tolerance.

8. Which of the following statements regarding phase II bio-transformation (conjugation) reactions is true?

a. Phase II reactions greatly increase the hydrophilicity of the xenobiotic.
b. Phase II reactions are usually the rate-determining step in the biotransformation and excretion of xenobiotics.
c. Carboxyl groups are very common additions of phase II reactions.
d. Most phase II reactions occur spontaneously.

e. Increased phase II reactions result in increased xenobiotic storage in adipocytes.

9. Where do most phase II biotransformations take place?
   a. mitochondria.
   b. ER.
   c. blood.
   d. nucleus.
   e. cytoplasm.

10. Which of the following is not an important cosubstrate for phase II biotransformation reactions?
   a. UDP-glucuronic acid.
   b. 3′-phosphoadenosine-5′-phosphosulfate (PAPS).
   c. S-adenosylmethionine (SAM).
   d. N-nitrosodiethylamine.
   e. acetyl CoA.

CHAPTER 7

Toxicokinetics

Kannan Krishnan

KEY POINTS

- *Toxicokinetics* is the study of the modeling and mathematical description of the time course of disposition (absorption, distribution, biotransformation, and excretion) of xenobiotics in the whole organism.
- The apparent volume of distribution (V_d) is the space into which an amount of chemical is distributed in the body to result in a given plasma concentration.
- Clearance describes the rate of chemical elimination from the body in terms of volume of fluid containing chemical that is cleared per unit of time.
- The half-life of elimination ($T_{1/2}$) is the time required for the blood or plasma chemical concentration to decrease by one half.

INTRODUCTION

Toxicokinetics refers to the quantitative study of absorption, distribution, metabolism, and excretion (ADME) of chemicals in biota through measurement and modeling of their concentrations or amounts in biological matrices (e.g., blood, plasma, excreta, exhaled air, tissues) as a function of time. Experimental studies of toxicokinetics facilitate characterization of the temporal profile of the concentration of chemicals and their metabolites in the target tissue or other biological matrices, reflecting the net effect of the rate and extent of absorption through one or more exposure routes, distribution to tissues and organs via systemic circulation, as well as disposition by metabolism and excretion. Toxicokinetic analyses constitute an essential part of systematic approaches to safety/risk evaluation of xenobiotics and other substances including

therapeutic drugs. The models in toxicokinetics range from a simple, one-compartment model to complex multicompartment models, with some explicitly incorporating mechanistic determinants of chemical uptake, distribution, and disposition. This chapter introduces toxicokinetic concepts, fundamentals of classic and physiologic toxicokinetic models, as well as their applications in toxicology, risk assessment, and biomonitoring.

TOXICOKINETIC DATA

Figure 7–1A presents the blood kinetic data collected in a group of animals as part of the toxicological evaluation of a xenobiotic following a single oral dose of 100 mg/kg body weight. The toxicokinetic curve indicates an initial phase of increasing concentration determined by the rate of absorption, then a peak or plateau during which absorption rate equals elimination rate, followed by a phase dominated essentially by elimination. In this hypothetical example, the mean blood concentration of the parent chemical in the treatment group corresponded to 0, 1.32, 2.74, 3.37, 3.55, 2.89, 1.67, and 0.37 mg/L at pre-exposure, or at 2, 4, 6, 8, 10, 12, and 24 hours post-exposure, respectively. The $C_{max,blood}$ is 3.55 mg/L for this treatment group. A common method of calculating the area under the curve (AUC_{blood}) involves summing the areas of trapezoids constituting the time-course curve. The trapezoidal method involves (i) averaging the blood concentrations found at two consecutive sampling times and (ii) multiplying it with the time interval between the two sampling periods (Figs. 7–1B and C). The lower panel illustrates the calculation of AUC for one trapezoid (4.56 (mg/L)·h), using the mean blood concentrations of 2.89 and 1.67 mg/L obtained at 10 and 12 hours post-dosing. For this particular dataset, $AUC_{0–24h}$ (= 41.65 (mg/L)·h) corresponds to the sum of areas of the seven trapezoids, that is, 1.32, 4.06, 6.11, 6.92, 6.44, 4.56, and 12.24 (mg/L)·h. For determining the AUC until complete elimination of the substance (i.e., 24 hours to infinity), the area of the last trapezoid is additionally computed by dividing the last measured blood concentration by the rate of elimination (AUC_{last}/K_{el} where K_{el} = first-order elimination rate constant, h^{-1}). The trapezoidal rule can also be used to calculate the area under the first moment curve (i.e., [concentration × time] vs. [time]). In turn, the ratio of the area under the first moment curve (AUMC) to the AUC is indicative of the mean residence time (MRT) spent by drug or chemical molecules in the body.

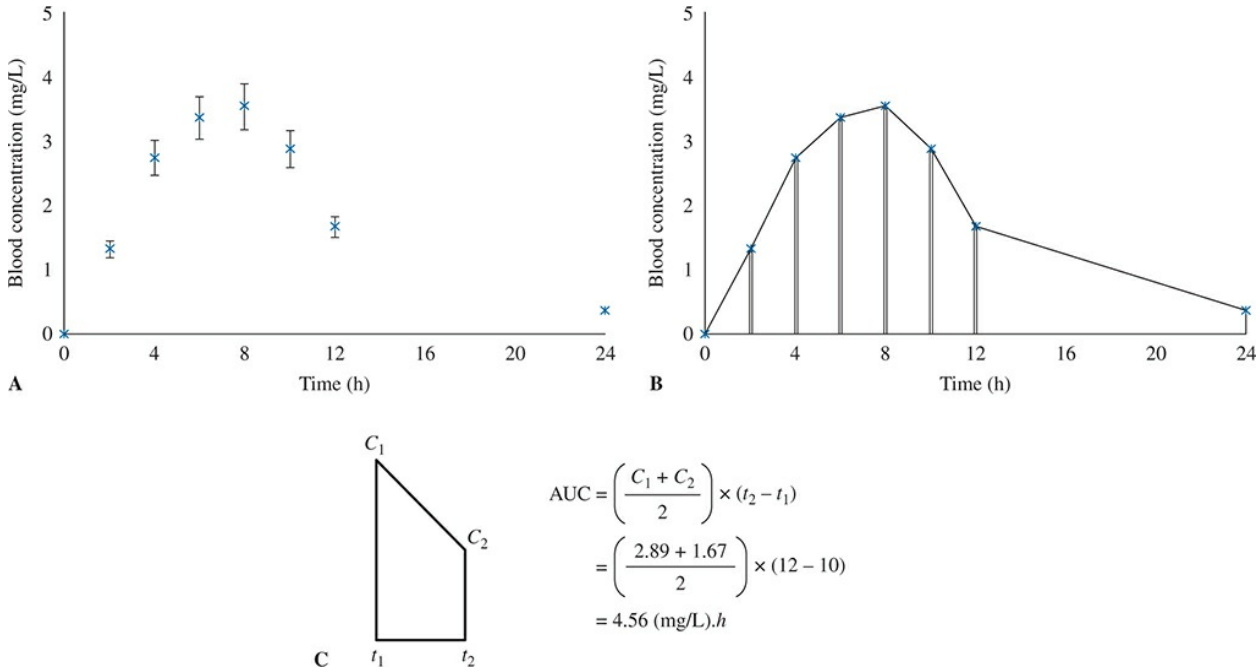

FIGURE 7–1 *Illustrations of* (***A***) *the measured blood concentration of a xenobiotic following a single oral dose,* (***B***) *the construction of trapezoids to calculate the area under the curve (AUC), and* (***C***) *the computation of the area under a single trapezoid corresponding to the blood concentrations (2.89 and 1.67 mg/L) at 10 and 12 hours post-dosing.*

The timing of sample collection and the time intervals between samples in toxicokinetic studies should be appropriate to enable reliable calculation of the dose metrics of interest. The time-course data collected should (i) capture the critical kinetic phases in the exposed animals, (ii) allow the estimation of fundamental toxicokinetic parameters, and (iii) permit, to the extent possible, the characterization of C_{max} and AUC. Chemical-specific toxicokinetic properties, limit of detection of the analytical method, and statistical considerations can facilitate the determination of the number of samples, sampling time intervals, and volume of biological samples to be collected. The number and volume of samples drawn should be such that they do not compromise the normal physiological status of the animal (e.g., not drawing more than 10% of the blood volume during the AUC sampling interval).

The accuracy of calculated dose metrics depends upon the number of data points collected in a toxicokinetic study, whereas their magnitude depends upon the dose administered, exposure route, single or multiple doses, as well as the rates and extent of ADME influenced by factors such as age and pathophysiological state of the test subject or animal. The choice of analysis or modeling method depends upon the underlying question(s) of the toxicological evaluation, the richness of the available data sets (toxicology, mode of action, and toxicokinetics), as well as the precision and predictive ability required for the end use.

TOXICOKINETIC CONCEPTS

Absorption and Bioavailability

The rate and extent of absorption of a chemical into the systemic circulation depends upon the dose, exposure route, as well as the volume and type of exposure matrix or dosing vehicle. The rate and efficiency with which a given chemical crosses the biological membrane barrier determine how quickly and how much of it appears in the systemic circulation. Whereas absorption might be incomplete when exposure occurs via extravascular routes, intravenous dosing assures 100% delivery of the dose into the systemic circulation and is used effectively as a benchmark of absorption by non-IV routes. The extent of absorption of a toxicant can be experimentally determined by comparing the time course of plasma toxicant concentration after IV and extravascular dosing. The AUC associated with the extravascular dose in reference to the AUC for the intravenous route is called *bioavailability* (*F*). In single-dose toxicokinetic studies, *F* is determined by using different IV and non-IV doses according to the following equation, provided the toxicant does not display dose-dependent or saturable kinetics:

$$F = \frac{(\mathrm{AUC}_{\text{non-IV}} / \mathrm{Dose}_{\text{non-IV}})}{(\mathrm{AUC}_{\text{IV}} / \mathrm{Dose}_{\text{IV}})}, \qquad (7\text{-}1)$$

where $\mathrm{AUC}_{\text{non-IV}}$, AUC_{IV}, $\mathrm{Dose}_{\text{non-IV}}$, and $\mathrm{Dose}_{\text{IV}}$ are the respective areas under the blood concentration versus time curves and doses for non-IV and IV administrations. A value of $F < 1$ indicates that less than 100% of the administered dose is able to reach the systemic circulation. Factors that alter this systemic availability include (1) limited absorption after oral dosing, (2) intestinal first-pass effect, (3) hepatic first-pass effect, and (4) mode of formulation, which affects dissolution rate or incorporation into micelles (for lipid-soluble compounds).

Volume of Distribution

The volume of distribution, V_d, corresponds to the apparent volume of a biological fluid in which a xenobiotic is diluted, and is expressed in units of volume (mL or L of blood, plasma, or plasma water). V_d would be approximately equal to the volume of blood if the chemical resided solely in blood and distributed uniformly among the blood components (plasma, erythrocytes). Hydrophilic substances exhibit smaller volumes of distribution, often close to 0.6 L/kg (or 60% of body weight) in adults, approximating the total body water in plasma (4%), interstitial fluid (21%), and intracellular fluid (35%). Those substances that are highly bound to plasma proteins and not showing any permeation in the blood capillaries or tissue membranes would result in a V_d that is reflective of the extracellular fluid. Xenobiotics exhibiting a marked affinity for tissue or fat depots have large V_d (exceeding physiological fluid spaces and body weight) leading to low plasma concentrations. The mechanisms of tissue sequestration not only include partitioning of a toxicant into tissue lipids but also involve binding to tissue pro-teins, concentrative uptake by active transporters, and trapping in specialized organelles.

Clearance

Clearance (CL) is the apparent volume of a physiological fluid (e.g., blood, plasma, plasma water) that is cleaned of the toxicant per unit time (i.e., mL/min or L/h). Thus, a CL value of 1 L blood/h is interpreted as 1 L of blood being completely cleared of a toxicant per hour.

Accordingly, high values of clearance indicate efficient and generally rapid removal of chemicals from the systemic circulation, whereas low clearance values indicate slow and less efficient removal. CL_{total} of a xenobiotic equals the sum of all individual clearance processes occurring in parallel contributing to its removal from systemic circulation:

$$CL_{total} = CL_{hepatic} + CL_{renal} + CL_{other}. \qquad (7\text{-}2)$$

The integral measure of the blood concentration $\left(AUC_0^\infty\right)$ in relation to the systemic dose (i.e., $Dose_{IV}$, or Dose via extravascular routes adjusted for bioavailability) is useful in computing CL_{total} as follows:

$$CL_{total} = \frac{Dose_{IV}}{AUC_0^\infty} = \frac{F \bullet Dose_{oral}}{AUC_0^\infty}. \qquad (7\text{-}3)$$

If non-hepatic clearances are negligible for a chemical, then Eq. (7-2) becomes $CL_{total} = CL_{hepatic}$. Further, as per Eq. (7-3), the inverse relationship between CL and AUC would suggest that for a given dose, the chemical with lower $CL_{hepatic}$ will exhibit a higher AUC and hence greater systemic exposure to the parent chemical.

Elimination Half-life

Elimination rate constant (K_{el}) represents the fraction of the amount of chemical in body that is removed from systemic circulation per unit time. This is analogous to the fraction of the volume of distribution that is subject to elimination by various clearance mechanisms (i.e., CL/V_d). For example, if the CL = 10 L/h and V_d = 100 L, then the K_{el} equals 0.1 h^{-1} implying that 10% of the V_d will be cleared of the chemical per hour. However, the actual amount eliminated in 1 hour would be slightly less given that the amount in the body is declining continuously (from time zero to 1 hour). Thus, in the case of first-order elimination kinetics, the fractional elimination is constant but the amount eliminated is proportional to the amount remaining. Because the percent eliminated over a given time period remains constant regardless of the dose, it is more intuitive and convenient to refer to an elimination half-life ($t_{1/2}$), that is, the time taken for the concentration of a chemical in a biological matrix such as blood or body to be reduced by half. Thus, after one elimination half-life, the initial blood concentration [C_0] would decline by 50%, that is, [C_0] × 0.5. After the second half-life, the remaining concentration would diminish by 50% due to elimination processes, that is, (0.5 × 0.5 × [C_0]) = 0.25 × [C_0], for a total decline of C_0 by a factor of 0.75 or 75%. After three, four, five, six, and seven half-lives, the initial concentration C_0 would diminish by 87.5%, 93.75%, 96.9%, 98.5%, and 99.2%, respectively. Elimination half-life, $t_{1/2,}$ thus reflects the rapidity of disappearance of a toxicant following discontinuation of exposure and it depends upon both V_d and CL as shown below:

$$t_{1/2} = \frac{0.693 \cdot V_d}{CL}. \qquad (7\text{-}4)$$

The above relationship among $t_{1/2}$, V_d, and CL emphasizes that care should be exercised in interpretation of data when relying upon $t_{1/2}$ as the sole representation of elimination of a chemical in toxicokinetic studies, since $t_{1/2}$ is influenced by both the V_d for the toxicant and the rate at which it is cleared from the blood. Thus, $t_{1/2}$ is not an independent toxicokinetic parameter.

CLASSIC TOXICOKINETIC MODELS

Model Structure

Classic toxicokinetics involves empirical analysis of the time-course data of a chemical and/or its metabolite(s) to derive parameters characteristic of their behavior in the exposed organism. This type of analysis uses one or more compartments to capture the toxicokinetics of a chemical in the organism (Fig. 7–2). The simplest structure of the classic toxicokinetic model represents the body as a homogeneous one-compartment system. When the log [C] versus time plot of the toxicokinetic data yields a curve as shown in Fig. 7–3 (top panel vs. bottom panel), it would indicate that differential distribution and equilibration processes exist among the model compartments (Figs. 7–3). Even though the number and nature of the compartments may not have exact anatomical or physiological correspondence, the resulting model structure and mathematical representation for a chemical would consistently fit with the available empirical data on the kinetics in one or more biological matrices such as whole blood, plasma, urine, exhaled breath, or tissues.

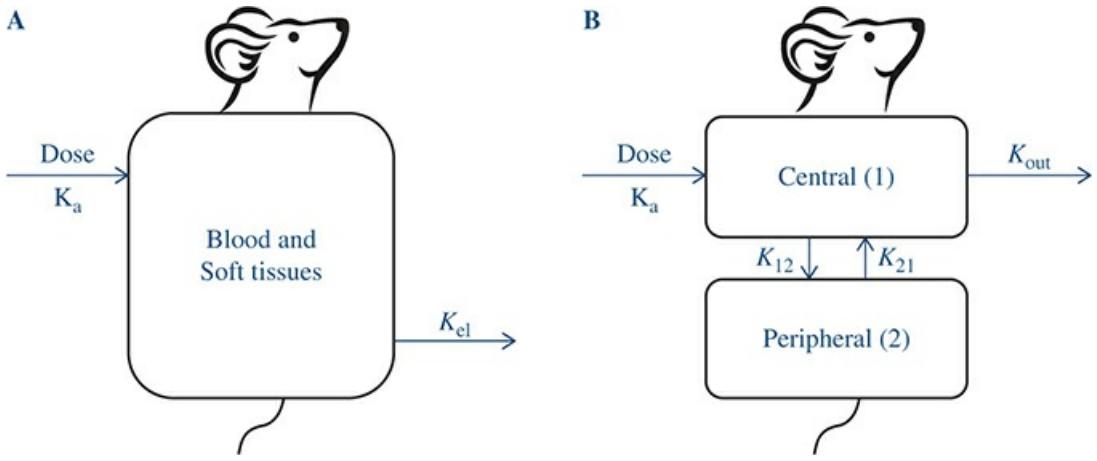

FIGURE 7–2 *Conceptual representations of compartmental toxicokinetic models.* Symbols for one-compartment model (**A**): $\boldsymbol{K_a}$ is the first-order absorption rate constant, and $\boldsymbol{K_{el}}$ is the first-order elimination rate constant. Symbols for two-compartment model (**B**): $\boldsymbol{K_a}$ is the first-order absorption rate constant into the central compartment (1), from the central compartment (1), $\boldsymbol{K_{12}}$ and $\boldsymbol{K_{21}}$ are the first-order rate constants for distribution between central (1) and

peripheral (2) compartments, whereas $\boldsymbol{K_{out}}$ is the first-order elimination rate constant from the central compartment.

Mathematical Representation

In the one-compartment toxicokinetic model reflecting homogeneous distribution of a chemical in the body, the rate of change in the amount ($d\text{Amount}_{body}/dt$, mg/h) equals the difference between the amount absorbed ($\text{Dose}_{absorbed}$) per unit time and amount eliminated from the body ($\text{Amount}_{eliminated}$) per unit time:

$$\frac{d\text{Amount}_{body}}{dt} = \frac{\text{Dose}_{absorbed}}{\text{h}} - \frac{\text{Amount}_{eliminated}}{\text{h}}. \qquad (7\text{-}5)$$

Notationally,

$$\frac{dA_{body}}{dt} = K_a D - K_{el} A_{body}, \qquad (7\text{-}6)$$

where A_{body} = amount in the body (mg), D = dose administered (mg), K_a = first-order absorption rate constant (h^{-1}), and K_{el} = first-order elimination rate constant (h^{-1}, equal to CL/V_d).

For the simple scenario of a single IV dose, because there is no absorption component, Eq. (7-6) reduces to the following form for describing change in chemical concentration in blood (C_b) due to first-order elimination:

$$\frac{dC_b}{dt} = -K_{el} C_b. \qquad (7\text{-}7)$$

Upon integration based on the initial condition or time zero blood concentration ($C_{b,0}$), the equation to calculate chemical concentration in blood at any given time t ($C_{b,t}$) becomes:

$$\ln C_{b,t} - \ln C_{b,o} = -K_{el} \cdot t. \qquad (7\text{-}8)$$

When more than one characteristic tissue(s) or tissue group(s) having different equilibration and elimination profiles, a biexponential equation of the following form may mathematically represent the kinetic behavior contained in the data:

$$C_t = A \cdot e^{-\alpha \cdot t} + B \cdot e^{-\beta \cdot t}, \qquad (7\text{-}9)$$

where A and B are coefficients in units of toxicant concentration, α is the exponential constant for the distribution phase, and β is the exponential constant for the elimination phase.

In Eq. (7-9), the α constant corresponds to the slope of the residual log-linear plot and not the

initial slope of the decline in the observed plasma toxicant concentration, that is, the initial rate of decline in plasma concentration approximates, but is not exactly equal to, the α rate constant (Fig. 7–3, bottom panel). It should be noted that distribution into and out of tissues and elimination of the toxicant from the systemic circulation occur at all times, that is, elimination does occur during the "distribution" phase, and distribution between compartments is still ongoing during the "elimination" phase.

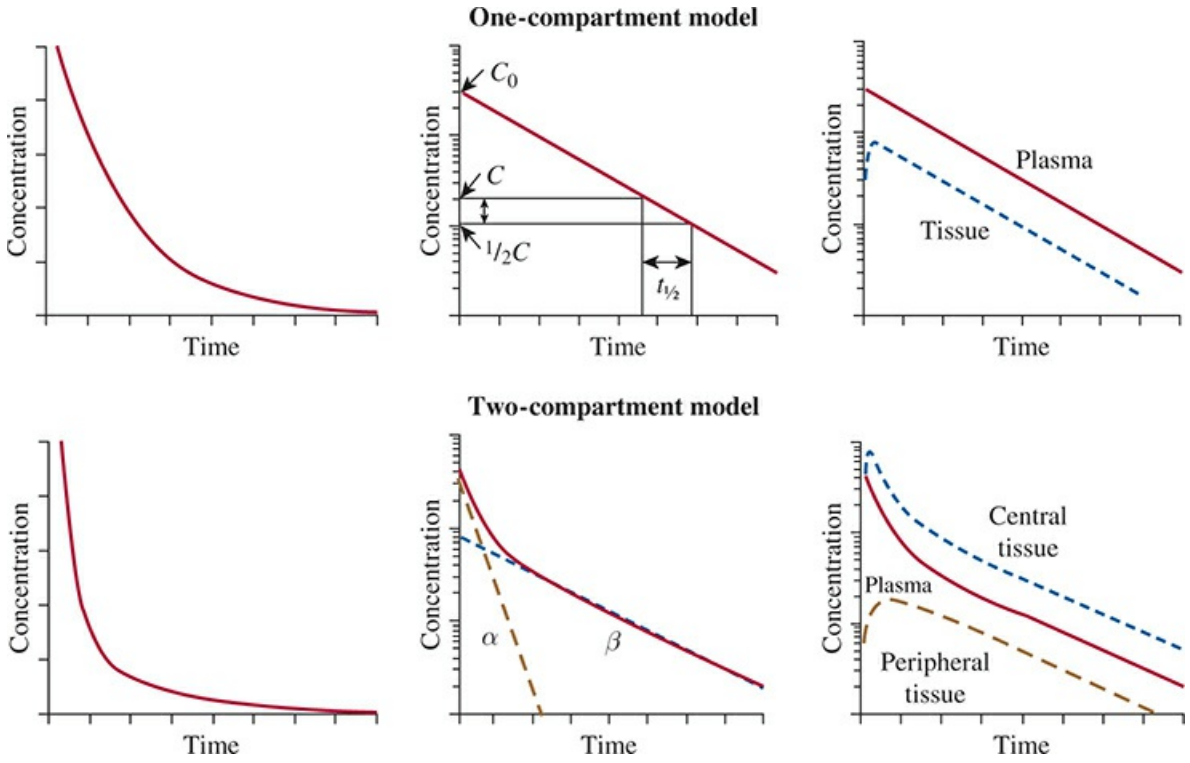

FIGURE 7–3 *Plasma concentration versus time curves of toxicants exhibiting kinetic behavior conforming to a one-compartment model (top row) and a two-compartment model (bottom row) following IV bolus injection.* Left and middle panels show the plots on a rectilinear and semilogarithmic scale, respectively. Right panels illustrate the relationship between tissue (dash lines) and plasma (solid line) concentrations over time. Note that tissue concentration can be higher, nearly the same, or lower than plasma concentration. Tissue concentration peaks almost immediately, and thereafter declines in parallel with plasma concentration. The right panel for the two-compartment model shows concentration–time profiles for typical tissues associated with the central (1) and peripheral (2) compartments as represented by short and long dash lines, respectively. For tissues associated with the central compartment, their concentrations decline in parallel with plasma. For tissues associated with peripheral compartment, toxicant concentration rises, while plasma concentration declines rapidly during the initial phase; it then reaches a peak and eventually declines in parallel with plasma in the terminal phase. Elimination rate constant K_{el} for one-compartment model and the terminal exponential rate constant β are determined from the slope of the log–linear concentration versus time curve. Half-life ($t_{1/2}$) is the time required for plasma toxicant concentration to decrease by one half. C_0 is the concentration of a toxicant for a one-compartment model at t= 0 determined by extrapolating the log–linear concentration–time

curve to the *y*-axis.

Data Analysis and Parameter Estimation

Figure 7–4A presents blood concentration versus time data, as measured in an experimental study following a single IV dose of 100 mg of a xenobiotic in the rat. Figures 7–4B and 7–4C depict the same data following $\log_{10}$ and natural log (ln) transformations. Given the behavior seen in these plots (i.e., a straight line in log transformed plots), a one-compartment model is fit to the concentration versus time-course data using Eq. (7-8). In this one-compartment model for a single IV dose, the initial concentration, C_0, corresponds to the intercept in Fig. 7–4C and equals the dose diluted in the apparent volume of distribution, V_d, as follows:

$$V_d = \frac{\text{Dose}}{C_0}. \qquad (7\text{-}10)$$

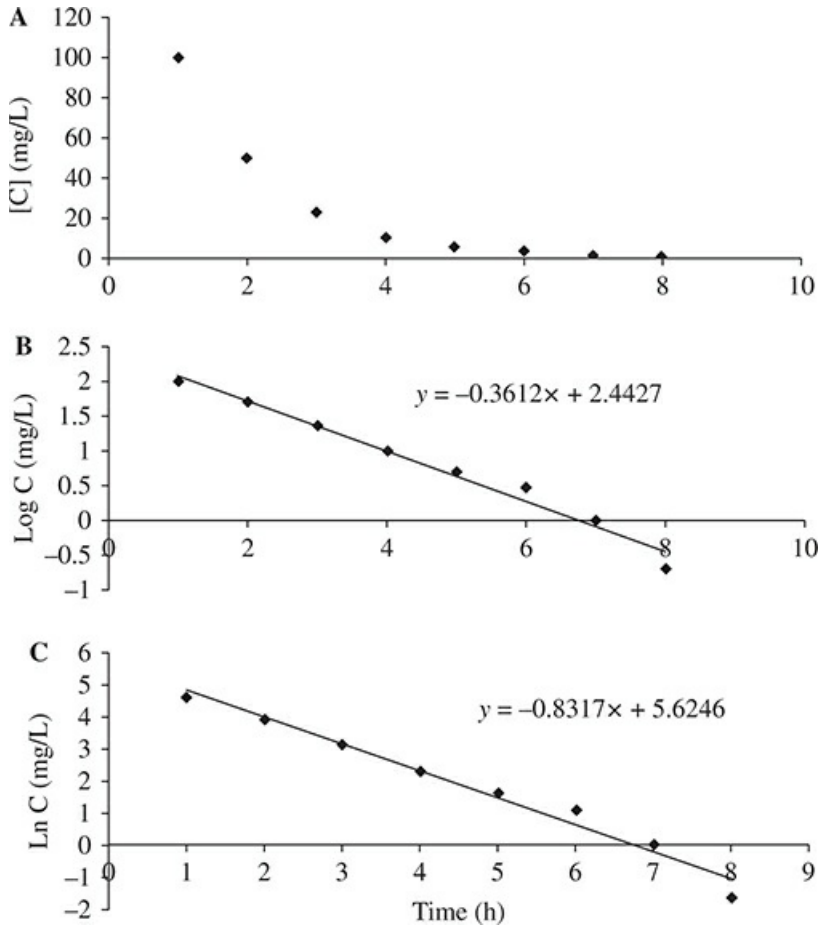

FIGURE 7–4 *Plots of the measured data (**A**) as well as the transformed data [log10(**B**) and natural log (**C**)] on the blood concentration versus time following a single IV dose.*

With a dose of 100 mg and the C_0 corresponding to the intercept is 5.62 (= 276 mg/L), the V_d

equals 0.36 L. The K_{el} value equals the slope of the natural log transformation plot (Fig. 7–4C) corresponding to the concentration difference (i.e., ln C_t − ln C_0) over a time period (Δt). Thus, the K_{el} associated with this dataset equals 0.832 h^{-1} (Fig. 7–4C) or 0.3612 × 2.303 (Fig. 7–4B). The $t_{1/2}$ for the initial concentration C_0 to become half its value (i.e., $0.5 \cdot C_0$) can be calculated from the K_{el} value as follows:

$$t_{1/2} = \frac{\ln C_0 - (\ln C_0 + \ln 0.5)}{K_{el}} = \frac{0.693}{K_{el}}. \qquad (7\text{-}11)$$

For the toxicant presented in Fig. 7–4, the $t_{1/2}$ value corresponds to 0.832 h. The toxicokinetic parameters discussed above, particularly K_{el} (or $t_{1/2}$) and V_d, can be used to calculate CL, which is an independent toxicokinetic determinant. For the chemical modeled after a single IV dose in Fig. 7–4, the CL equals 0.3 L/h (i.e., V_d of 0.36 L and K_{el} of 0.832 h^{-1}). The relationship among these parameters does not imply that CL is dependent on V_d or $t_{1/2}$; rather $t_{1/2}$ depends upon CL and V_d.

For a two-compartment model, CL is equal to V_d times β. In multicompartment models, V_d is calculated according to Eq. (7-12) based on the computation of area under the toxicant concentration–time curve:

$$V_d = \frac{\text{Dose}_{IV}}{\beta \cdot \text{AUC}_0^{\infty}}, \qquad (7\text{-}12)$$

where Dose_{IV} is the IV dose or known amount of toxicant in the body at time zero, β is the elimination rate constant, and is the area under the toxicant concentration versus time curve from time zero to infinity. Because the concept of an overall apparent volume of distribution is strictly applicable to the terminal exponential phase, some investigators refer to it as V_β (for a two-compartment model) or V_z (for a general multicompartment model).

PHYSIOLOGIC TOXICOKINETIC MODELS

Physiologically based pharmacokinetic (PBPK) models or physiologically based toxicokinetic (PBTK) models describe the interrelationships among the key determinants of uptake and disposition to simulate the time-course behavior of xenobiotics and their metabolites in blood and various tissues. PBPK models permit: (i) simulation of the concentration of chemicals and their metabolites in blood and in any other tissue of interest including the target organ, (ii) evaluation of the nature and magnitude of the potential impact of physiological, physicochemical, and metabolic parameters on the toxicokinetics of chemicals, (iii) prediction of the toxicokinetics of chemicals across species and populations using relevant input parameters, (iv) accounting for temporal change in physiological and metabolic parameters, complex dosing regimens, and saturable processes, and (v) simulation of toxicokinetic datasets collected under various dosing situations (exposure routes, doses, and scenarios) with a single set of input

parameters and equations. The range of values and distributions of input parameters are not always well defined for many chemicals and their metabolites in all species and pathophysiological states of interest. PBPK modeling frequently uses a priori parameter estimates in mechanism-based equations to simulate the toxicokinetics of chemicals in biota.

The scope and intended application of the PBPK model is designed up-front to ensure that the model development process is concordant with the required level of precision and extent of evaluation. The construction of PBPK models begins with development of the model structure, mathematical representation of key processes, and estimation of model parameters. The PBPK model equations and parameters are then written as a computer program, solved to provide toxicokinetic simulations, and compared with available data to guide further refinement or analysis (sensitivity, uncertainty, variability) in view of assessing the suitability of the model for the intended application.

Model Structure

The structure of a PBPK model for a given chemical corresponds to a diagrammatic representation of key processes and pathways determining its toxicokinetics in the organism (i.e., species or individual). The PBPK model is represented as boxes and arrows, with the boxes corresponding to the organs (individual or groups) and the arrows reflecting the physiological flows and clearance processes. A PBPK model structure requires striking a balance between the principles of parsimony (i.e., minimal but essential elements) and plausibility (i.e., reflective of physiological reality), while being consistent with the current state of knowledge on the toxicokinetics and mode of action of the chemical under study. The complexity of PBPK models increases only when it is required to explain the behavior contained in the toxicokinetic data. In PBPK models, increments of complexity generally involve the addition of compartments with different time-constants (i.e., blood flow divided by the distribution volume specific to each tissue compartment) or mathematical representations of mechanisms to account for specific accumulation or disposition behavior of the chemical under study. The level of detail relates to the compartments (e.g., whole body, target tissue, or intracellular concentrations) and to the chemical forms tracked within the model (e.g., parent chemical, metabolites, mixture components).

Mathematical Representation

The following paragraphs provide sample descriptions of the computation of concentrations of neutral, nonionized chemicals in metabolizing and non-metabolizing organs as well as in systemic circulation.

Computing Tissue Concentrations—The rate of change in the amount of chemical in the tissue compartments (dA/dt) is proportional to the concentration difference ($C_{in} - C_{out}$) and the membrane permeability coefficient (k) per Fick's first law as described below:

$$\frac{dA}{dt} = k \cdot (C_{in} - C_{out}). \qquad (7\text{-}13)$$

Chemical input to the tissue (amount per unit time) equals the influx clearance (CL_{in}) times incoming concentration (C_{in}), whereas chemical removal (amount per unit time) equals efflux clearance (CL_{out}) and the concentration leaving the compartment (C_{out}). The mass balance differential equation (MBDE) can be written as follows:

$$\frac{dA}{dt} = (CL_{in} \cdot C_{in}) - (CL_{out} \cdot C_{out}). \qquad (7\text{-}14)$$

The concentration of free chemical leaving a tissue compartment, C_{out}, depends upon the amount of chemical in that particular compartment (A_t) and its distribution volume (i.e., tissue volume, V_t, multiplied by tissue:blood partition coefficient, P_{tb}), such that the above equation becomes:

$$\frac{dA}{dt} = (CL_{in} \cdot C_{in}) - \left(CL_{out} \cdot \frac{A_t}{V_t \cdot P_{tb}}\right). \qquad (7\text{-}15)$$

The clearance terms in the above equation depend upon the membrane permeability and tissue blood flow. For small molecular weight compounds, blood flow rather than the membrane diffusion is the limited factor, such that the above MBDE can be rewritten as follows:

$$\frac{dA}{dt} = Q_t(C_a - C_{vt}), \qquad (7\text{-}16)$$

where C_a = concentration of chemicals in arterial blood entering the tissue compartment and C_{vt} = concentration of chemical in venous blood leaving the tissue. For high-molecular-weight compounds, membrane diffusion rather than tissue blood flow is the limiting factor such that their flux through the cellular matrix and tissue blood needs to be accounted for separately. If the diffusion of a chemical from tissue blood to cellular matrix is slow with respect to tissue blood flow, then both the extracellular and intracellular components are characterized (Figs. 7–5 and 7–6). However, when the tissue perfusion rate (i.e., blood flow) is small compared to membrane diffusion, then the tissues are described as homogeneous, well-mixed compartments. Consequently, the rate of change in the amount of chemical in the tissue is described with a single equation for the whole tissue mass (Eq. 7-16), followed by using the output of this equation to calculate concentration in the tissue and venous blood leaving the tissue.

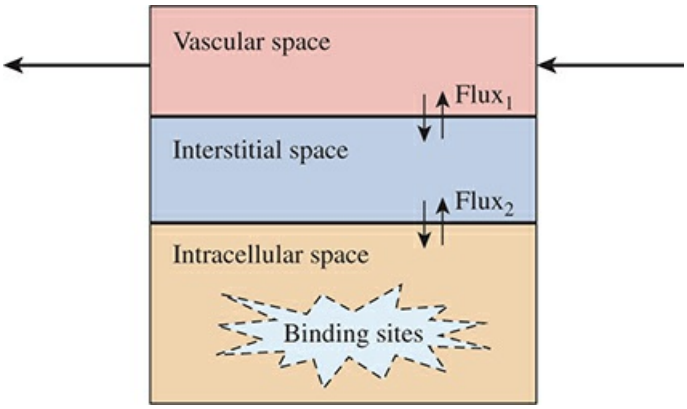

FIGURE 7–5 *Schematic representation of a lumped tissue compartment in a physiologically based toxicokinetic model.* The blood capillary and cell-barriers separating the vascular, interstitial, and intracellular subcompartments are depicted in heavy black lines. The vascular and interstitial subcompartments are often combined into a single extracellular subcompartment.

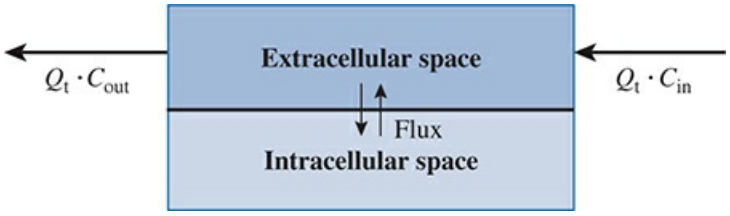

FIGURE 7–6 *Schematic representation of a tissue compartment that features membrane-limited uptake kinetics.* Perfusion of blood into and out of the extracellular compartment is depicted by longer arrows. Transmembrane transport (flux) from the extracellular to the intracellular subcompartment is depicted by shorter arrows. ***Q*t** is blood flow, ***C*in** is toxicant concentration entering the compartment, and ***C*out** is toxicant concentration leaving the compartment.

For compartments exhibiting metabolic capacity, the rate of the amount cleared by metabolism (dA_{met}/dt) is also included such that the MBDE becomes:

$$\frac{dA}{dt} = Q_t(C_a - C_{vt}) - \frac{dA_{met}}{dt}. \qquad (7\text{-}17)$$

The rate of metabolism in PBPK models is described as a first-order, second-order, or saturable process (Table 7–1). When the amount metabolized per unit time is directly proportional to the input variable (e.g., free concentration of chemical in liver), metabolism is described as a first-order process. The second-order equation is employed when the rate of metabolism depends on the available concentration of both the cofactor and the chemical. The saturable metabolism, often described as a Michaelis–Menten reaction, reflects first-order behavior at low substrate concentrations and zero-order behavior at concentrations that are far excessive of the Michaelis parameter, K_m. All the metabolism descriptions presented in Table 7–1 are based on venous blood concentration (i.e., free concentration) of a xenobiotic, reflecting the

"venous equilibration model" descriptions. Because calculation of the rate of metabolism is integrated within Eq. (7-17), the blood flow (i.e., perfusion) limitation of hepatic metabolism is automatically included. Alternatively, the blood flow to liver and hepatic extraction ratio can be used explicitly to describe metabolism in PBPK models (as $Q_h \cdot E_h \cdot C_a$, where Q_h = liver blood flow rate, E_h = hepatic extraction ratio, and C_a = arterial blood concentration), but the E_h may be dose-dependent when saturation occurs in the dose range of interest.

TABLE 7–1 Examples of Equations Used for Calculating the Rate of the Amount of Chemical Metabolized (dA_{met}/dt) in Physiologic Toxicokinetic Models

Reaction Type	Equation
First-order process	$\frac{dA_{met}}{dt} = K_f \cdot C_{vt} \cdot V_t$
Second-order process	$\frac{dA_{met}}{dt} = K_s \cdot C_{vt} \cdot V_t \cdot C_{cf}$
Saturable process	$\frac{dA_{met}}{dt} = \frac{V_{max} \cdot C_{vt}}{K_m + C_{vt}}$

Abbreviations: C_{cf}, concentration of the cofactor; C_{vt}, concentration of chemical in venous blood leaving the tissue; K_f, first-order reaction rate; K_m, Michaelis constant; K_s, second-order reaction rate constant; V_{max}, maximal velocity; V_t, volume of metabolizing tissue.

Analogous to the hepatic clearance, the MBDE can accommodate biliary and fecal clearances by considering the rate of bile flow, the rate of transfer into and reabsorption from the bile, as per the molecular weight of the chemical or its conjugated metabolite. Urinary excretion is modeled as a function of the rates of filtration, reabsorption, and secretion. The amount of chemical filtered (dF/dt) and the rate of change in the concentration of chemical or its metabolite in the urine (dU/dt) are calculated as follows:

$$\frac{dF}{dt} = \text{GFR} \cdot C_v, \qquad (7\text{-}18)$$

$$\frac{dU}{dt} = U_o \cdot C_u, \qquad (7\text{-}19)$$

where GFR = glomerular filtration rate, U_o = urinary output per unit time, C_v = unbound blood concentration of xenobiotic, and C_u = xenobiotic concentration in urine. The amount of chemical secreted (or reabsorbed) then equals the difference between the amount in urine and the amount filtered.

Computing Blood Concentrations—The rate of change in the amount of chemical in blood compartment in PBPK models can be calculated conceptually as per Eq. (7-16). Alternatively, the mass conservation equations can be solved to compute blood concentrations. In this regard, chemical input to the lung compartment equals the amount inhaled ($Q_p \times C_{inh}$) plus the amount returning to lungs via mixed venous blood ($Q_c \times C_v$), whereas the output equals the amount leaving the lungs via arterial blood ($Q_c \times C_a$) plus the amount exhaled ($Q_p \times C_{alv}$). Notationally,

$$Q_p C_{inh} + Q_c C_v = Q_c C_a + Q_p C_{alv}, \qquad (7\text{-}20)$$

where C_{inh} = inhaled concentration, C_v = mixed venous blood -concentration, C_a = arterial concentration, C_{alv} = alveolar concentration, Q_p = alveolar ventilation rate, and Q_c = cardiac output.

Given that lung equilibrates vapor between alveolar air and blood ($C_{alv} = C_a/P_b$), the above equation can be rewritten to compute arterial blood concentrations:

$$C_a = \frac{Q_p C_{inh} + Q_c C_v}{\left(Q_c + \frac{Q_p}{P_b}\right)}. \qquad (7\text{-}21)$$

The above equation simplifies to $C_a = C_v$ for highly nonvolatile chemicals (with a negligible value of C_{inh} and very large P_b) not metabolized in the lung compartment. In such situations, the conducting airways (i.e., nasal passages, larynx, trachea, bronchi, and bronchioles) are considered as inert tubes that carry the chemical to the pulmonary region where diffusion occurs. When simple continuous ventilation-equilibration models are insufficient to describe the uptake behavior (e.g., regional uptake of highly soluble polar solvents), the adsorption and/or dissolution in the surface of the respiratory epithelium during inhalation as well as desorption during exhalation can be taken into account along with the cyclic nature of respiratory exchange.

The mixed venous blood concentration, C_v, entering the lung compartment results from the venous concentration of chemicals leaving the various tissue compartments following their equilibration as a function of the tissue:blood partition coefficients. The C_v is not a simple average of C_{vt} of the tissue compartments, as the fraction of cardiac output flowing into and out of the tissues is not identical across the compartments.

Parameters: Significance and Estimation

The equations of the PBPK models contain a number of input parameters, which are conveniently grouped as physiological, physicochemical, or biochemical parameters including rate constants of reactions and clearance processes relevant to the chemical being modeled.

Physiological Parameters—The physiological parameters consist of volumes and flows; the volumes are required for the tissues or tissue groups represented as boxes, whereas flows are associated with the arrows in the model, which include alveolar ventilation rate, cardiac output,

and organ-specific blood flow rates. Tissue volumes along with their composition, relative to that of blood, determine the extent to which chemicals are diluted within each compartment. The greater the volume of tissue and lipid content, the larger the V_d of lipophilic xenobiotics and lower blood concentrations.

Physicochemical Parameters—PBPK modeling requires partition coefficients, which represent the relative distribution of a nonionized chemical between two matrices at equilibrium. Of these, the tissue to blood partition coefficient (P_{tb}) is a key determinant of the volume of distribution specific to each tissue compartment of a PBPK model, whereas the blood:air partition coefficient (P_b) is a determinant of the pulmonary uptake and clearance of volatile organic chemicals. The pulmonary absorption of chemicals possessing a relatively high P_b is limited by alveolar ventilation rate, whereas that of chemicals with low P_b is limited by cardiac output.

Rate Constants and Biochemical Parameters—Values required for PBPK modeling include oral absorption rate constant, dermal permeability coefficient, membrane diffusion coefficient, maximal velocity for metabolism, Michaelis parameter, binding association constant, and urinary/biliary excretion rate. These parameters have often been determined based on time-course data collected in vivo or in vitro.

In silico approaches utilize the rich database for pharmaceutical substances regarding the determinants of oral absorption (i.e., lipophilicity, p*K*a, solubility, particle size, permeability, release kinetics, and dissolution kinetics), as well as mathematical models and algorithms to simulate the rate of drug absorption in humans. For the metabolism constants, there are increasing examples of scaling to in vivo parameters obtained in vitro with microsomes, post-mitochondrial fractions preparations, and freshly isolated hepatocytes.

Model Simulation

The equations in a PBPK model are solved to obtain simulation of the toxicokinetics of xenobiotics for a given species, dose, and exposure scenario. In order to solve the MBDE of each tissue compartment, the arterial (input) blood concentration needs to be known, which depends upon mixed venous concentration as well as the chemical input resulting from absorption. The mixed venous concentration in turn depends upon the chemical concentration in venous blood leaving the tissue compartments such that the input and output of all compartments within the PBPK model are interconnected in a loop-type calculation. Fundamental to the implementation of these calculations is the integration of the differential equations in the PBPK model.

Model Verification and Evaluation

Model verification focuses on whether the PBPK model is correctly coded (error-free) and the toxicokinetic simulations could be reproduced in a different programming software or computational platform. Model evaluation focuses on comparing the simulations (usually continuous) with experimental toxicokinetic data (usually discrete) and ascertaining whether the model with its structure and parameters is fit to provide output required for a specific end use. Given that both the PBPK model simulations and experimental data are subject to uncertainty and variability, it is not realistic to expect an exact match between every single data point and the

simulation output. Therefore, PBPK models providing predictions within a factor of 2 of the experimental data are generally considered adequate. Principles determining the level of confidence in a model are listed in Table 7–2. The species, developmental stage, exposure route, and dose range corresponding to the toxicity study and the PBPK modeling study should be compared to select the appropriate model for use in interpreting a toxicity study.

TABLE 7–2 Checklist for Characterizing the Level of Confidence in Physiologically Based Toxicokinetic (PBPK) Models

Biological Basis of Pbpk Models	Performance of Pbpk Models	Reliability of Pbpk Models
• The sum total of the tissue blood flow rates equals the cardiac output specified within the model. • The sum total of the volumes of compartments equals the soft tissue volumes or body weight, as the case may be. • The values of tissue volumes and tissue blood flow rates (individual or group) specified in the model are within the documented range for the particular species and life stage. • The ventilation:perfusion ratio specified in the model is within physiological limits. • The allometric scaling of parameters, if applicable, is consistent with the known physiological principles. • The major sites/processes of absorption, distribution, and clearance are included in the model. • The mathematical equations of ADME do not violate the known theoretical or biological principles. • The input parameters adequately reflect the characteristics of the subject, population, chemical, and exposure pathway.	• The PBPK model consistently reproduces the general trend of the toxicokinetic data (i.e., peaks, bumps, and valleys, saturation of metabolism). • The PBPK model predictions are within an acceptable level of correspondence with the experimental data (e.g., within a factor of two). • The PBPK model reproduces the chemical-specific toxicokinetic data under various experimental conditions or exposure scenarios.	• The PBPK model is capable of providing reliable predictions of the concentration–time course of the candidate dose metrics in the target organ or a suitable surrogate compartment (e.g., blood). • The data used for calibrating and/or evaluating the PBPK model are reliable. • The sensitivity of the output (dose metric) to change in numerical values of input parameters has been characterized for relevant human or animal exposures. • The uncertainty in PBPK model predictions of dose metric has been assessed for the relevant exposure conditions.

TOXICOKINETIC TOPICS AND APPLICATIONS

Nonlinear Kinetics

The distribution and clearance of most chemicals follow first-order kinetics in the low-dose region. Accordingly, the K_{el}, V_d, CL, and $t_{1/2}$ are expected to be in the low-dose range (i.e., no dose-dependent change in kinetic parameters occurs under first-order condition). Under first-order conditions, the dose metrics (e.g., C_{max}, AUC) will be nearly identical when compared on the basis of unit dose (e.g., per µg/kg/day) or exposure concentration (per ppm). A semilogarithmic plot of blood concentration versus time over a range of doses will show a set of parallel lines such that blood concentration at a given time remains proportional to dose. However, for some toxicants, as the dose increases, its V_d and/or CL may change, showing nonlinear or dose-dependent kinetics. Enzyme-mediated metabolism, active transport processes, and protein binding have finite capacities and therefore can be saturated.

The transition from first-order to zero-order (or saturation) kinetics is important in toxicology because it can lead to prolonged persistence of a compound in the body after a single dose or an acute exposure, and excessive accumulation during repeated or chronic exposure. Some salient characteristics of nonlinear kinetics include: (i) the decline in the concentrations of the parent

chemical in the body is not exponential, (ii) amount metabolized are not proportional to the dose, (iii) V_d, CL, K_{el} (or β), or $t_{1/2}$ change with increasing dose, (iv) the composition of excretory products changes quantitatively or qualitatively with the dose, and (v) dose–response curves depict a larger increase in response to an increasing dose, starting at the dose at which saturation effects become evident. A common cause of time-dependent kinetics is autoinduction of metabolizing enzymes through activation of gene transcription by certain chemicals.

PBPK models facilitate high-dose to low-dose extrapolation of tissue dosimetry by accounting for the mechanism(s) underlying the nonlinear kinetic behavior of chemicals (e.g., saturable metabolism, enzyme induction, enzyme inactivation, protein binding, and depletion of glutathione reserves). Quantitative descriptions of such mechanisms are essential for any toxicokinetic model to facilitate extrapolation or interpolation of dose metrics from high dose to low dose.

Single versus Repeated Dose Kinetics

Continuous or chronic exposure to a chemical can lead to its accumulation in the body depending upon its elimination half-life and interval between the doses. Lipophilic chemicals with large V_d and small CL exhibit long half-lives and accumulate as the exposure continues. On the contrary, for a chemical with a small $t_{1/2}$ compared to the time interval between doses, it is likely to be eliminated almost completely before the next dose such that there would be no significant accumulation. Typically, the increase in blood concentration is relatively rapid after dosing, as it is governed by the early (distribution) half-life and is slower at later times when the terminal (elimination) half-life becomes dominant. Therefore, when the doses are given at certain intervals of time (and not in a continuous fashion), the fraction eliminated between two dosing intervals needs to be accounted for computing the dose metrics. Knowledge of the fraction eliminated during the time interval between doses and the extent to which C_{max} and C_{min} fluctuate during a dosing interval are key considerations in designing toxicity studies.

Compartmental models are useful in estimating blood and tissue concentrations associated with a single dose or repeated doses given by various routes, and this can frequently be accomplished using a single set of parameters. Re-estimation or adjustment of parameter values might be necessary to accommodate the observed or anticipated change in input parameters with time (e.g., change in V_d due to body fat content, change in CL_{int} due to enzyme induction). Fundamentally, the rate of change in the amount of chemical in non-metabolizing tissues in PBPK models becomes equal to zero at steady state, such that the tissue concentrations remain stable during repeated exposures.

Metabolite Kinetics

Metabolism of a parent chemical can result in bioactivation or detoxication, with the resulting products subjected to further metabolism to more active or inactive moieties. When the toxicity is attributed to the biotransformation product(s), the formation and subsequent disposition kinetics of a toxic metabolite are of interest. As the parent chemical is transformed into a metabolite, the blood concentration of the metabolite rises depending upon its reactivity, V_d, and CL. The metabolite might exhibit reactivity/stability, be subjected to further biotransformation

reactions to form other metabolites, or undergo excretion via the kidneys or bile; hence at some point in time, the blood concentration of the metabolite exhibits a peak and falls thereafter. A biologically active metabolite assumes toxicological significance when it is cleared much less efficiently than the parent compound and/or exhibits accessibility and reactivity in the target site.

Cross-Route Dose Extrapolation

Comparison or extrapolation of toxicity data across exposure routes can be conducted for systemically acting chemicals based on administered dose or toxicokinetic considerations. The data on route-specific bioavailable fraction are used to conduct route-to-route extrapolation of toxicokinetically equivalent doses. The bioavailable dose for each exposure route can be calculated by multiplying the administered dose with the route-specific bioavailability (F_{route}). The extrapolation of equivalent dose between two exposure routes (e.g., oral to dermal route in units of mg/kg/day) is then determined based on the ratio of the route-specific bioavailable fraction (F_{route1}/F_{route2}):

$$\text{Dose}_{route2} = \text{Dose}_{route1} \frac{F_{route1}}{F_{route2}}. \qquad (7\text{-}22)$$

PBPK models, by accounting for species- and route-specific rate and magnitude of absorption as well as first-pass effect, facilitate the route to route extrapolation based on equivalent tissue dose of toxic moiety (Fig. 7–7). Accordingly, given that the total clearance (sum of renal, hepatic, and pulmonary clearances, expressed as L/day) is the same regardless of the exposure route, the extrapolation of oral dose (mg/kg/day) to inhalation (inh) concentration (mg/m^3) can be calculated as follows:

$$\text{Dose}_{inh} = \frac{\text{Dose}_{oral} \times \text{BW} \times F_{oral}}{Q_{alv}}, \qquad (7\text{-}23)$$

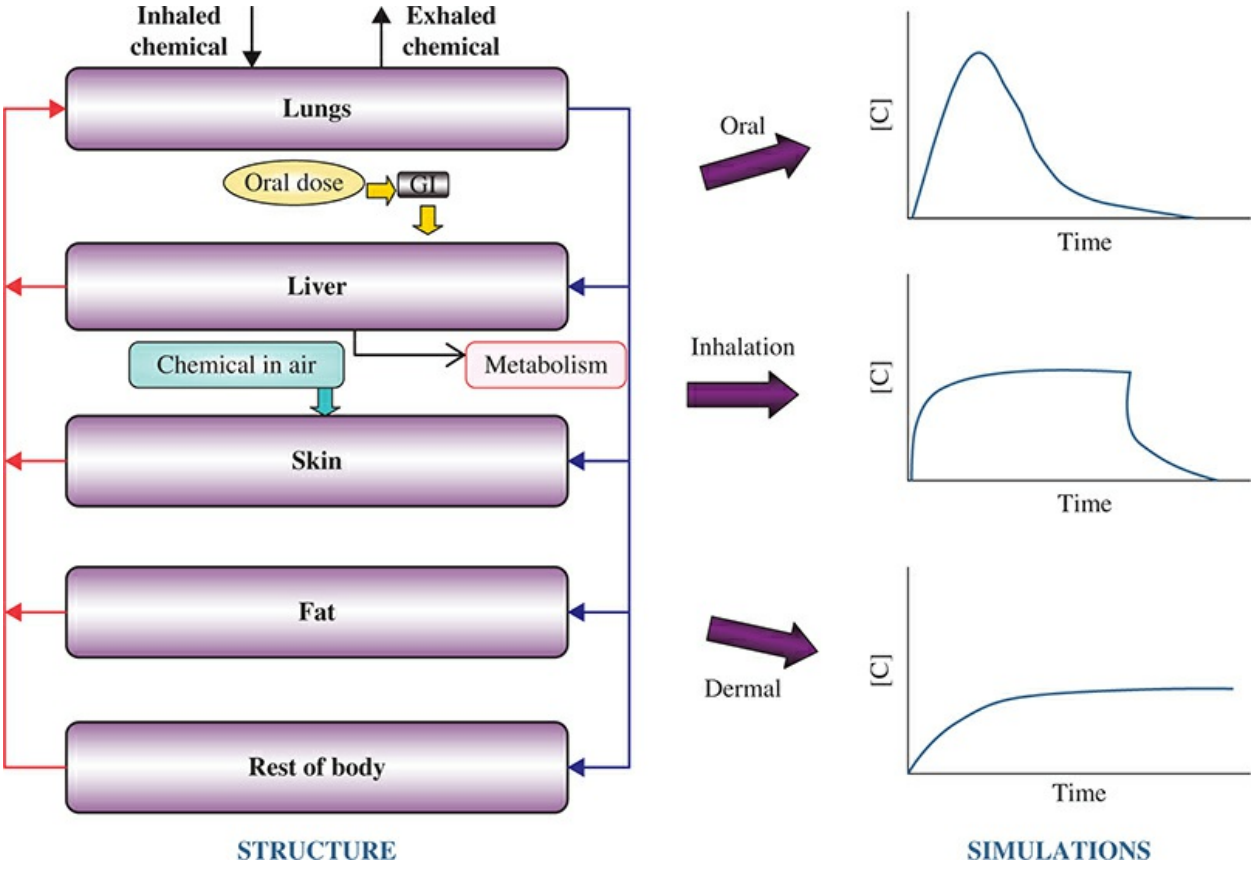

FIGURE 7–7 *Illustration of the use of physiologic toxicokinetic model to simulate the blood concentration [C] as a function of time for three routes of exposure: oral, inhalation, and dermal.* For simulating the route-specific toxicokinetics of the chemical, only the route-specific input parameters are specified along with the chemical concentration in the corresponding exposure medium.

where BW = body weight, F_{oral}= bioavailable fraction for the oral route (accounting for first-pass effect and absorption by the oral route) associated with the relevant dose (e.g., NOAEL), and Q_{alv}= alveolar ventilation rate (m^3/day).

Interspecies Dose Extrapolation

The extrapolation of a toxicologically equivalent dose from one species to another (typically from test animal species to humans) has been historically addressed using a factor of 10. The default value of the toxicokinetic component is equal to interspecies ratio of BW to a fractional power (0.67 to 0.75), which is consistent with the observations that several physiological parameters (GFR, breathing rate, cardiac output) and volume of extracellular water are more closely related to body surface rather than body weight of mammals. Such allometric dose adjustments $\left(\text{e.g., } \text{Dose}_{\text{human}}\ (\text{mg/kg/d}) = \text{Dose}_{\text{animal}}\ (\text{mg/kg/d})\left(\frac{\text{BW}_{\text{Animal}}}{\text{BW}_{\text{Human}}}\right)^{0.33}\right)$ reflect interspecies differences in body surface area and physiological flow rates and also provide toxicokinetically

equivalent doses across species as long as the parent chemical is the potential toxic moiety and AUC_{blood} is the appropriate measure of dose to target tissue. It may be necessary to conduct interspecies extrapolation based on chemical-specific data on the dose metrics of relevance to the mode of action (AUC, C_{max}) or the associated toxicokinetic parameter (CL).

Interindividual Kinetic Variability

The toxicokinetic parameters (e.g., CL, $t_{1/2}$) and dose metrics (e.g., C_{max}, AUC) for a xenobiotic may vary between individuals and subgroups in the human population. Interindividual variability in toxicokinetics is characterized by the human kinetic adjustment factor (HK_{AF}), which is the ratio between the upper percentile value (i.e., 95th, 99th) and its central tendency value in the entire population of a relevant variable for a given chemical. PBPK models can estimate the population distribution of physiological parameters, metabolizing enzymes (including polymorphism), and rates of toxicokinetic processes.

Kinetic Interactions During Mixed Exposures

Toxicokinetic interactions during mixed exposures result when one chemical alters the absorption, distribution, metabolism, or excretion of another chemical. Such interactions result in an increase or decrease in the internal dose per unit administered dose during mixed exposures in comparison with the individual chemical exposures. During mixed exposures, xenobiotics may compete directly with each other for an enzymatic binding site (competitive inhibition), bind directly to the enzyme-xenobiotic complex but not to the free enzyme (uncompetitive inhibition), or both (noncompetitive inhibition). The toxicokinetic analysis should account for the nonlinearity arising from the saturable metabolism of individual mixture components and the impact of the metabolic inhibition as a function of the concentration and affinity of one mixture component (substrate) in comparison with the free concentration and potency of another mixture component (inhibitor). When induction of metabolizing enzymes occurs, the toxicokinetic analysis would indicate that the impact is more pronounced in cases where hepatic metabolism is capacity-limited (e.g., high doses, xenobiotics exhibiting low hepatic extraction ratio).

Interpretation of Human Biomonitoring Data

Human biological monitoring refers to the systematic sampling of body fluids (e.g., blood, urine, exhaled air), and at times body tissues, for the purpose of estimating an individual's internal dose from exposure to chemicals in the workplace or in the environment. Biomonitoring accounts for all exposure sources and routes (oral, dermal, and inhalation), individual differences in toxicant absorption and disposition, and critical personal variables (body size and composition), workload that affects pulmonary ventilation, or cigarette smoking that could affect the metabolic status of an individual. Although biomonitoring provides a measure of internal exposure, the data obtained must be relatable to health guidance values. For each chemical of concern, it is useful to establish a reference biomonitoring value below which a reasonable assurance of safety exists. The tools for interpreting biomonitoring data use forward dosimetry or reverse dosimetry approaches. Forward dosimetry refers to the use of toxicokinetic data and models to develop concentrations of exposure biomarkers corresponding to the existing health guidance values

(e.g., occupational exposure limit, reference dose, tolerable daily intake) or point of departure in a dose–response curve (e.g., NOAEL). Reverse dosimetry uses toxicokinetic parameters and models to reconstruct/estimate exposure levels associated with the measured concentrations of biomarkers in biological matrices (urine, blood, plasma, exhaled air).

CONCLUSION

Toxicokinetic data and models are central to the selection of doses and species for toxicity testing, interpretation of dose–response relationships, and efficient design of animal experiments. Toxicokinetic principles are also key to the design and interpretation of human biomonitoring studies. Emerging toxicity testing and risk assessment paradigms as well as extensive ongoing human biomonitoring programs will require toxicokinetic data and tools for numerous chemicals. Increasing focus on the development of high throughput (HTP) approaches to derive toxicokinetic parameters and PBPK models for new chemicals, mixtures, and particles is required.

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QUESTIONS

1. Regarding the two-compartment model of classic toxicokinetics, which of the following is true?
   - **a.** There is rapid equilibration of chemical between central and peripheral compartments.
   - **b.** The logarithm of plasma concentration versus time data yields a linear relationship.
   - **c.** There is more than one dispositional phase.
   - **d.** It is assumed that the concentration of a chemical is the same throughout the body.
   - **e.** It is ineffective in determining effective doses in toxicity studies.

2. When calculating the fraction of a dose remaining in the body over time, which of the following factors need not be taken into consideration?
   - **a.** half-life.
   - **b.** initial concentration.
   - **c.** time.
   - **d.** present concentration.
   - **e.** elimination rate constant.

3. All of the following statements regarding apparent volume of distribution (V_d) are true EXCEPT:

a. V_d relates the total amount of chemical in the body to the concentration of chemical in the plasma.
b. V_d is the apparent space into which an amount of chemical is distributed in the body to result in a given plasma concentration.
c. A chemical that usually remains in the plasma has a low V_d.
d. V_d will be low for a chemical with high affinity for tissues.
e. V_d can be used to estimate the amount of chemical in the body if the plasma concentration is known.

4. Chemical clearance:
a. is independent of V_d.
b. is unaffected by kidney failure.
c. is indirectly proportional to V_d.
d. is performed by multiple organs.
e. is not appreciable in the GI tract.

5. A chemical with which of the following half-lives ($T_{1/2}$) will remain in the body for the longest period time when given equal dosage of each?
a. $T_{1/2}$ = 30 min.
b. $T_{1/2}$ = 1 day.
c. $T_{1/2}$ = 7 h.
d. $T_{1/2}$ = 120 s.
e. $T_{1/2}$ = 1 month.

6. With respect to first-order elimination, which of the following statements is FALSE?
a. The rate of elimination is directly proportional to the amount of the chemical in the body.
b. A semilogarithmic plot of plasma concentration versus time shows a linear relationship.
c. Half-life ($T_{1/2}$) differs depending on the dose.
d. Clearance is dosage-independent.
e. The plasma concentration and tissue concentration decrease similarly with respect to the elimination rate constant.

7. The toxicity of a chemical is dependent on the amount of chemical reaching the systemic circulation. Which of the following does NOT *greatly* influence systemic availability?
a. absorption after oral dosing.
b. intestinal motility.
c. hepatic first-pass effect.
d. intestinal first-pass effect.
e. incorporation into micelles.

8. Which of the following is NOT an advantage of a physiologically based toxicokinetic

model?

a. Complex dosing regimens are easily accommodated.
b. The time course of distribution of chemicals to any organ is obtainable.
c. The effects of changing physiologic parameters on tissue concentrations can be estimated.
d. The rate constants are obtained from gathered data.
e. The same model can predict toxicokinetics of chemicals across species.

9. Which of the following will not help to increase the flux of a xenobiotic across a biological membrane?

a. decreased size.
b. decreased oil:water partition coefficient.
c. increased concentration gradient.
d. increased surface area.
e. decreased membrane thickness.

10. Which of the following statements is true regarding diffusion-limited compartments?

a. Xenobiotic transport across the cell membrane is limited by the rate at which blood arrives at the tissue.
b. Diffusion-limited compartments are also referred to as flow-limited compartments.
c. Increased membrane thickness can cause diffusion-limited xenobiotic uptake.
d. Equilibrium between the extracellular and intracellular space is maintained by rapid exchange between the two compartments.
e. Diffusion of gases across the alveolar septa of a healthy lung is diffusion-limited.

UNIT 3 NON-ORGAN-DIRECTED TOXICITY

CHAPTER 8

Chemical Carcinogenesis

James E. Klaunig and Zemin Wang

KEY POINTS

- The term *cancer* describes a subset of neoplastic lesions.
- A *neoplasm* is defined as a heritably altered, relatively autonomous growth of tissue with abnormal regulation of gene expression.
- *Metastases* are secondary growths of cells from the primary neoplasm.
- A *carcinogen* is an agent whose administration to previously untreated animals leads to a statistically significant increased incidence of neoplasms of one or more histogenetic types as compared with the incidence in appropriate untreated animals.
- *Initiation* requires one or more rounds of cell division for the "fixation" of the DNA damage.
- *Promotion* results from the selective functional enhancement of the initiated cell and its progeny by the continuous exposure to the promoting agent.
- *Progression* is the transition from early progeny of initiated cells to the biologically malignant cell population of the neoplasm.

INTRODUCTION

Cancer is a disease characterized by genomic mutation, modified gene expression, cell proliferation, and aberrant cell growth. It is one of the leading causes of death in the world. Multiple causes of cancer include infectious agents, radiation, and chemicals. Estimates suggest that 70% to 90% of all human cancers have a link to environmental, dietary, and behavioral factors.

Definitions

Neoplasia is defined as new growth or autonomous growth of tissue. A neoplastic lesion is referred to as a neoplasm (Table 8–1).

TABLE 8–1 Terminology

Neoplasia	New growth or autonomous growth of tissue
Neoplasm	The lesion resulting from the neoplasia
Benign	Lesions characterized by expansive growth, frequently exhibiting slow rates of proliferation that do not invade surrounding tissues
Malignant	Lesions demonstrating invasive growth, capable of metastases to other tissues and organs
Metastases	Secondary growths derived from a primary malignant neoplasm
Tumor	Lesion characterized by swelling or increase in size, may or may not be neoplastic
Cancer	Malignant neoplasm
Carcinogen	A physical or chemical agent that causes or induces neoplasia
Genotoxic	Carcinogens that interact with DNA resulting in mutation
Non-genotoxic	Carcinogens that modify gene expression but do not damage DNA

For benign neoplasms, the tissue of origin is frequently followed by the suffix “oma”; for example, a benign fibrous neoplasm would be termed fibroma, and a benign glandular epithelium termed an adenoma. Malignant neoplasms from epithelial origin are called carcinoma while those derived from mesenchymal origin are referred to as sarcoma. Thus, a malignant neoplasm of fibrous tissue would be a fibrosarcoma while that derived from bone would be an osteosarcoma.

The term cancer describes a subset of neoplasia that represents malignant neoplasms. Carcinogens that induce cancer include chemicals, viruses, hormones, radiation, or solid materials. Genotoxic carcinogens interact with DNA to damage or change its structure. Non-genotoxic carcinogens do not directly interact with nuclear DNA, but may change gene expression, modify normal cell function, bind to or modify cellular receptors, or increase the number of cells in the target tissue. These agents work through epigenetic mechanisms that modify DNA and proteins associated with DNA, thereby altering gene expression or the cellular phenotype without modifying or directly damaging its structure. Common features of genotoxic and non-genotoxic carcinogens are shown in Table 8–2.

TABLE 8–2 Features of Genotoxic and Non-genotoxic Carcinogens

Genotoxic carcinogens
Mutagenic
Can be complete carcinogens
Tumorigenicity is dose responsive
No theoretical threshold
Non-genotoxic carcinogens
Nonmutagenic
Threshold, reversible
Tumorigenicity is dose responsive
May function at tumor promotion stage
No direct DNA damage
Species, strain, tissue specificity

MULTISTAGE CARCINOGENESIS

Carcinogenesis involves a series of definable stages, including initiation, promotion, and progression (Fig. 8–1). These steps follow a temporal sequence of events demonstrable by histopathology and observed in many target tissues. The defining characteristics of each stage are outlined in Table 8–3. Once a neoplasm is formed, additional intracellular and extracellular changes occur in the process of the development of a malignant cancer.

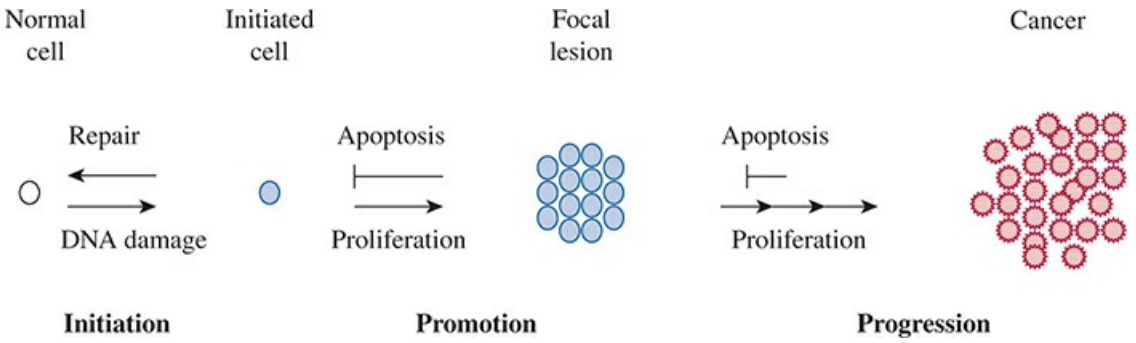

FIGURE 8–1 Multistage model carcinogenesis.

TABLE 8–3 Characteristics of the Stages of Carcinogenesis Process

Initiation
DNA modification
Mutation
Genotoxic
One cell division necessary to lock-in mutation
Modification is not enough to produce cancer
Nonreversible
Single treatment can induce mutation
Promotion
No direct DNA modification
Non-genotoxic
No direct mutation
Multiple cell divisions necessary
Clonal expansion of the initiated cell population
Increase in cell proliferation or decrease in cell death (apoptosis)
Reversible
Multiple treatments (prolonged treatment) necessary
Threshold
Progression
DNA modification
Genotoxic event
Mutation, chromosome disarrangement
Changes from preneoplasia to neoplasia benign/malignant
Irreversible
Number of treatments needed with compound unknown (may require only single treatment)

Initiation

The first stage of the cancer process involves initiation, a process that is defined as a stable, heritable change (Fig. 8–1). This stage is a relatively rapid, irreversible process that results in a carcinogen-induced mutational event. Chemical and physical agents that function at this stage are referred to as initiators or initiating agents. Initiating agents lead to genetic changes including mutations and deletions. Chemical carcinogens that covalently bind to DNA and form adducts that result in mutations are initiating agents. The initiating event becomes "fixed" when the DNA adducts or other damage to DNA is not correctly or completely repaired prior to DNA synthesis.

Once initiated cells are formed: (1) the initiated cell can remain in a static nondividing state through influences by growth control either via normal surrounding cells or through endocrine influence; (2) the initiated cell may possess mutations incompatible with viability or normal function and be deleted through apoptotic mechanisms; or (3) the cell, through stimuli such as intrinsic factors or from chemical exposure, may undergo cell division resulting in the proliferation of the initiated cell. Besides production of an initiated cell through carcinogen binding and misrepair, additional evidence shows that induction of continual stress, resulting in continual cell proliferation, can also produce new mutated, initiated cells.

Promotion

Stage 2 of the carcinogenesis process (the promotion stage) involves the selective clonal expansion of initiated cells to produce a preneoplastic lesion. Exogenous and endogenous agents that function at this stage are frequently referred to as tumor promoters, which are not mutagenic and generally are not able to induce tumors by themselves. They act through mechanisms that involve changes in gene expression that in turn result in sustained cell number in the target tissue either through increases in cell proliferation and mitogenesis and/or the inhibition of apoptosis. This stage involves the modulation of gene expression through receptor or non–receptor-mediated processes. With repeated applications of the chemical only, initiated cells continue to clonally expand and divide into a focal lesion (Fig. 8–1). Tumor promotion is a dose-dependent and reversible process; with removal of the promotional agent, focal cells cease proliferation and may return to single initiated cells. In addition, agents that function at the promotion stage demonstrate a dose-dependent threshold for their effects. Below a certain dose or frequency of application the chemical is unable to induce cell growth. Carcinogens that function at the tumor promotion stage in general are organ specific. For example, phenobarbital functions at the tumor promotion stage selectively in the liver.

Progression

Progression involves conversion of preneoplastic lesions into a neoplasm. In this stage, additional genotoxic events may further DNA damage including chromosomal damage such as aberrations and translocations. In the neoplastic state, cells accumulate mutations and epigenetic changes that cause cells to lose normal growth control. Progression is irreversible, whether the formed neoplasm is benign or malignant, and autonomous growth and/or lack of growth control is achieved. Spontaneous progression can occur from spontaneous karyotypic changes that occur in mitotically active initiated cells during promotion. An accumulation of nonrandom chromosomal aberrations and karyotypic instability are hallmarks of progression.

MECHANISMS OF ACTION OF CHEMICAL CARCINOGENS

The formation of a neoplasm is a multistage, multistep process that involves the ultimate release of the neoplastic cells from normal growth control processes and creating a tumor microenvironment. The major characteristics of a neoplasm as it progresses into a malignant state include (1) sustaining cell proliferation, (2) resisting cell death (apoptosis), (3) inducing angiogenesis, (4) enabling replicative immortality, (5) activating invasion and metastasis, (6) evading growth suppressors, (7) reprogramming of energy metabolism, and (8) evading immune destruction. Thus, tumors are not just a collection of clonal neoplastic cells but a complex tissue with multiple cell populations that interact with one another and function as a unique tissue. This tumor microenvironment involves the recruitment of normal stromal and inflammatory cells that contribute to the growth and development of the neoplasm.

Genotoxic/DNA-Reactive Compounds

Genotoxic compounds interact with nuclear DNA of a target cell producing DNA damage that, if not repaired, is inherited in subsequent daughter cells. DNA-reactive carcinogens can be further subdivided according to whether they are active in their parent form (i.e., direct-acting chemicals that directly bind to DNA without being metabolized) and those that require metabolic activation (i.e., indirect-acting carcinogens that require metabolism in order to react with DNA).

Direct-Acting (Activation-Independent) Carcinogens— These carcinogens are highly reactive electrophilic molecules that can interact with and bind to nucleophiles such as cellular macromolecules including DNA. Some common electrophilic species are shown in Fig. 8–2. Generally, chemicals containing these moieties frequently cause tumor formation at the site of chemical exposure.

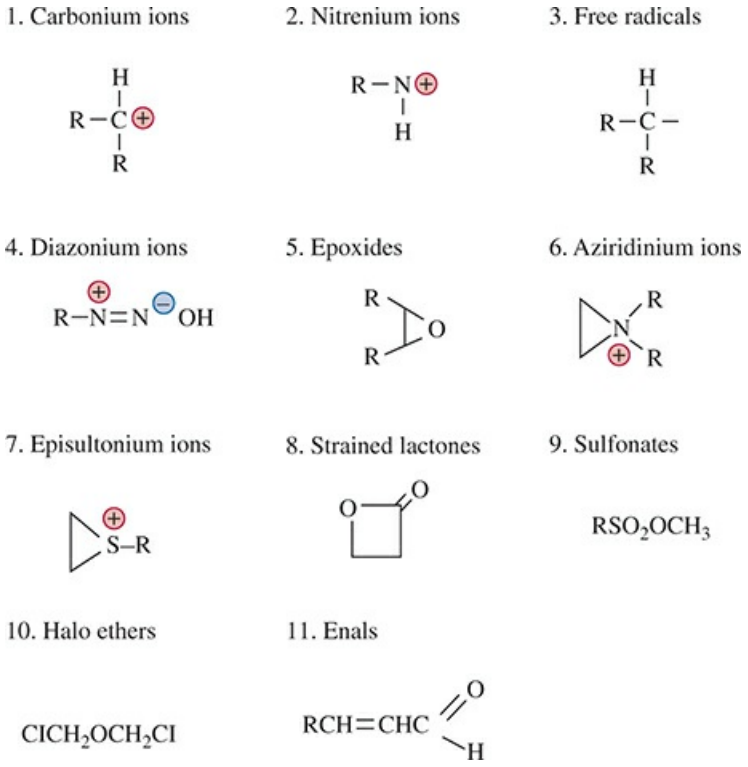

FIGURE 8–2 Structures of reactive electrophiles.

The relative carcinogenic strength of direct-acting carcinogens depends in part on the relative rates of interaction between the chemical and genomic DNA, as well as competing reactions with the chemical and other cellular nucleophiles. Chemical stability, transport, and membrane permeability determine the carcinogenic activity of the chemical. Direct-acting carcinogens are typically carcinogenic at multiple sites and in all species examined.

Indirect-Acting Genotoxic Carcinogens—The majority of DNA-reactive carcinogens are found as parent compounds, or procarcinogens that require subsequent metabolism to be carcinogenic. The terms procarcinogen, proximate carcinogen, and ultimate carcinogen have been coined to define the parent compound (procarcinogen) and its metabolite form, either

intermediate (proximate carcinogen) or final (ultimate carcinogen) that reacts with DNA. The ultimate form of the carcinogen is most likely the chemical species that result in mutation and neoplastic transformation. The ultimate form of carcinogenic chemicals may or may not be known, or there may be more than one ultimate carcinogenic metabolite depending on the metabolic pathway. Detoxification pathways may also inactivate the carcinogen. Indirect-acting genotoxic carcinogens usually produce their neoplastic effects at the target tissue where the metabolic activation of the chemical occurs.

Mutagenesis

Effects of mutations depend on when in the cell cycle the DNA adducts are formed, where the adducts are formed, and the type of repair process used in response to the damage. Mutagenesis may result from misread DNA through transitions and transversions, frame-shifting or broken DNA strands.

Damage by Alkylating Electrophiles

As noted, most chemical carcinogens require metabolic activation to exert a carcinogenic effect. The ultimate carcinogenic forms of these chemicals are frequently strong electrophiles (Fig. 8–2) that can readily form covalent adducts with nucleophilic targets. Generally, the stronger electrophiles display a greater range of nucleophilic targets (i.e., they can attack weak and strong nucleophiles), whereas weak electrophiles are only capable of alkylating strong nucleophiles (e.g., S: atoms in amino acids).

An important and abundant source of nucleophiles is contained not only in the DNA bases but also in the phosphodiester backbone (Fig. 8–3). Different electrophilic carcinogens will often display different preferences for nucleophilic sites in DNA and different spectra of damage.

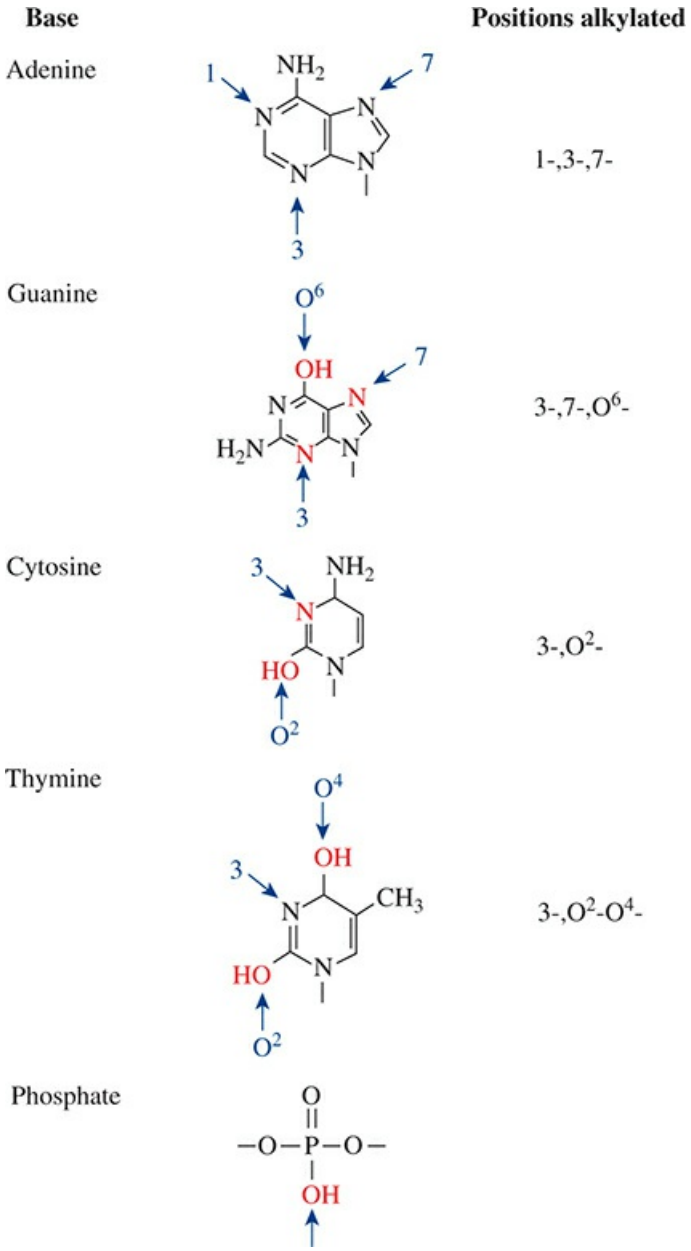

FIGURE 8–3 Examples of cellular nucleophiles and sites of possible adduct formation.

Another common modification to DNA is the hydroxylation of DNA bases. Oxidative DNA adducts have been identified in all four DNA bases (Fig. 8–4). The source of oxidative DNA damage is typically formed from free radical reactions that occur endogenously in the cell or from exogenous sources.

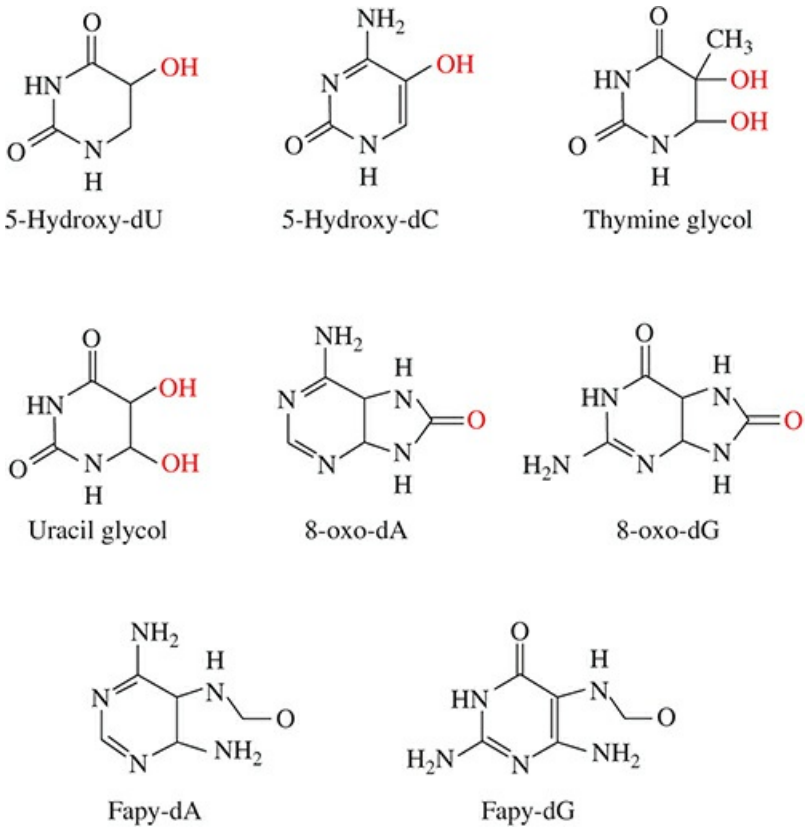

FIGURE 8–4 Structures of selected oxidative bases.

Although a large number of adducts can be formed following exposure to chemicals, whether a particular DNA adduct will result in mutation and participate in the carcinogenesis process is dependent in part on the persistence of the adduct through the process of DNA replication, which is also in part dependent upon DNA repair.

DNA Repair

Following the formation of a carcinogen–DNA adduct, the persistence of the adduct is a major determinant of the outcome. This persistence depends on the ability of the cell to repair the altered DNA. However, the presence of a DNA adduct is not sufficient for the carcinogenesis process to proceed. The relative rates or persistence of particular DNA adducts may be an important determinant of carcinogenicity. Differences in susceptibility to carcinogenesis are likely the result of various factors, including DNA replication within a tissue and repair of a DNA adduct. The development of cancer following exposure to chemical carcinogens is a relatively rare event because of a cell's ability to recognize and repair DNA. The DNA region containing the adduct is removed and a new patch of DNA is synthesized, using the opposite intact strand as a template. The new DNA segment is then spliced into the DNA molecule in place of the defective one. To be effective in restoring a cell to normal, repair of DNA must occur prior to cell division.

DNA Repair Mechanisms

Although cells possess mechanisms to repair many types of DNA damage, these are not always completely effective, and residual DNA damage can lead to the insertion of an incorrect base during DNA replication, followed by transcription and translation of the mutated templates, ultimately leading to the synthesis of altered protein. Mutations in an oncogene, tumor-suppressor gene or gene that controls the cell cycle, can result in a clonal cell population with a proliferative or survival advantage. The development of a tumor requires many such events, occurring over a long period of time, and for this reason human cancer induction often takes place within the context of chronic exposure to chemical carcinogens.

Cells have several mechanisms for repairing DNA damage. Repair of DNA damage does not always occur prior to cell replication, and repair of DNA damage by some chemicals is relatively inefficient. As such, exposure to chemicals can increase the probability of acquiring mutations that ultimately lead to cancer development.

Mismatch Repair of Single-Base Mispairs— Spontaneous mutations may occur through normal cellular DNA replication mistakes such as point mutations, a change in a single base pair in the DNA sequence, or a small insertion or deletion or a frame shift mutation of some modest size. Depurination is a fairly common occurrence and spontaneous event in mammals, and results in the formation of apurinic sites. All mammalian cells possess apurinic endonucleases that function to cut DNA near apurinic sites. The cut is then extended by exonucleases, and the resulting gap repaired by DNA polymerase and ligase.

Excision Repair— DNA regions containing chemically modified bases, or DNA chemical adducts, are typically repaired by excision repair processes. Proteins that slide along the surface of a double-stranded DNA molecule recognize the irregularities in the shape of the double helix and affect the repair of the lesion.

Homologous Recombination and Nonhomologous End-Joining Repair of DNA—A cell that has double-strand breaks can be repaired by joining the free DNA ends. The joining of broken ends from different chromosomes, however, will lead to the translocation of DNA pieces from one chromosome to another. Translocations have the potential to enable abnormal cell growth by placing a proto-oncogene next to, and, therefore, under the control of another gene promoter. Homologous recombination is one of two mechanisms responsible for the repair of double-strand breaks. In this process, the double-strand break on one chromosome is repaired using the information on the homologous, intact chromosome.

The predominant mechanism for double-stranded DNA repair in multicellular organisms is nonhomologous repair and involves rejoining the ends of the two DNA molecules. Although this process yields a continuous double-stranded molecule, several base pairs are lost at the joining point. This type of deletion may produce a possible mutagenic coding change.

Classes of Genotoxic Carcinogens

Polyaromatic Hydrocarbons—PAHs are found at high levels in charcoal-broiled foods, cigarette smoke, and in diesel exhaust.

Alkylating Agents—Although some alkylating chemicals are direct-acting genotoxic chemicals,

many require metabolic activation to produce electrophilic metabolites that can react with DNA. Alkylating compounds readily react with DNA at more than 12 sites. The N^7 position of guanine and the N^3 position of adenine are the most reactive sites for alkylating chemicals to bind to DNA.

Aromatic Amines and Amides—Aromatic amines and amides encompass a class of chemicals with varied structures. Classically, exposure to these chemicals was through use in the dye industry. Today, exposure still occurs through cigarette smoke and environmental sources. Phase I cytochrome P450-mediated reactions yield hydroxylated metabolites that are often associated with adduct formation in proteins and DNA and produce liver and bladder carcinogenicity.

Classes and Mode of Action of Non-genotoxic (Epigenetic) Carcinogens

Organ and tissue targets induced by non-genotoxic carcinogens are often in tissues where a significant incidence of background, spontaneous tumors is seen in the animal model. Prolonged exposure to relatively high levels of chemicals is usually necessary for tumor production by this mechanism. Examples include chemicals that function via sustained cytotoxicity, receptor-mediated (e.g., CAR, peroxisome proliferator–activated receptor alpha [PPARα], aryl hydrocarbon receptor [AhR]) effects, hormonal perturbation, induction of oxidative stress and modulation of methylation status (Table 8–4).

TABLE 8–4 Proposed Modes of Action for Selected Non-genotoxic Chemical Carcinogens

Mode of Actoin	Example
Cytotoxicity	Chloroform Melamine
α2u-Globulin binding	D-Limonene 1,4-Dichlorobenzene
Receptor-mediated	
CAR	Phenobarbital Toxaphene
PPARa	Trichloroethylene Perchloroethylene Diethylhexylphthalate Fibrates (e.g., clofibrate)
AhR	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) Polychlorinated biphenyls (PCBs) Polybrominated biphenyls (PBBs)
Hormonal	Biogenic amines Steroid and peptide hormones Diethylstilbestrol (DES) Phytoestrogens (Bisphenol-A) Tamoxifen Phenobarbital
Altered methylation	Phenobarbital Choline deficiency Diethanolamine
Immunosuppression	Atrazine Bisphenol A Phthalates
Oxidative stress inducers	Ethanol TCDD Lindane Dieldrin Acrylonitrile

Cytotoxicity—Chemicals that function through this mechanism produce sustained cell death that is accompanied by persistent regenerative growth, resulting in the potential for the acquisition of "spontaneous" DNA mutations and allowing mutated cells to accumulate and proliferate. This process then gives rise to preneoplastic focal lesions that upon further expansion can lead to tumor formation. The induction of cytotoxicity may be observed with many carcinogens both genotoxic and non-genotoxic when high toxic exposures occur. Thus, the induction of cytotoxicity with compensatory hyperplasia may contribute to the observed tumorigenicity of many carcinogenic chemicals at high-dose levels.

Receptor-Mediated

CAR Receptor-Mediated (Phenobarbital-Like Carcinogens)— Phenobarbital is a commonly studied non–DNA-reactive compound that is known to cause tumors by a non-genotoxic mechanism involving liver hyperplasia. Induction of Cyp2b is mediated by activation of the constitutive androstane receptor (CAR), a member of the nuclear receptor family. Other CAR-dependent phenobarbital responses that are critical for tumor formation include increased cell proliferation, inhibition of apoptosis, inhibition of gap junctional communication, hypertrophy, and development of preneoplastic focal lesions in the liver. CAR can be activated by both direct ligand binding and ligand-independent (indirect) mechanisms. Phenobarbital activates CAR indirectly by inhibiting epidermal growth factor receptor (EGFR) binding to EGF and preventing downstream events leading to increased gene expression.

Peroxisome Proliferator–Activated Receptor α—A wide array of chemicals increase the number and volume of peroxisomes in the cytoplasm of cells. These peroxisome proliferators include lipid-lowering fibrate drugs (e.g., ciprofibrate, clofibrate), herbicides (e.g., 2,4-dichlorophenoxyacetic acid), chlorinated solvents (e.g., trichloroethylene and perchloroethylene), perfluorinated compounds (e.g., perfluorooctane sulfonate and perfluorooctanoic acid), plasticizers (e.g., diethylhexylphthalate and other phthalates), and natural products. PPARα is highly expressed in cells that have active fatty acid oxidation capacity (e.g., hepatocytes, cardiomyocytes, enterocytes). PPARα plays a central role in lipid metabolism and acts as a transcription factor to modulate gene expression following ligand activation.

Aryl Hydrocarbon Receptor—Activators of the AhR including TCDD and selected polychlorinated- and brominated-biphenyl (PCBs and PBBs) compounds bind to the AhR, the ligand bound AhR translocates to the nucleus, dimerizes with the Ah receptor nuclear translocator (ARNT), and binds to aryl hydrocarbon response elements (AREs also known as dioxin response elements [DRE] and xenobiotic response elements [XRE]). AhR-ARNT–dependent genes include cytochrome P450 family members, NAD(P)H:quinone oxidoreductase, a cytosolic aldehyde dehydrogenase 3, a UDP-glucuronosyltransferase, and a glutathione transferase.

Hormonal Mode of Action—Hormonally active chemicals include steroids and peptide hormones that produce tissue-specific changes through interaction with a receptor. Many non–DNA-reactive chemicals induce neoplasia through receptor-mediated mechanisms, and/or perturbation of hormonal balance. Trophic hormones induce cell proliferation at their target organs, which may lead to tumor development when the mechanisms of hormonal control are disrupted.

Estrogenic Agents—Estrogen and estrogen-mimetic chemicals can induce tumors in estrogen-dependent tissue. Epidemiological data on breast and ovarian cancer indicate that individuals with higher circulating estrogen levels and those with exposure to the potent estrogenic chemical diethylstilbestrol (DES) are at increased risk for cancer development. DES has been causally associated with the higher incidence of adenocarcinomas of the vagina and cervix in daughters of women treated with the hormone during pregnancy. The mechanism of action for DES is believed to function through its ability to induce changes in the cell cycle and microtubule function, which may lead to an abnormal number of chromosomes in a cell.

Estrogenic substances in plants (phytoestrogens) include genistein, daidzein, glycitein, equol, and their metabolites found in soy products and various lignan derivatives. In addition, a number of environmental nonsteroidal synthetic compounds demonstrate apparent estrogenic activity

(e.g., nonyl-phenol, bisphenol-A, chlorinated hydrocarbons). The potential for these chemicals to induce cancer in humans remains a highly debated topic.

***Thyroid Hormone*—**A reduction of thyroid hormone concentrations (T4 and/or T3) and increased thyroid-stimulating hormone (TSH) have been shown to induce neoplasia in the rodent thyroid. TSH increases proliferative activity in the thyroid. After chronic treatment chemical-induced increases in TSH lead to follicular cell hypertrophy, hyperplasia, and eventually neoplasia.

DNA Methylation and Carcinogenesis—Post-DNA synthetic methylation of the five position on cytosine (5-methylcytosine; 5mC) is a naturally occurring modification to DNA in higher eukaryotes that influence gene expression. Under normal conditions, DNA is methylated symmetrically on both strands. Immediately following DNA replication, the newly synthesized double-stranded DNA contains hemimethylated sites that signal DNA maintenance methylases to transfer methyl groups from *S*-adenosylmethionine to cytosine residues on the new DNA strand. The degree of methylation within a gene inversely correlates with the expression of that gene; hypermethylation of genes is associated with gene silencing, whereas hypomethylation results in enhanced expression of genes. Several chemical carcinogens are known to modify DNA methylation, methyltransferase activity, and chromosomal structure. During carcinogenesis, both hypomethylation and hypermethylation of the genome have been observed.

Reactive oxygen species can also modify DNA methylation through interfering with the ability of methyltransferases to interact with DNA resulting in changes in DNA methylation profiles. Hypomethylation of CpG sites allows the expression of normally quiescent genes. Also, the abnormal methylation pattern observed in cells transformed by chemical oxidants may contribute to an overall aberrant gene expression and promote the tumor process.

MicroRNA and Chemical Carcinogenesis—MicroRNAs (miRNAs) are small noncoding RNAs, usually consisting of 21 to 25 nucleotides, that typically regulate gene expression through repression or degradation of targeted messenger RNAs (mRNAs), controlling genes involved in multiple cellular processes. Dysregulation of mRNAs has been implicated in the development of initiation, promotion, and progression of cancer. Many miRNAs function as antitumor agents (or tumor suppressor genes), others behave as oncogenes, commonly known as oncomiRNAs (Fig. 8–5). The carcinogenic effect of a carcinogen can be dependent on the balance between these two groups of miRNAs. The alterations in miRNAs expression following exposure to carcinogens are usually tissue and chemical specific. The miRNA profiles and miRNAs specific to carcinogen exposure may potentially be used as indicators of the carcinogenic process, biomarkers for carcinogen exposure, and for the identification of potential chemical carcinogens.

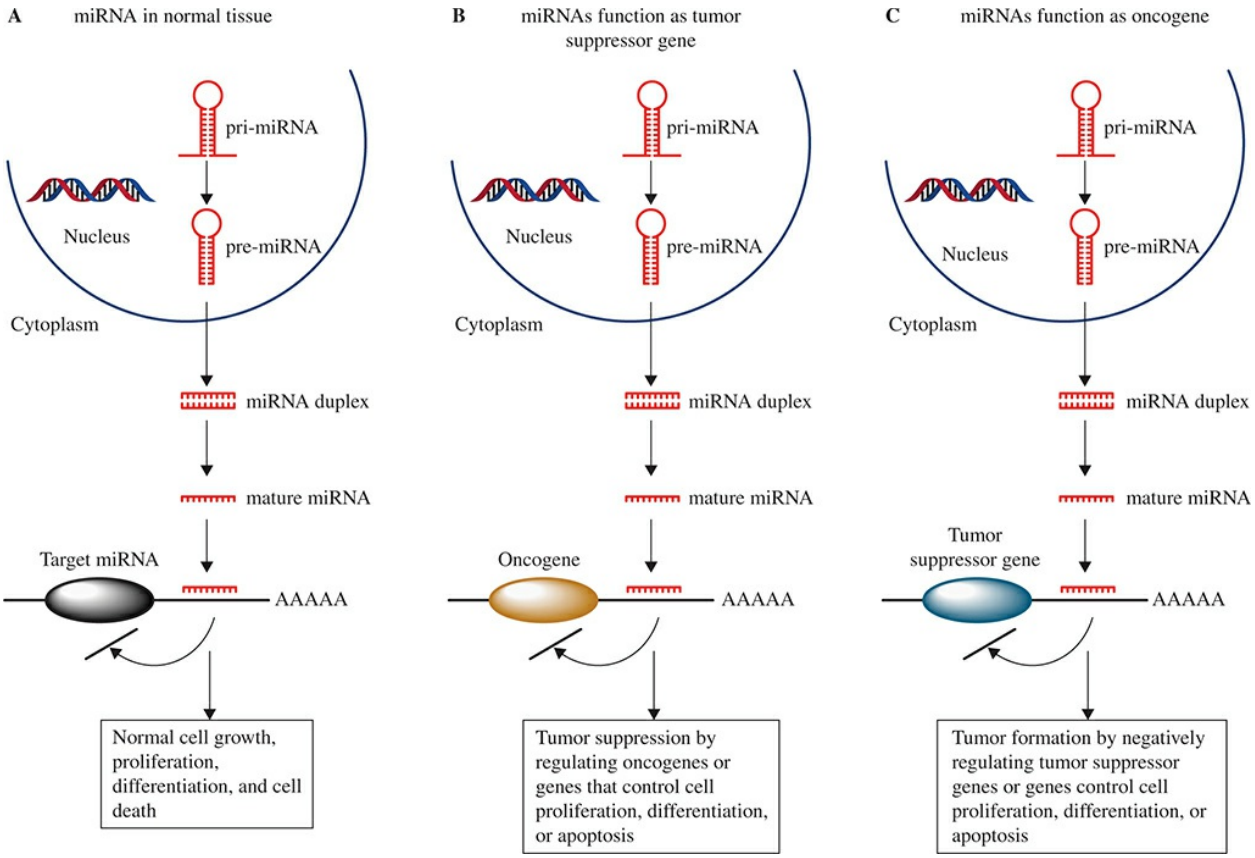

FIGURE 8–5 Role of microRNA in carcinogenesis.

Immunosuppression and Carcinogenesis—Cancer immune surveillance is an essential host protection mechanism that inhibits carcinogenesis by identifying and removing early preneoplastic cells from the body and to maintain cellular homeostasis. Evading immune recognition and destruction is one hallmark of cancer. Immune suppression has also been proposed as a possible mode of action of non-genotoxic carcinogens.

Prolonged inflammation can destroy immune cells leading to immune suppression. This mechanism of immunosuppression has been suggested to be involved in the carcinogenesis of environmental immune disruptors such as bisphenol A, atrazine, phthalates, and PBDEs.

Oxidative Stress and Chemical Carcinogenesis— Reactive oxygen species, including the superoxide anion (O_2•–), hydroperoxyl radical (HO_2•), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\bullet}OH$), produced from both endogenous and exogenous sources are typically counterbalanced by antioxidants. Antioxidant defenses are both enzymatic (e.g., superoxide dismutase, glutathione peroxidase, and catalase) and nonenzymatic (e.g., vitamin E, vitamin C, β-carotene, and glutathione). Endogenous sources of reactive oxygen species include oxidative phosphorylation, P450 metabolism, peroxisomes, and inflammatory cell activation. An increase in H_2O_2 production often accompanies exposure to chemicals that stimulate the number and activity of peroxisomes. Several agents that induce cancer also cause reactive oxygen species formation, oxidative stress, and frequently oxidative damage to the cell.

Oxidative DNA Damage and Carcinogenesis—Reactive oxygen species left unbalanced by

antioxidants can result in damage to cellular macromolecules. Reactive oxygen species can produce single- or double-stranded DNA breaks, purine, pyrimidine, or deoxyribose modifications, and DNA cross-links. Persistent DNA damage can result in either arrest or induce transcription, induce signal transduction pathways, replication errors, and genomic instability: these events are potentially involved in carcinogenesis.

Oxidative damage to mitochondrial DNA and mutations in mitochondrial DNA have been identified in several cancers. Compared to nuclear DNA, the mitochondrial genome is relatively susceptible to oxidative base damage owing to (1) close proximity to the electron transport system, a major source of reactive oxygen species; (2) lack of mitochondrial DNA protection by histones; and (3) limited DNA repair capacity in the mitochondria.

Aside from oxidized nucleic acids, oxygen radicals can also damage cellular biomembranes resulting in lipid peroxidation. Peroxidation of biomembranes generates a variety of products including reactive electrophiles, such as epoxides and aldehydes, and malondialdehyde (MDA).

Oxidative Stress and Cell Growth Regulation—Reactive oxygen species production and oxidative stress can affect both cell proliferation and apoptosis. High concentrations of reactive oxygen species can initiate apoptosis, whereas low levels influence signal transduction pathways and alter gene expression. By increasing cellular levels of oxidants, many xenobiotics alter gene expression through activation of cAMP-mediated cascades, calcium-calmodulin pathways, and transcription factors such as AP-1, Nrf2, Hif1, and NF-κB as well as signaling through mitogen-activated protein (MAP) kinases (extracellular signal-regulated kinases [ERK], c-Jun N-terminal kinases [JNK], and the p38 kinases). Activation of these signaling cascades by reactive oxygen species induced by chemical carcinogens ultimately leads to altered gene expression for genes affecting proliferation, differentiation, apoptosis, and others.

Gap Junctional Intercellular Communication and Carcinogenesis

Cells within an organism communicate variously including through gap junctions, which are aggregates of connexin proteins that form a conduit between two adjacent cells. Gap junctional intercellular communication is important in the regulation of cell growth and cell death, in part, through the ability to exchange small molecules (<1 kDa) between cells. If cell communication is blocked between tumor and normal cells, the exchange of growth inhibitory signals from normal cells to initiated cells is prevented, potentially allowing unregulated growth and clonal expansion of initiated cell populations.

Inorganic Carcinogens

Metals—Several metals exhibit carcinogenicity in experimental animals and/or exposed humans, including arsenic, beryllium, cadmium, chromium, nickel, and lead. The carcinogenic manifestations vary and include increased risk for skin, lung, and liver tumors. Additional discussion of selected metals is in Chapter 23.

Modifiers of Chemical Carcinogenic Effects

Genetic and environmental factors have a significant impact on the way in which individuals and/or organisms respond to carcinogen exposure. As with most genes, enzymes that metabolize carcinogens are expressed in a tissue-specific manner, and the enzymatic profile can vary with tissue or cell type or display differential localization within cells. Further, differential expression of carcinogen metabolizing enzymes among species may help explain the differential responses to chemical carcinogens across species.

Polymorphisms in Carcinogen Metabolism and DNA Repair

Genetic polymorphisms where a gene has more than one allele arise from human genetic variability. The human genome project noted that base variations occurred approximately once in every 1000 base pairs. Therefore, there may be over one million genetic variations between any two individuals. A single nucleotide polymorphism (SNP) is a variant in DNA sequence found in greater than 1% of the population. By definition, changes in DNA sequence go from mutation to polymorphism when a unique genotype is seen in over 1% of the population. Over 3 million candidate SNPs have been identified to date with up to 10 million being estimated to be present within the human genome. Genetic polymorphisms may account for the susceptibility of some individuals to certain cancers.

Proto-Oncogenes and Tumor-Suppressor Genes

Proto-oncogenes and tumor-suppressor genes encode a wide array of proteins that function to control cell growth and proliferation. Common characteristics of oncogenes and tumor-suppressor genes are shown in Table 8–5. Mutations in both oncogenes and tumor-suppressor genes contribute to the progressive development of human cancers. Accumulated damage to multiple oncogenes and/or tumor-suppressor genes can result in altered cell proliferation, differentiation, and/or survival of cancer cells.

TABLE 8–5 Characteristics of Proto-Oncogenes, Cellular Oncogenes, and Tumor Suppressor Genes

Proto-Oncogenes	Oncogenes	Tumor Suppressor Genes
Dominant	Dominant	Recessive
Broad tissue specificity for cancer development	Broad tissue specificity for cancer development	Considerable tissue specificity for cancer development
Germline inheritance rarely involved in cancer development	Germline inheritance rarely involved in cancer development	Germline inheritance frequently involved in cancer development
Analogous to certain viral oncogenes	No known analogues in oncogenic viruses	No known analogues in oncogenic viruses
Somatic mutations activated during all stages of carcinogenesis	Somatic mutations activated during all stages of carcinogenesis	Germline mutations may initiate, but mutation to neoplasia occurs only during progression stage

Retroviruses—The *Rous sarcoma virus* (RSV) can produce sarcomas. The genome of RSV and other retroviruses consists of two identical copies of mRNA, which is then reverse transcribed into DNA and incorporated into the host-cell genome. Oncogenic transforming viruses such as RSV contain the v-*src* gene, a gene required for cancer induction. Normal cells contain a gene closely related to v-*src* in RSV. This discovery highlighted that cancer may be induced by the action of normal, or nearly normal, genes.

DNA Viruses—Six major classes of DNA tumor viruses have been identified: simian virus 40 (SV40), polyoma virus, hepatitis B virus, papilloma viruses, adenoviruses, herpes viruses, and poxviruses. Unlike retroviral oncogenes, which are derived from normal cellular genes and have no function for the virus, the known oncogenes of DNA viruses are integral parts of the viral genome required for viral replication. Infection by small DNA viruses is lethal to most non-host animal cells; however, a small proportion integrates the viral DNA into the host-cell genome. The cells that survive infection become permanently transformed due to the presence of one or more oncogene in the viral DNA. Papilloma viruses can infect and cause tumors in humans.

Proto-Oncogenes—An oncogene encodes a protein that is capable of transforming cells in culture or inducing cancer in animals. Of the known oncogenes, the majority appear to have been derived from normal genes (i.e., proto-oncogenes) and are involved in cell signaling cascades. Because most proto-oncogenes are essential for maintaining viability, they are highly conserved. Activation of proto-oncogenes arises through mutational events occurring within proto-oncogenes. Numerous chemical carcinogens are capable of inducing mutations in proto-oncogenes. Oncogene products can operate at levels of signaling cascades, including ligand,

receptor, second messengers, and transcription factor stages of transduction.

Tumor-Suppressor Genes

***Retinoblastoma (Rb) Gene*—**In contrast to oncogenes, the proteins encoded by most tumor-suppressor genes act as inhibitors of cell proliferation or cell survival (Table 8–6). The prototype tumor-suppressor gene, Rb, was identified by studies of inheritance of retinoblastoma. Loss or mutational inactivation of Rb contributes to the development of many human cancers. In its unphosphorylated form, Rb binds to the E2 factor (*E2F*) transcription factor preventing E2F-mediated transcriptional activation of several genes whose products are required for DNA synthesis. When Rb becomes phosphorylated during late G_1 and dissociates from E2F, E2F induces synthesis of DNA replication enzymes resulting in a commitment into the cell cycle.

TABLE 8–6 Examples of Tumor Suppressor Genes and Cancer Association

Tumor Suppressor	Disorder	Neoplasm
Rb1	Retinoblastoma	Small-cell lung carcinoma
p53	Li–Fraumeni syndrome	Breast, colon, lung cancers
BRCA1	Unknown	Breast carcinoma
WT-1	Wilms tumor	Lung cancer
p16	Unknown	Melanoma

***p53 Gene*—**The p53 protein is a tumor-suppressor that is essential for checkpoint control and arrests the cell cycle in cells with damaged DNA in G_1. Cells with functional p53 arrest in G_1 when exposed to DNA-damaging agents such as irradiation, whereas cells lacking functional p53 are unable to block the cell cycle. p53 is activated by a wide array of stressors including UV light, γ irradiation, heat, and many carcinogens. In most cells, accumulation of p53 also leads to induction of proteins that promote apoptosis, thereby preventing proliferation of cells that are likely to accumulate multiple mutations. When the p53 checkpoint control does not operate properly, damaged DNA can replicate, producing mutations and DNA rearrangements that contribute to the development of transformed cells.

***BRCA1 Gene*—**Genetic analysis of breast tumors has revealed a hereditary predisposition for breast cancer linked to BRCA1 (breast cancer gene 1), a tumor-suppressor gene. Mutation of a single BRCA1 allele results in a 60% probability of developing breast cancer by age 50. Germ line mutations lead to loss of function of the BRCA1 gene, perhaps by acting as a transcription factor through binding at a zinc finger domain. However, no mutations have been observed in sporadic breast cancers, suggesting that BRCA1 gene silencing may occur through nonmutational mechanisms.

Wilms Tumor Gene (WT1)—Wilms tumor occurs in the developing kidney at a rate of approximately one per 10,000 children. The WT1 gene is likely responsible for tumor development and is thought to function as a transcription factor.

p16 Gene—The proteins that function as cyclin-kinase inhibitors are important in cell cycle regulation. Mutations, especially deletions of the p16 gene, that inactivate the ability of p16 to inhibit cyclin D–dependent kinase activity are common in several human cancers including a high percentage of melanomas. Loss of p16 would mimic cyclin D1 overexpression, leading to Rb hyperphosphorylation and release of active E2F transcription factor. Thus, p16 normally acts as a tumor suppressor. As with the BRCA1 gene, relatively few mutations have been found in this gene, and some researchers have speculated that epigenetic mechanisms such as gene silencing by DNA methylation may occur during tumorigenesis.

Hormesis, Dose Response, and Carcinogenesis

Hormesis is a U, J, or inverted U-shaped dose–response with low-dose exposures often resulting in beneficial rather than harmful effects. Adaptive mechanisms have been proposed to explain the hormetic effects observed by chemical carcinogens. Adaptive responses usually involve actions of the chemical on cellular signaling pathways that lead to changes in gene expression, resulting in enhanced detoxification and excretion of the chemical as well as preserving the cell cycle and programmed cell death. Following very low doses of chemicals, the upregulation of these mechanisms is proposed to overcompensate for cell injury such that a reduction in tumor promotion and/or tumor development is seen, which would explain the U- or J-shaped response curves obtained following carcinogen exposure. A common feature of chemical carcinogens for which hormetic effects have been proposed is the formation of reactive oxygen species and the induction of cytochrome P450 isoenzymes.

Chemoprevention

The study of chemicals that prevent, inhibit, or slow down the process of cancer is referred to as chemoprevention. Many chemicals, including drugs, antioxidants, foodstuffs, and vitamins, inhibit or retard components of the cancer process in both in vitro and in vivo models. A basic assumption in chemoprevention is that treating early stages of the malignant process will halt or delay the progression to neoplasia. Cancer chemopreventive chemicals may function as inhibitors of carcinogen formation, blocking agents, and/or as suppressing agents. Blocking agents serve to prevent the metabolic activation of genotoxic or non-genotoxic carcinogens by either inhibiting their metabolism or enhancing detoxification mechanisms. Chemopreventive chemicals increase tissue resistance function in the target tissue, usually an early preneoplastic lesion by increasing tissue maturation and/or decreasing cell proliferation. Suppressing chemicals induce tissue differentiation, may counteract oncogenes, enhance tumor-suppressor gene activities, inhibit proliferation of premalignant cells, or modify the effect of the carcinogen on the target tissue.

ASSESSING CARCINOGENICITY OF

CHEMICALS

Numerous in vivo and in vitro experimental systems are available to assess potential carcinogenicity of chemicals. The types of tests to identify chemicals with carcinogenic potential can be classified into general categories, based on the duration required to conduct the test. Short-term tests are typically of the duration of days to a few weeks, intermediate-term tests last from weeks up to a year, whereas chronic long-term tests usually encompass 6 months to 2 years. These bioassays use bacterial and mammalian targets.

Short-Term Tests for Mutagenicity

Short-term tests for mutagenicity were developed to identify potentially carcinogenic chemicals based on their ability to induce mutations in DNA. A variety of in vivo and in vitro short-term tests are available. Most of these tests quantify the mutagenicity of chemicals as a surrogate for carcinogenicity. Therefore, although they are usually very predictive of indirect (if a metabolic source is provided) and direct acting agents, these tests routinely fail to detect non-genotoxic carcinogens.

In Vitro Gene Mutation Assays—The most widely used short-term test is the Ames assay. *Salmonella typhimurium* strains, deficient in DNA repair and unable to synthesize histidine, are treated with several doses of the test compound. In the presence of a mutagenic chemical, the defective histidine gene can be mutated back to a functional state (*back mutation*), resulting in a restoration of bacterial growth in medium lacking histidine. The mouse lymphoma assay is used to determine whether a chemical is capable of inducing mutation in eukaryotic cells. The ability of cells in culture to acquire resistance to trifluorothymidine (the result of forward mutation at the thymidine kinase locus) is quantified. The Chinese hamster ovary (CHO) test is commonly used to assess the potential mutagenicity of chemicals with the hypoxanthine–guanine phosphoribosyltransferase (HGPRT) gene as the endpoint.

In Vivo Gene Mutation Assays—In vivo tests have advantages over the in vitro test systems in that they take into account whole animal processes such as absorption, tissue distribution, metabolism, and excretion of chemicals and their metabolites. The commonly used in vivo models include transgenic rodent mutation assay systems based on the genes of the *lac* operon, (MutaMouse, Big Blue, and Pig-a gene mutation assay). Following exposure of mice to test chemicals, high-molecular-weight DNA isolated from the tissue under investigation is analyzed for mutations. The ratio of mutants to the total population provides a mutation frequency for each chemical and each organ tested. As with the other short-term tests that have been discussed, it is unlikely that in vivo genotoxicity test systems will identify non-genotoxic/non–DNA-reactive compounds.

The Pig-a gene mutation assay is primarily performed in rats and is based on the X-linked Pig-a gene (phosphatidylinositol *N*-acetylglucosaminyltransferase, subunit A), which is involved in the production of glycosylphosphatidylinositol (GPI) anchor proteins on the cell surface. Currently, the assay is optimized for measuring the Pig-a mutant phenotype in peripheral blood erythrocytes by quantification of CD59-negative reticulocytes and red blood cells. The Pig-a gene is highly conserved in humans, rats, mice, and monkeys, allowing comparison of mutation

induction across multiple species.

Chromosomal Alterations—Chromosomal alterations are quite common in malignant neoplasms. Both in vivo and in vitro assays are available. To assess induction of chromosomal alterations, cells are harvested in their first mitotic division after the initiation of chemical exposure. Cells are stained with Giemsa and scored for completeness of karyotype (21 ± 2 chromosomes). The classes of aberrations recorded include breaks and terminal deletions, rearrangements and translocations, as well as despiralized chromosomes.

Sister chromatid exchanges (SCEs) are a measure of DNA damage and increased levels of DNA damage, events that are associated with mutation induction and cancer. SCEs reflect an interchange of DNA between different chromatids at homologous loci within a replicating chromosome. Second-division metaphase cells are scored to determine the frequency of SCE/cell for each dose level. Disruption of the DNA replication process or damage to chromosomes by chemicals can alter the genetic material distributed to either of the two daughter nuclei. When this occurs, the genetic material that is not incorporated into a new nucleus may form its own "micronucleus," which is clearly visible with a microscope. For this assay, animals are exposed to chemicals and the frequency of micronucleated cells is determined at some specified time after treatment.

***DNA Damage*—**Primary DNA damage represents possible premutational events that can be detected, either directly or indirectly, using mammalian cells in culture or using rodent tissue. Unscheduled DNA synthesis (UDS) is a commonly used assay that measures the ability of a test article to induce DNA lesions by quantifying the increase in DNA repair. Among the available techniques is the measurement of DNA strand that breaks both in vivo and in vitro.

Short-Term Tests: Transformation Assays—Various in vitro test methods have been developed to assess the carcinogenic potential of chemicals. The C3H/10T½ cell line has been widely used for the transformation assay. It was originally derived from fibroblasts taken from the prostate of a C3H mouse embryo. The cells are approximately tetraploid but the chromosome number in the cells varies widely. As such, these cells are chromosomally abnormal and have already passed through some of the stages that might be involved in the production of a cancerous cell. These cells will stop growing when their density is sufficiently high (contact growth inhibition). However, the contact inhibition can fail, resulting in cell piling that forms a transformed colony. Following exposure to xenobiotics, this assay assesses carcinogenic potential based on the percentage of colonies that are transformed.

The most frequently used endpoint for cell transformation is morphological transformation of mammalian cell fibroblasts in culture. Transformation assays using Syrian hamster embryo (SHE) cells are available for the assessment of the carcinogenic potential of chemicals. The SHE cell assay, a diploid cell transformation assay, measures carcinogenic potential of xenobiotics by assessing transformed colonies based on morphological criterion.

Chronic Testing for Carcinogenicity

***Chronic (Two Year) Bioassay*—**Two-year studies in laboratory rodents remain the primary method by which chemicals or physical agents are identified as having the potential to be hazardous to humans. In the chronic bioassay, two or three dose levels of a test chemical (up to

the maximum tolerated dose) and a vehicle control are administered to 50 males and 50 females (mice and rats), beginning at 8 weeks of age, continuing throughout their lifespan. During the study, food consumption and bodyweight gain are monitored and the animals are observed clinically on a regular basis; at necropsy, the tumor number, location, and diagnosis for each animal are thoroughly assessed.

Organ-Specific Bioassays and Multistage Animal Models—Many tissue-specific bioassays were developed to produce a sensitive and reliable assay that could be conducted in a time frame shorter in duration than the two-year chronic bioassay. These assays are commonly used to detect carcinogenic activity of chemicals in various target organs.

Carcinogenicity Testing in Liver—The liver represents a major target organ for chemical carcinogens. It has been estimated that nearly half of the chemicals tested in the two-year chronic bioassay by the National Toxicology Program showed an increased incidence of liver cancer. Liver carcinogenesis assays have been developed to study and distinguish chemicals that affect the initiation or promotion stage of hepatocarcinogenesis.

Carcinogenicity Testing in Skin—The mouse skin model is one of the most extensively studied and used models for understanding multistage carcinogenesis. This model exploits many of the unique properties of mouse skin, one major advantage being that the development of neoplasia can be followed visually. In addition, the number and relative size of papillomas and carcinomas can be quantified as the tumors progress. Both initiating and promoting activities of chemical carcinogens can be assessed using this model. Grossly, initiated cells of the skin appear identical to normal skin. Because terminally differentiated cells in skin are no longer capable of undergoing cell division, only initiated cells retain their proliferative capacity and thus represent the cell populations that give rise to tumors. Upon repeated application of tumor promoters, selective clonal expansion of initiated keratinocytes occurs, resulting in skin papillomas, which over time can progress to carcinomas.

Carcinogenicity Testing in Lung—Strain A mice are genetically susceptible to the development of lung tumors, with lung tumors being observed in control animals as early as 3 to 4 weeks of age with a steady increase to nearly 100% by 24 months of age. In this model, carcinogenicity is typically assessed as an acceleration of tumor development rather than an increase in tumor incidence. The strain A mouse lung tumor assay is sensitive to chemicals, such as PAHs, nitrosamines, nitrosoureas, carbamates, aflatoxin, certain metals, and hydrazines.

Carcinogenicity Testing in Other Organs—Test systems are available to examine the ability of a chemical to promote neoplastic development in the kidney, bladder, pancreas, stomach, colon, small intestine, and oral cavity. These models vary in the initiating carcinogen used, and frequency, duration, and site of application, as well as duration of promoting chemical exposure.

Transgenic Animals in Carcinogenicity Assessment

Animal models with genetic alterations that invoke a susceptibility to carcinogenesis by chemicals include the Tg.AC and rasH2 transgenic mice, and $p53^{+/-}$ and $XPA^{-/-}$ knockout mice. Transgenic animal models as an alternative to the two-year chronic bioassay may be useful as screening models for assessment of chemical carcinogenicity; however, they do not provide

definitive proof of potential human carcinogenicity. Coupled with information on genotoxicity, particularly DNA reactivity, structure–activity relationships, results from other bioassays, and mechanistic investigations including toxicokinetics, metabolism, and mechanistic information, these alternate mouse models appear to be useful for assessing the carcinogenicity of chemicals.

New Approaches—A major concern for the correct evaluation of the safety of chemicals is the need to obtain reliable and pertinent scientific information on which to develop proper risk evaluation and assessment. Research during the last decade of the 20th century and the turn of the 21st century has dramatically increased our knowledge of the cellular and molecular pathways that contribute to the induction and prevention of cancer. Technological advances in high-throughput assays and computational science using cells, cell lines, and components of cells, as well as in silico computer models are being used to evaluate the effects of chemicals on biological processes and pathways that are important in toxicity (including cancer).

CHEMICAL CARCINOGENESIS IN HUMANS

Infectious agents, lifestyle, medical treatments, environmental, and occupational exposure account either directly or indirectly for the majority of cancers seen in humans. Of these, the component that contributes the most to human cancer induction and progression is lifestyle: tobacco use, alcohol use, and poor diet (Table 8–7). Tobacco usage either through smoking tobacco, chewing tobacco, or tobacco snuff-type products is estimated to be responsible for 25% to 40% of all human cancers. A particularly strong correlation between tobacco usage and mouth, larynx, lung, esophageal, and bladder cancer exists. The induction of pancreatic cancer also appears to have a linkage to tobacco use. Alcohol consumption also contributes anywhere from 2% to 4% of cancers of the esophagus, liver, and larynx.

TABLE 8–7 Carcinogenic Factors Associated with Lifestyle

Chemical(S)	Neoplasm(S)
Alcohol beverage	Esophagus, liver, oropharynx, and larynx
Aflatoxins	Liver
Betel chewing	Mouth
Dietary intake (fat, protein, calories)	Breast, colon, endometrium, gallbladder
Tobacco smoking	Mouth, pharynx, larynx, lung, esophagus, bladder

Poor diets whether high-fat, low-protein, high-calories or diets lacking in needed antioxidants and minerals account for anywhere from 10% to 70% of human cancers. There is substantial evidence that overnutrition either through excess calories and/or high-fat diets contributes to

breast, colon, and liver cancer in humans. The method of cooking may also influence the production of carcinogens produced in the cooking process. Carcinogenic heterocyclic amines and PAHs are formed during broiling and grilling of meat. Acrylamide, a suspected human carcinogen, has been found in fried foods at low concentrations.

Occupations that have been associated with the development of specific cancers are listed in Table 8–8.

TABLE 8–8 Occupational Human Carcinogens

Agent	Industrial Process	Neoplasms
Asbestos	Construction, asbestos mining	Peritoneum, bronchus
Arsenic	Mining and smelting	Skin, bronchus, liver
Alkylating agents (mechlorethamine hydrochloride and bis[chloromethyl] ether)	Chemical manufacturing	Bronchus
Benzene	Chemical manufacturing	Bone marrow
Benzidine, β-naphthylamine	Dye and textile	Urinary bladder
Chromium and chromates	Tanning, pigment making	Nasal sinus, bronchus
Nickel	Nickel refining	Nasal sinus, bronchus
Polynuclear aromatic hydrocarbons	Steel making, roofing, chimney cleaning	Skin, scrotum, bronchus
Vinyl chloride monomer	Chemical manufacturing	Liver
Wood dust	Cabinet making	Nasal sinus
Beryllium	Aircraft manufacturing, electronics	Bronchus
Cadmium	Smelting	Bronchus
Ethylene oxide	Production of hospital supplies	Bone marrow
Formaldehyde	Plastic, textile, and chemical	Nasal sinus, bronchus
Polychlorinated biphenyls	Electrical-equipment production and maintenance	Liver

A number of drugs and medical diagnostic tools have also been linked to the induction of human cancer (Table 8–9).

TABLE 8–9 Human Carcinogenic Chemicals Associated with Medical Therapy and Diagnosis

Chemical Or Drug	Associated Neoplasms
Alkylating agents (cyclophosphamide, melphalan)	Bladder, leukemia
Azathioprine	Lymphoma, reticulum cell sarcoma, skin, Kaposi sarcoma
Chloramphenicol	Leukemia
Diethylstilbestrol	Vagina (clear cell carcinoma)
Estrogens	Liver cell adenoma, endometrium, skin
Phenacetin	Renal pelvis (carcinoma)
Phenytoin	Lymphoma, neuroblastoma
Thorotrast	Liver (angiosarcoma)

EVALUATION OF CARCINOGENICITY IN HUMANS

The assessment and designation of a chemical or a mixture of chemicals as carcinogenic in humans is evaluated by various agencies worldwide. The evaluation usually encompasses epidemiological, experimental animal, and in vitro data utilizing assays as described earlier in this chapter. One of the first schemes for the classification of an agent's carcinogenicity was devised by the International Agency on Research in Cancer (IARC) (Table 8–10). The IARC approach assigns the chemical or mixture to one of five groupings based upon strength of evidence for the chemical's possible, probable, or definite carcinogenicity to humans. Similar classifications exist for the USEPA, the Food & Drug Administration, and the European Community (EC).

TABLE 8–10 IARC Classification of the Evaluation of Carcinogenicity for Human Beings

Group	Evidence
1. Agent is carcinogenic to humans	Human data strong Animal data strong
2A. Agent is probably carcinogenic to humans	Human epidemiology data suggestive Animal data positive
2B. Agent is possibly carcinogenic to humans	Human epidemiology data weak Animal data positive
3. Agent is not classifiable as to carcinogenicity to humans	Human and animal data inadequate
4. Agent is probably not carcinogenic to humans	Human and animal data negative

CONCLUSION

Cancer is a multistage process that involves initial mutational events followed by changes in gene expression leading to the selected clonal proliferation of the precancerous cell. Neoplasia appears to exhibit multiple characteristics including increased selective lesion growth (through sustained cell proliferation and/or resistance to apoptosis), the induction of angiogenesis, enabling replicative immortality, activation of factors that influence invasion and metastasis, evasion of normal growth suppression, modulation of energy metabolism, and the avoidance of attack by the immune system. The multistage nature and characteristics of the process have been extensively examined with regard to molecular, cellular, tissue, and organ events.

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QUESTIONS

1. There is evidence that certain dietary components are carcinogenic. Which of the following is NOT tabbed as a dietary carcinogen?
   a. excessive caloric intake.

b. excessive alcohol consumption.
c. aflatoxin B1 (a food contaminant).
d. insufficient caloric intake.
e. nitrites (found in some lunchmeats).

2. Which of the following statements regarding mechanisms of chemical carcinogenesis is FALSE?
   a. Procarcinogens require metabolism in order to exert their carcinogenic effect.
   b. Free radicals are highly reactive molecules that have a single, unpaired electron.
   c. DNA adducts interfere with the DNA replication machinery.
   d. Mutations in the DNA and failure to repair those mutations can be highly carcinogenic.
   e. Biological reduction of molecular oxygen is the only way free radicals can be formed.

3. In addition to being necessary for transcription to occur, which of the following transcription factors also plays a crucial role in nucleotide excision repair?
   a. TFIIA.
   b. TFIIB.
   c. TFIID.
   d. TFIIF.
   e. TFIIH.

4. Which of the following statements regarding DNA repair is true?
   a. Base excision repair requires the removal of a longer piece of DNA in comparison with nucleotide excision repair.
   b. The repair of double-stranded DNA breaks is more prone to error than is base excision repair.
   c. Dimerization of pyrimidines is repaired via base excision repair.
   d. Mismatch repair can only recognize normal nucleotides that are paired with a noncomplementary nucleotide.
   e. Nucleotide excision and base excision are tolerance mechanisms used to respond to DNA damage.

5. Which of the following statements is a characteristic of the initiation stage of carcinogenesis?
   a. Initiation is reversible in viable cells.
   b. The dose–response exhibits an easily measurable threshold.
   c. Cell division is required for the fixation of the process.
   d. All initiated cells survive over the lifespan of the organism.
   e. Spontaneous initiation of cells is a rare occurrence.

6. Tumor suppressor genes are mutated in a majority of cancers. Which of the following is NOT a characteristic of a tumor suppressor gene?
   a. A mutation in a tumor suppressor gene is dominant.
   b. Germ line inheritance of a mutated tumor suppressor gene is often involved with cancer

development.

c. There is considerable tissue specificity for cancer development.
d. The *p53* gene is a tumor suppressor gene that also acts as a transcription factor.
e. Mutations in tumor suppressor genes can result in loss of cell cycle control.

7. Which of the following molecules does NOT play an important role in cell cycle regulation?
   a. p53.
   b. cyclin-D.
   c. MAPK.
   d. MHC.
   e. E2F.

8. Which of the following environmental factors is proportionally responsible for the LEAST amount of cancer deaths?
   a. tobacco.
   b. infection.
   c. diet.
   d. sexual behavior.
   e. alcohol.

9. The evidence of the carcinogenicity of dietary intake is sufficient to include one's diet as associated with neoplasms of all of the following EXCEPT:
   a. colon.
   b. breast.
   c. pancreas.
   d. endometrium.
   e. gallbladder.

10. Which of the following is the correct definition of a complete carcinogen?
    a. a chemical capable only of initiating cells.
    b. a chemical possessing the ability of inducing cancer from normal cells, usually possessing properties of initiating, promoting, and progression agents.
    c. a chemical capable of converting an initiated cell or a cell in the stage of promotion to a potentially malignant cell.
    d. a chemical capable of causing the expansion of initiated cell clones.
    e. a chemical that will cause cancer 100% of the time that it is administered.

CHAPTER 9

Genetic Toxicology

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KEY POINTS

- Genetic toxicology assesses the effects of chemical and physical agents on the hereditary material (DNA) and on the genetic processes of living cells.
- *Oncogenes* are genes that stimulate the transformation of normal cells into cancer cells.
- Genetic toxicology assays serve to identify mutagens for purposes of hazard identification, and to characterize dose–response relationships and mutagenic mechanisms.
- A broad range of short-term assays for genetic toxicology serve to identify many mutagens and address the relationship between mutagens and cancer-causing agents.

INTRODUCTION

Genetic toxicology is the study of genetic damage to the hereditary material that results in genetic alterations, including mutagenicity, transmissible genetic alterations, and genotoxicity. Genotoxicity covers a broad spectrum of endpoints, including DNA damage such as DNA strand breaks and DNA adduct biomarkers (both pro-mutagenic and non-mutagenic), unscheduled DNA synthesis (UDS), and sister chromatid exchanges (SCEs). Genotoxicity also encompasses the mechanisms by which DNA damage occurs and resulting cellular responses. This chapter describes the field of genetic toxicology, the cellular pathways that counteract DNA damage on a daily basis, the use of genetic toxicology data in cancer and genetic risk assessments, the mechanisms underlying genetic toxicology assays, the assays that can be used for detecting genotoxic endpoints, the use of the same assays for better understanding of the mechanisms of mutagenesis, and new methods for the assessment of genetic alterations.

HEALTH IMPACT OF GENETIC ALTERATIONS

Mutations are permanent, transmissible alterations in DNA sequences that result in changes to the amount or structure of genetic material in the cell. Both small (i.e., one or a few base-pair changes of a single gene) and large scale (i.e., changes affecting multiple genes) DNA alterations can lead to mutations. Mutagenicity refers to the induction of mutations; substances that induce mutations are known as mutagens. Clastogenicity refers to the process of inducing chromosomal breaks, and clastogens are substances that induce chromosomal breakage, such as chromosomal

deletions and rearrangements. These events can lead to mutations provided they are not lethal to the cell harboring them. Substances that induce loss or gain of whole chromosome(s) are called aneugens, and aneugenicity arises through changes in the ploidy (i.e., aneuploidy) of the normal chromosome complement of the cell. The importance of mutations and chromosomal alterations for human health is evident from their roles in genetic disorders, including birth defects and cancer.

Somatic Cells

Somatic mutations arise in the genomes of all normal dividing and transformed cells as a result of endogenous and exogenous processes and stressors. An association between mutation and cancer has long been evident, and the average cancer genome contains about 10^3 to 10^4 point mutations, 10 to 10^2 small insertions or deletions, and 1 to 10 large-scale chromosome rearrangements (including copy-number alterations). The so-called “cancer genes” exhibit attributes that confer growth advantages such as the capacity to generate their own mitogenic signals, the evasion of apoptosis, the resistance of exogenous growth-inhibitory signals, reprogramming of energy metabolism, evading immune destruction, cellular proliferation without limits, and acquisition of angiogenic, invasive, and metastatic properties.

Oncogenes stimulate the transformation of normal cells into cancer cells. They originate when genes called proto-oncogenes, which are involved in normal cellular growth and development, are genetically altered. Normal regulation of cellular proliferation requires a balance between factors that promote growth and those that restrict it. Mutational alteration of proto-oncogenes can lead to overexpression of their growth-stimulating activity, whereas mutations that inactivate tumor-suppressor genes, which normally restrain cellular proliferation, free cells from their inhibitory influence.

At least three stages have been defined in carcinogenesis: initiation, promotion, and progression. *Initiation* involves the induction of a genetic alteration, *promotion* involves cellular proliferation in an initiated cell population, and *progression* involves the accumulation of additional irreversible genetic changes and it is marked by increasing genetic instability and malignancy. More recent studies are leading to the concept of acquired capabilities.

Eight acquired characteristics essential for the formation of all tumors irrespective of tumor type and species include self-sufficiency in growth signals, insensitivity to antigrowth signals, evading apoptosis, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis, reprogramming of energy metabolism, and evading immune destruction. It seems probable that there is no specific order for obtaining these characteristics. In addition, genome instability and inflammation may be general features that underlie these eight hallmarks.

Genomic instability is a feature of all cancers. Gene mutations, chromosome aberrations, and aneuploidy are all implicated in the development of cancer. Mutagens and clastogens contribute to carcinogenesis as initiators, but mutagens, clastogens, and aneugens may also contribute to the multiple genetic alterations that characterize progression or the development of acquired capabilities. Other agents that contribute to carcinogenesis, such as promoters, need not be mutagens. However, the role of mutations is critical, and analyzing mutations and mutagenic effects is essential for understanding and predicting chemical carcinogenesis.

Germ Cells

The relevance of gene mutations to health is evident from the many disorders often caused by base-pair substitutions or small deletions that are inherited as simple Mendelian characteristics. Disorders such as cystic fibrosis, phenylketonuria, and Tay-Sachs disease are caused by the expression of recessive mutations. These mutations are mainly inherited from previous generations and are expressed when an individual inherits the mutant gene from both parents.

Besides causing diseases that exhibit Mendelian inheritance, gene mutations undoubtedly contribute to human disease through the genetic component of disorders with a complex etiology such as heart disease, hypertension, and diabetes. Sensitive cytogenetic methods have led to the discovery of minor variations in chromosome structure that have no apparent effect or that cause fetal death or serious abnormalities. Aneuploidy (gain or loss of one or more chromosomes) also contributes to fetal deaths and causes disorders such as Down syndrome. Most of the adverse effects of chromosomal abnormalities occur prenatally. Among the abnormalities that have been observed, aneuploidy is the most common, followed by polyploidy. Most chromosomal anomalies observed in newborns arise de novo in the germ cells of the parents. New advances in next generation sequencing, together with identification of genetic sequence variants associated with common diseases, have the potential to provide estimates of personal disease risk and germline genetic risk to more precisely guide medical management.

CANCER AND GENETIC RISK ASSESSMENTS

Cancer Risk Assessment

Somatic mutations are brought about by the combination of genetic effects and cellular proliferation either by (1) increasing genetic damage so there are more errors produced during DNA replication or by (2) increasing the DNA replication rate, which increases the likelihood that spontaneous errors in DNA become fixed in daughter cells. For carcinogen risk assessment, it is important to understand impacts on genotoxicity and cellular proliferation rates. For non-DNA-reactive substances, mechanisms that can increase cell proliferation include receptor-mediated responses (e.g., enzyme induction, CAR, PXR, AhR) and non-receptor mediated MOA (e.g., cytotoxicity leading to regeneration). In a weight-of-evidence approach, the most weight is allocated to high-quality in vivo studies of gene and chromosome mutations in humans and mammals exposed via physiologically relevant oral, dermal, or inhalation routes.

Risk assessment requires application of default approaches for extrapolation, for example, from laboratory animals to humans, from high to low exposures, from intermittent to chronic lifetime exposures, and from route to route of exposure. Considerations for causality include (1) biological concordance; (2) essentiality of key events; (3) dose–response concordance, temporal concordance, and incidence concordance of empirical observation; (4) consistency among different biological contexts; and (5) analogy (consistency across structurally significant chemicals) to promote better common understanding, consistency in use, as well as confidence in particular MOA. Finally, adverse outcome pathway frameworks can provide a basis for research, regulatory toxicology testing and risk assessment, and regulatory decision making.

Genetic Risk Assessment

For risk assessment with DNA-reactive chemicals, key elements and factors should be identified: (1) hazard assessment, (2) assessment of exposure to genotoxicants, (3) methods for dose and effect assessment, (4) risk characterization strategies for genotoxic environmental chemicals, and (5) monitoring of environmental genotoxicant levels.

Quantitative approaches for analyzing dose–response curves for genotoxic effects and deriving point-of-departure (PoD) metrics have been evaluated and include the no-observed-genotoxic-effect-level (NOGEL) or the highest dose tested for which there is no significant increase in genotoxic effect compared to control; the threshold effect level (BPD or Td) or the dose at which the slope changes from zero (horizontal) to positive, with its standard error forming the confidence bounds (90% CI); the benchmark dose (BMD_{10}) or the dose that produces 10% increase over the fitted background; BMD_{1SD} (the change in response corresponding to one standard deviation) or 10% excess risk for individuals below and above the 2nd and 98th percentiles, respectively; and slope-transition-dose (STD) or the lowest dose for which the lower bound of the 95% confidence interval of the slope exceeds zero.

The mechanistic information used by authoritative bodies in their risk assessment has included critical involvement of non-DNA targets, contribution of DNA repair mechanisms, exceeded detoxification capacity, disruption of enzymes involved in DNA synthesis or replication, chemical reactivity or properties unlikely to occur in vivo, inadequate uptake or toxicokinetics limiting distribution to target, mutational spectrum in tumor genes similar to those in untreated animals, structural similarities to similar threshold-acting chemicals, secondary or indirect origin of the observed damage, and species and tumor-specific non-genotoxic MOA. Parameters and safety factors evaluated to minimize the uncertainty in the assessment include (a) species differences and allometric scaling; (b) differences in absorption, distribution, metabolism, and pharmacokinetics; (c) differences in duration of exposure; (d) severity of toxicity endpoint; (e) variability among individuals; and (f) uncertainty in PoD. Knowledge of exposure, metabolism, and pharmacokinetics can help refine this extrapolation and reduce the assessment factor values by replacement with the experimentally determined parameters. This approach will facilitate the derivation of reference doses below which there is no additional significant risk to human health and margin of exposure values that are useful for evaluating human health risk and regulatory decision making.

FORMATION OF DNA DAMAGE AND DNA DAMAGE RESPONSE PATHWAYS

DNA Damage

All organisms sustain a certain number of background mutations as a result of limited DNA chemical stability, numerous stressors, cellular processes, and interactions with the environment. Endogenous and exogenous chemicals produce a wide array of DNA lesions such as covalent DNA base adducts, cross-links between DNA bases or between DNA bases and proteins, and single- and double-strand breaks. DNA replication, transcription, or erroneous incorporation of ribonucleotides into DNA can also be a source of spontaneous mutation and genome instability.

Ubiquitously present ultraviolet radiation, ionizing radiation, and exogenous hydrocarbons also present threats to genomic stability (Fig. 9–1).

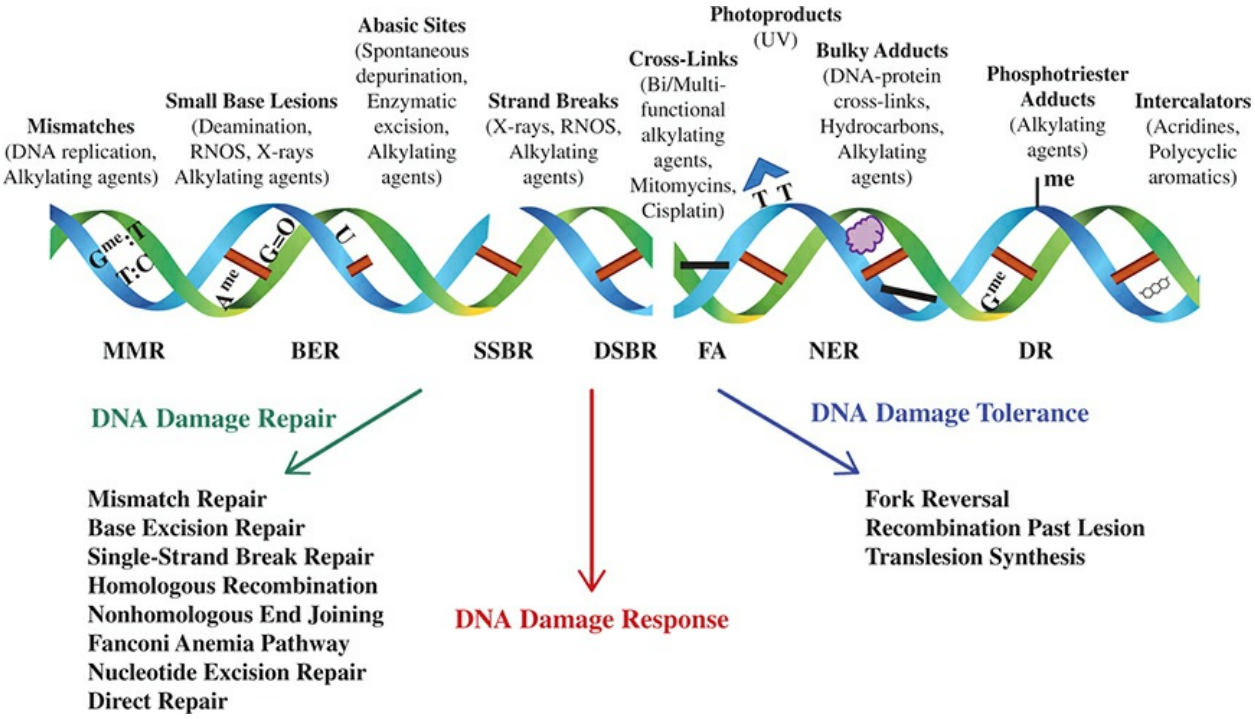

FIGURE 9–1 *Cellular mechanisms of DNA repair and damage tolerance to spontaneous and induced DNA damage.*

Background DNA Damage—Genomes sustain a continuous damage load due to numerous stressors, cellular metabolism, and limited chemical stability of DNA. Endogenous processes produce oxidized and alkylated bases, single- and double-strand breaks, interstrand cross-links, hydrolytic and enzymatic deamination of cytosine, and 5-methylcytosine leading to uracil and thymine, respectively.

The process of DNA replication itself is error-prone as one in about 10^6 replicated bases can be mis-inserted by replicative polymerases, or the polymerase can slip at repetitive sequences resulting in small insertion–deletion loops (IDLs). Living cells reduce the rate of polymerase error appreciably through error recognition and repair processes. The result is a low spontaneous mutation rate of the DNA replication process.

Ionizing Radiation (IR)—X-rays, γ-rays, neutrons, and α and β particles can produce radiochemical DNA damage by either direct or indirect mechanisms. IR exposure typically induces clustered damaged sites associated with double-strand breaks and oxidized base lesions on abasic sites or with single-strand breaks and oxidized base lesions on abasic sites, Multiple lesions can be formed in DNA from the same radiation energy deposition event.

Ultraviolet Light—Ultraviolet (UV) light is a type of electromagnetic nonionizing radiation with the wavelength range shorter than that of the violet end of the visible spectrum, but longer than that of x-rays. UV radiation typically induces pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs) as well as their Dewar valence isomers.

Chemical Agents—Chemicals can produce DNA alterations either directly by DNA-reactive mechanisms of adduct formation or indirectly by intercalation of polycyclic, aromatic, and planar chemicals between the base pairs. The conversion of DNA adducts or other structural modifications into gene and chromosomal mutations by errors of DNA replication on the damaged template, or occasionally by errors of DNA repair, can lead to cells being initiated along the key events pathway to the adverse outcome of cancer.

Many electrophilic chemicals react with nucleophilic sites within DNA, forming covalent addition products called biomarker DNA adducts, or some other DNA structural modifications. Some alkylated bases can mispair, causing mutations when DNA is replicated, other adducts are cytotoxic, while others can be both mutagenic and cytotoxic.

DNA Repair and Tolerance Pathways

The thousands of steady-state background DNA lesions are readily repaired in cells by an integrated network that includes direct repair (DR), base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), nonhomologous end-joining of double-strand breaks (NHEJ), homologous repair pathway (HR), and Fanconi Anemia (FA) pathway (Fig. 9–1). Among these repair activities, base excision repair is considered one of the most active, handling thousands of abasic sites, alkylated, oxidized, and deaminated bases, single-strand breaks and nicks per day. These pathways also have overlapping repair substrates. The basic principles underlying DNA repair processes involve damage recognition, followed by either direct reversal of the damage (sealing of strand breaks or removal of alkyl adducts from DNA bases) or excision of damage that involves sequential steps of repair DNA synthesis, and ligation of strands. DNA repair is usually error-free. In contrast, if the sustained damage is extensive, the cell can undergo apoptosis (programmed cell death), effectively avoiding it becoming a mutant cell. This chapter presents a brief outline of the major classes of DNA repair and tolerance pathways (Fig. 9–2).

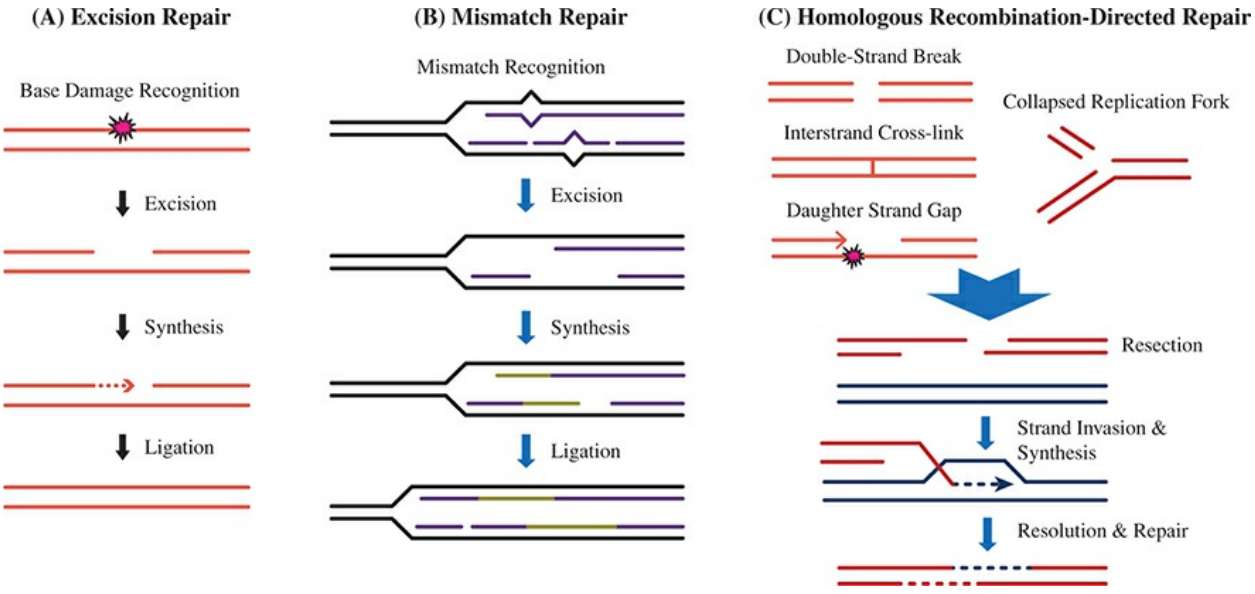

FIGURE 9–2 *Schematics of basic steps of key DNA repair pathways.* (**A**) Excision repair; (**B**) mismatch repair; (**C**) homologous recombination-directed DNA repair.

Base Excision Repair (BER)—The base excision repair (BER) pathway is one of the most active DNA repair pathways in cells operating at all cell cycle stages. BER repairs non-bulky oxidative, deaminated, and alkylated modifications to bases or the sugar phosphate backbone as well as abasic sites in both the nucleus and mitochondria. Key enzymatic steps (Fig. 9–2A) to remove the initial DNA lesion and restore the original sequence start with (i) lesion recognition and excision of damaged base or inappropriate base by *N*-glycosylase, (ii) incision of the phosphodiester backbone at the resulting abasic site by apurinic/apyrimidinic (AP) endonuclease, (iii) termini clean-up to permit repair synthesis and/or nick ligation, (iv) gap-filling to replace the excised nucleotide by DNA polymerase, and (v) sealing of the final nick by DNA ligase enzymes.

Nucleotide Excision Repair (NER)—The nucleotide excision repair (NER) pathway is a highly conserved pathway that recognizes and removes bulky DNA lesions that distort DNA double helix and impede progress of DNA and RNA polymerases, causing replication fork collapse or stalled transcription, respectively. The basic four steps of NER involve (i) damage recognition and incision on both sides of the lesion, (ii) excision, (iii) repair synthesis, and (iv) ligation of the nicks (Fig. 9–2A). NER consists of a general pathway termed global genomic repair (GGR) that removes lesions from the entire genome and a specialized pathway referred to as transcription-coupled repair (TCR). These pathways differ in the mechanism of damage recognition, but share the same mechanism for removal and repair of the damage.

Double-Strand Break Repair—Cell survival and genome integrity are seriously compromised by the presence of broken chromosomes. Unrepaired double-strand breaks activate DNA damage repair (DDR) networks that allow either recognition of the break, activation of cellular checkpoints and DNA repair, or induction of apoptosis. Recent evidence also suggests that small non-protein-coding RNAs are generated at the sites of DNA damage and control DDR network responses.

Homologous Recombination (HR)—The initiating step involves the processing of the DNA ends immediately adjacent to the double-strand break and the production of a 3′-single-stranded tail by exonucleases or helicase activity. With strand invasion and D-loop formation, and branch migration whereby the single-stranded tail invades, an undamaged homologous DNA molecule and Holliday junction DNA complex is formed. Holliday junction is cross-shaped structure that forms when two double-stranded DNA molecules become separated into four strands in order to exchange segments of genetic information. DNA synthesis and the resolution of the Holliday junction by its cleavage produce two DNA molecules (with or without a structural crossover).

Nonhomologous End-Joining (NHEJ)—Classical nonhomologous end-joining (NHEJ) joins two broken DNA ends in close spatial proximity to each other by direct re-ligation. NHEJ entails three main steps: recognition of the two-ended strand breaks, processing to remove non-ligatable termini, and joining of the two ends.

Fanconi Anemia (FA) Pathway—Fanconi anemia (FA) DNA excision repair involves the repair of interstrand cross-links (ICLs). ICLs can interfere with cellular processes requiring separation of the two DNA strands, such as DNA replication and transcription. Unrepaired ICLs can lead to mutations, chromosome breakage, and mitotic catastrophe. In addition to recognition and repair of ICLs, the complex FA pathway orchestrates cooperation of other DNA repair

processes to complete ICLs repair.

Mismatch Repair (MMR)—MMR maintains genome integrity by correcting base pair mismatches and IDLs that escape the proofreading activity of DNA polymerases, and mismatches that occur after HR events. The principal steps of prokaryotic and eukaryotic MMR are damage recognition and binding to the mismatch, stabilizing of the binding by the addition of other proteins, cutting the DNA at a distance from the mismatch, excision past the mismatch, resynthesis, and ligation (Fig. 9–2B).

Direct Repair (DR)—The error-free direct repair (DR) mechanism is defined as the elimination of DNA and RNA damage using chemical reversion that does not require a nucleotide template, breaking of the phosphodiester backbone, or DNA synthesis. There are two distinct DR mechanisms in most organisms: photoreactivation of UV-induced pyrimidine dimers by photolyases, and the removal of the alkyl groups from DNA and RNA by methyltransferases and dioxygenases.

Methylguanine DNA Methyltransferase (MGMT)—*O*^{6}-Alkylguanines are cytotoxic and pro-mutagenic adducts produced by many endogenous and exogenous alkylating chemicals. If the cells enter S phase prior to O^6-methylguanine (*O*^{6}-MeG) repair, processive DNA polymerases commonly mispair *O*^{6}-MeG with dT resulting in *O*^{6}-MeG:T mismatch and fixation of a G:C-to-A:T transition mutation in the second round of replication. The ubiquitous methylguanine DNA methyltransferases (MGMT) remove alkyl groups from *O*^{6} position of dG. In all organisms, the alkyl group (such as methyl, ethyl, propyl, butyl, benzyl, and 2-chloroethyl) is transferred via a one-step reaction to the repair protein by covalent binding to a highly conserved, active site cysteine directly restoring the DNA base and inactivating the protein.

Alkbh Fe(II)/α-Ketoglutarate-Dependent Dioxygenases— Direct repair of *N3*-cytosine and *N1*-adenine alkyl adducts in DNA and RNA has been associated with the action of alkylation protein B (AlkB) family of dioxygenases. The dealkylation reaction catalyzed by AlkB and ALKBH occurs via transformation of α-ketoglutarate into succinate and restoration of the undamaged base.

Translesion Synthesis (TLS)—Translesion synthesis (TLS) is carried out by 11 highly conserved and specialized DNA polymerases that are lesion- and species-specific. TLS involves a temporary switch from the replicative, high-fidelity polymerases to specialized, error-prone polymerases that help the cells bypass and tolerate the unrepaired damage. Common characteristics of TLS polymerases are large active sites that accommodate the damage, fewer contacts with DNA, low processivity, and lack of 3′–5′ exonuclease activity.

DNA Damage Response (DDR)—DNA damage response (DDR) networks effectively remove thousands of background DNA loads and maintain a very low level of spontaneous mutagenesis. DNA repair enzymes and signaling networks are regulated by DDR kinases including ATM (ataxia-telangiectasia mutated), ATR (ATM- and Rad3-Related), and DNA-PKcs (DNA-dependent protein kinases) at the sites of DNA damage via posttranslational protein modification cascades. As most mutations in DNA occur when a cell with unrepaired damage enters the S phase of the cell cycle, DDR checkpoints arrest the progression of the cell cycle to allow additional time for excision repair of the damage. Should the checkpoints fail to prevent

polymerase stalling, or if the damage is too extensive, cells can undergo apoptosis to eliminate damaged cells.

MECHANISMS OF INDUCTION OF GENETIC ALTERATIONS

Formation of Gene Mutations

Somatic Cells—Small DNA sequence changes, such as point mutations, involve alterations in a single base pair that affect the function of only one gene, and small deletions/additions in any DNA region. Changes in a single base pair may produce a: *missense mutation*, which results in a protein with one amino acid substituted for another; *nonsense mutation*, in which a stop codon replaces an amino acid codon, leading to premature termination of translation; and *frameshift mutation*, which causes a change in the reading frame by an addition or deletion of one or a few base pairs (not in multiples of three) in protein-coding regions, leading to pre-mature termination of the protein. Base substitutions can be further subdivided as transitions where the change is purine for purine or pyrimidine for pyrimidine, and transversions where the change is purine for pyrimidine and vice versa.

The great majority of spontaneous mutations arise from polymerase slippage, DNA biosynthetic errors, and replication errors of an altered template, which can be a result of oxidative and alkylated damage, or the deamination of bases eventually leading to G:C-to-A:T transitions after two rounds of DNA replication. Gene mutations induced by a majority of chemicals and nonionizing radiations are base substitutions, frameshifts, and small deletions produced by errors of DNA replication on a damaged template.

Germ Cells—An important consideration for assessing gene mutations induced by chemicals in germ cells is the relationship between exposure and the timing of DNA replication. The spermatogonial stem cell is present throughout the reproductive lifetime of an individual; each time it divides, it produces a differentiating spermatogonium and a stem cell that can accumulate genetic damage. The DNA damage incurred during late spermatid/spermatozoa stages can be repaired post-fertilization using the egg's repair proteins. The oocyte is resistant to the induction of gene mutations by nonradiomimetic chemicals because DNA repair occurs in oocytes, and there is no further S phase until the zygote.

Formation of Chromosomal Alterations

Somatic Cells

***Structural Chromosome Aberrations*—**Large-scale chromosomal mutations, or abnormalities, can involve deletion or insertion of several contiguous genes, inversion of genes on a chromosome, or the exchange of large segments of DNA between nonhomologous chromosomes. Aberrations induced by ionizing radiations are generally formed by errors of DNA repair, whereas those produced by nonradiomimetic chemicals result from errors of DNA replication on a damaged DNA template.

The DNA repair errors that lead to the formation of chromosome aberrations following

ionizing radiation (and radiomimetic chemical) exposure arise from misligation of double-strand breaks or interaction of coincidentally repairing regions during excision repair of damaged bases. Incorrect rejoining of chromosomal pieces during repair leads to chromosomal exchanges within (e.g., inversions and interstitial deletions) and between (e.g., dicentrics and reciprocal translocations) chromosomes. Failure to rejoin double-strand breaks or to complete the repair of other types of DNA damage leads to terminal deletions. The failure to incorporate an acentric fragment into a daughter nucleus at anaphase/telophase, or the failure of a whole chromosome to segregate at anaphase to the cellular poles, can result in the formation of a membrane-bound micronucleus that resides in the cytoplasm. Errors of DNA replication on a damaged template can lead to the deletion or exchange of individual chromatids (chromatid-type aberrations).

***Numerical Chromosome Changes*—**Monosomies, trisomies, and ploidy changes can arise from errors in chromosomal segregation. Alteration of various cellular components can result in failure to segregate the sister chromatids to separate daughter cells or in failure to segregate a chromosome to either pole. The mechanisms underlying chromosomal loss are pertinent to those involved in the formation of micronuclei. Aneuploidy is a known cause of human disease and represents a major cause of infertility, pregnancy failure, and serious genetic disorders in the offspring.

Germ Cells—The formation of chromosomal alterations in germ cells occurs via misrepair for ionizing radiations and radiomimetic chemicals for treatments in G_1 and G_2, and by errors of replication for all radiations and chemicals for DNA damage present during the S phase. The types of aberrations formed in germ cells are the same as those formed in somatic cells (e.g., deletions, inversions, translocations). The specific segregation of chromosomes during meiosis influences the probability of recovery of an aberration, particularly a reciprocal translocation, in the offspring of a treated parent.

ASSAYS FOR DETECTING GENETIC ALTERATIONS

Introduction to Assay Design

Genetic toxicology assays serve to (1) identify mutagens for purposes of hazard identification and (2) characterize dose–response relationships and mutagenic mechanisms, both of which contribute to an understanding of genetic and carcinogenic risks. Table 9–1 lists key assays that have a prominent place in genetic toxicology.

TABLE 9–1 Overview of Genetic Toxicology Assays

ASSAYS

I. Prediction of genotoxicity
  A. Interpretation of chemical structure
    Structural alerts to genotoxicity
  B. In silico predictive models
    Computational and structural programs: MCASE, TOPKAT, DEREK
    Quantitative structure–activity relationship (QSAR) modeling

II. DNA damage and repair assays
  A. Direct detection of DNA damage:
    Alkaline elution assays for DNA strand breakage in hepatocytes
    Comet assay (single-cell gel electrophoresis) for DNA strand breakage
    CometChip
    Nonmammalian comets in ecotoxicology
    Assays for chemical adducts in DNA
  B. DNA repair, recombination, and genotoxic stress responses as indicators of damage:
    Differential killing of repair-deficient and wild type bacteria
    Induction of the bacterial SOS system
    "Green Screen" for *GADD45a* gene induction in TK6 human cells
    Unscheduled DNA synthesis (UDS) in isolated rat hepatocytes or rodents in vivo
    Induction of mitotic recombination

III. Prokaryote gene-mutation assays
  A. Bacterial reverse mutation assays:
    Salmonella/mammalian microsome assay (Ames test)
    E. coli WP2 tryptophan reversion assay
    Salmonella-specific base-pair substitution assay (Ames II assay)
    E. coli lacZ-specific reversion assay
  B. Bacterial forward mutation assays:
    E. coli lacI assay
    Resistance to toxic metabolites or analogs in *Salmonella*

IV. Assays in nonmammalian eukaryotes:
  A. Fungal assays:
    Forward mutations, reversion, and small deletions
    Mitotic crossing over, gene conversion, and homology-mediated deletions in yeast
    Genetic detection of mitotic and meiotic aneuploidy in yeast
  B. Plant assays:
    Gene mutations affecting chlorophyll in seedlings, the *waxy* locus in pollen, or *Tradescantia* stamen-hair color
    Chromosome aberrations and micronuclei in mitotic and meiotic cells of corn, *Tradescantia*, and other plants
  C. *Drosophila* assays:
    Sex-linked recessive lethal test in germ cells
    Heritable translocation assays
    Mitotic recombination and LOH in eyes or wings

V. Mammalian gene-mutation assays
  A. In vitro assays for forward mutations:
    tk mutations in mouse lymphoma or human cells
    hprt or *xprt* mutations in Chinese hamster or human cells
    CD59 mutations in CHO-human hybrid A_L cells
  B. In vivo assays for gene mutations in somatic cells:
    Mouse spot test (somatic cell specific-locus test)
    hprt mutations (6-thioguanine-resistance) in rodent lymphocytes
    Pig-a mutations (immunological detection of mutations blocking glycosylphosphatidylinositol synthesis)
  C. Transgenic assays:
    Mutations in the bacterial *lacI* gene in Big Blue® mice and rats
    Mutations in the bacterial *lacZ* gene in the Muta™ Mouse
    Mutations in the phage *cII* gene in *lacI* or *lacZ* transgenic mice
    Point mutations and deletions in the *lacZ* plasmid mouse
    Point mutations and deletions in *gpt delta* mice and rats
    Forward mutations and reversions in ΦX174 transgenic mice
    Inversions and deletions arising in pKZ1 mice by intrachromosomal recombination

VI. Mammalian cytogenetic assays
- A. Chromosome aberrations:
  - Metaphase analysis in cultured Chinese hamster or human cells
  - Metaphase analysis of rodent bone marrow or lymphocytes in vivo
  - Chromosome painting and other FISH applications in vitro and in vivo
- B. Micronuclei:
  - Cytokinesis-block micronucleus assay in human lymphocytes
  - Micronucleus assay in mammalian cell lines
  - In vivo micronucleus assay in rodent bone marrow or blood
  - In vivo micronucleus assay in tissues other than marrow or blood
- C. Sister chromatid exchange:
  - SCE in human cells or Chinese hamster cells
  - SCE in rodent tissues, especially bone marrow
- D. Aneuploidy in mitotic cells:
  - Hyperploidy detected by chromosome counting or FISH in cell cultures or bone marrow
  - Micronucleus assay with centromere/kinetochore labeling in cell cultures
  - Altered parameters in flow-cytometric detection of micronuclei in CHO cells
  - Mouse bone marrow micronucleus assay with centromere labeling

VII. Germ cell mutagenesis
- A. Measurement of DNA damage
  - Molecular dosimetry based on mutagen adducts in reproductive cells
  - UDS in rodent germ cells
  - Alkaline elution assays for DNA strand breaks in rodent testes
  - Comet assay in sperm and gonadal tissue
- B. Gene mutations
  - Mouse specific-locus test for gene mutations and deletions
  - Mouse electrophoretic specific-locus test
  - Dominant mutations causing mouse skeletal defects or cataracts
  - ESTR assay in mice
  - Germ cell mutations in transgenic assays
- C. Chromosomal aberrations
  - Cytogenetic analysis of oocytes, spermatogonia, spermatocytes, or zygotes
  - Direct detection in sperm by FISH
  - Micronuclei in mouse spermatids
  - Mouse heritable translocation test
- D. Dominant lethal mutations
  - Mouse or rat dominant lethal assay
- E. Aneuploidy
  - Cytogenetic analysis for aneuploidy arising by nondisjunction
  - Sex chromosome loss test for nondisjunction or breakage
  - Micronucleus assay in spermatids with centromere labeling
  - FISH with probes for specific chromosomes in sperm

Some assays for gene mutations detect forward mutations whereas others detect reversion. Forward mutations, such as those detected in the *thymidine kinase* (*tk*) gene in the widely used

assay in mouse lymphoma cells, are genetic alterations in a wild type gene that are detected by a change in phenotype caused by the alteration or loss of gene function. In contrast, reversion mutations are those that restore gene function in a mutant, such as the histidine revertants detected in the Ames assay in *Salmonella*, bringing about a return to the wild type phenotype.

The simplest gene mutation assays rely on selection techniques to detect mutations. By imposing experimental conditions under which only cells or organisms that have undergone mutation can grow, selection techniques greatly facilitate the identification of rare cells that have experienced mutation among the many cells that have not.

Studying mutagenesis in intact animals requires more complex assays, which range from inexpensive short-term tests that can be performed in a few days to complicated assays for mutations in mammalian germ cells. There remains a gradation in which an increase in relevance for human risk entails more elaborate and costly tests.

Many substances that are not themselves mutagenic or carcinogenic can be activated into mutagens and carcinogens by metabolism. Such chemicals are called promutagens and procarcinogens. The most widely used metabolic activation system in microbial and cell culture assays is a postmitochondrial supernatant from a rat liver homogenate, along with appropriate buffers and cofactors. Most of the short-term assays in Table 9–1 require exogenous metabolic activation to detect promutagens. Exceptions are assays in intact mammals and a few simpler assays that have a high level of endogenous cytochrome P450 metabolism, such as the detection of UDS or DNA strand breakage in cultured hepatocytes.

Despite their usefulness, in vitro metabolic activation systems cannot mimic mammalian metabolism perfectly. There are differences among tissues in reactions that activate or inactivate foreign compounds, and organisms of the normal flora of the intestines can contribute to metabolism in intact mammals. Chemicals that induce enzyme systems or otherwise alter the physiological state can also modify the metabolism of toxicants, and the balance between activation and detoxification reactions in vitro may differ from that found in vivo.

Structural Alerts and In Silico Assays

The first indication that a chemical is a mutagen often lies in chemical structure. Potential electrophilic sites in a molecule serve as an alert to possible mutagenicity and carcinogenicity because such sites confer reactivity with nucleophilic sites in DNA. Computer-based systems for predicting genotoxicity based on chemical structure properties are sometimes called in silico assays, which include computational and structural programs and the modeling of quantitative structure–activity relationships although there is much skepticism that such approaches can replace biological testing, they hold promise of improving the efficiency of integrated testing strategies and reducing current levels of animal use.

DNA Damage and Repair Assays

Some assays measure DNA damage itself through such indicators as chemical adducts or strand breaks in DNA, or indirectly, through the measurement of biological repair processes. Adducts in DNA are detected by ^{32}P-postlabeling, high-performance liquid chromatography, fluorescence-based methods, mass spectrometry, immunological methods using antibodies against specific adducts, isotope-labeled DNA binding, and electrochemical detection.

DNA strand breakage can be measured by alkaline elution and electrophoretic methods. Single-cell gel electrophoresis, also called the comet assay, is a widely used, rapid method of measuring DNA damage. In this assay, cells are incorporated into agarose on slides, lysed to liberate their DNA, and subjected to alkaline electrophoresis. The DNA is stained with a fluorescent dye for observation and image analysis. Broken DNA fragments in the tail migrate more quickly than larger pieces of DNA of the head producing a blur of fragments (a "comet") when the DNA is extensively damaged. The extent of DNA damage can be estimated from the intensity of the comet tail relative to the total intensity (head plus tail) that reflects the amount of DNA breakage. The comet assay has broad applicability among diverse species, including plants, worms, mollusks, fish, and amphibians.

The occurrence of DNA repair can serve as an indicator of DNA damage. The measurement unscheduled DNA synthesis (UDS), which is a measure of excision repair, is a mammalian DNA repair assay indicating that DNA has been damaged. UDS has been used with cultured hepatocytes with endogenous cytochrome P450 enzyme activities as well as tissues of intact animals, including hepatocytes and germinal tissue. The absence of UDS, however, does not provide evidence that DNA has not been damaged because some classes of damage are not readily excised.

Gene Mutations in Prokaryotes

The most common means of detecting mutations in microorganisms is selecting for reversion in strains that have a specific nutritional requirement differing from wild type members of the species; such strains are called auxotrophs. In the Ames assay, one measures the frequency of histidine-independent bacteria that arise in a histidine-requiring strain in the presence or absence of the chemical being tested. Auxotrophic bacteria are treated with the chemical of interest and plated on medium that is deficient in histidine. The assay is conducted using genetically different strains so that reversion by base pair substitutions and frameshift mutations in several DNA sequence contexts can be detected and distinguished. Since *Salmonella* does not metabolize promutagens in the same way as mammalian tissues, the assay is generally performed in the presence and absence of a rat liver S9 metabolic activation system. Equivalent information can be obtained from the WP2 tryptophan reversion assay in *E. coli* whereby mutations are detected by selecting for reversion of a *trpE* allele from Trp^- to Trp^+. Bacterial forward mutation assays, such as selections for resistance to arabinose or to purine or pyrimidine analogs in *Salmonella*, are also used in research and testing.

Genetic Alterations in Nonmammalian Eukaryotes

Gene Mutations and Chromosome Aberrations—The fruit fly, Drosophila, has long occupied a prominent place in genetic research because of the sex-linked recessive lethal (SLRL) test. The SLRL test permits the detection of recessive lethal mutations at 600 to 800 different loci on the X chromosome by screening for the presence or absence of wild type males in the offspring of specifically designed crosses. The SLRL test yields information about mutagenesis in germ cells, which is lacking in all microbial and cell culture systems. Genetic and cytogenetic assays in plants find use in special applications, such as in situ monitoring for mutagens and exploration of the metabolism of promutagens by agricultural plants.

Mitotic Recombination—Recombinogenic effects in yeast have long been used as a general indicator of genetic damage, and interest in induced recombination has increased because of their implication in the etiology of cancer. Widely used assays for recombinogens detect mitotic crossing over and mitotic gene conversion in the yeast *Saccharomyces cerevisiae*. Strategies have also been devised to detect recombinogenic effects in human lymphocytes, other mammalian cells, mice, chickens, plants, and mycelial fungi.

Gene Mutations in Mammalian Cells

The most widely used assays for gene mutations in mammalian cells detect forward mutations that confer resistance to a toxic chemical. Instead of using selective media, flow cytometry can be used to detect gene mutations by immunological methods in mammalian cell cultures and intact animals.

Gene Mutations In Vivo

In vivo assays involve treating intact animals and analyzing appropriate tissues for genetic effects. The choice of suitable doses, treatment procedures, controls, and sample sizes is critical in the conduct of in vivo tests. Mutations may be detected either in somatic cells or in germ cells. The mouse spot test is a traditional genetic assay for gene mutations in somatic cells. Visible spots of altered phenotype in mice heterozygous for coat-colored genes indicate mutations in the progenitor cells of the altered regions.

Mutation assays can provide information on mechanisms of mutagenesis. Base pair substitutions and large deletions can be differentiated with probes for the target gene and Southern blotting as base substitutions are too subtle to be detected on blots. Gene mutations have been characterized at the molecular level by DNA sequence analysis both in transgenic rodents and in endogenous mammalian genes.

Transgenic Assays—Transgenic animals contain foreign DNA sequences that have been added to the genome. The foreign DNA is represented in all the somatic cells of the animal and is transmitted in the germ line to progeny. Mutagenicity assays in transgenic animals combine in vivo metabolic activation and pharmacodynamics with simple microbial detection systems, and they permit the analysis of mutations induced in diverse mammalian tissues. The transgenic animals that have figured most heavily in genetic toxicology are rodents that carry *lac* genes from *E. coli*. The bacterial genes were introduced into mice or rats by injecting a vector carrying the genes into fertilized oocytes generating grown up transgenic animals containing multiple copies of chromosomally integrated plasmid or phage shuttle vectors. After mutagenic treatment of the transgenic animals, the *lac* genes are recovered from the animal, packaged in phage λ, and transferred to *E. coli* for mutational analysis. Mutant plaques are identified based on phenotype, and mutant frequencies can be calculated for various tissues of the treated animals.

Mammalian Cytogenetic Assays

Chromosome Aberrations—Cytogenetic assays use microscopy for the direct observation of the effects of interest. This approach differs sharply from the indirectness of traditional genetic

assays in which one observes a phenotype and reaches conclusions about genes. In conventional cytogenetics, metaphase analysis is used to detect chromosomal anomalies, especially unstable chromosome and chromatid aberrations. Cells should be treated during a sensitive period of the cell cycle (typically S), and aberrations should be analyzed at the first mitotic division after treatment so that the sensitivity of the assay is not reduced by unstable aberrations being lost during cell division. Examples of chromosome aberrations are shown in Fig. 9–3.

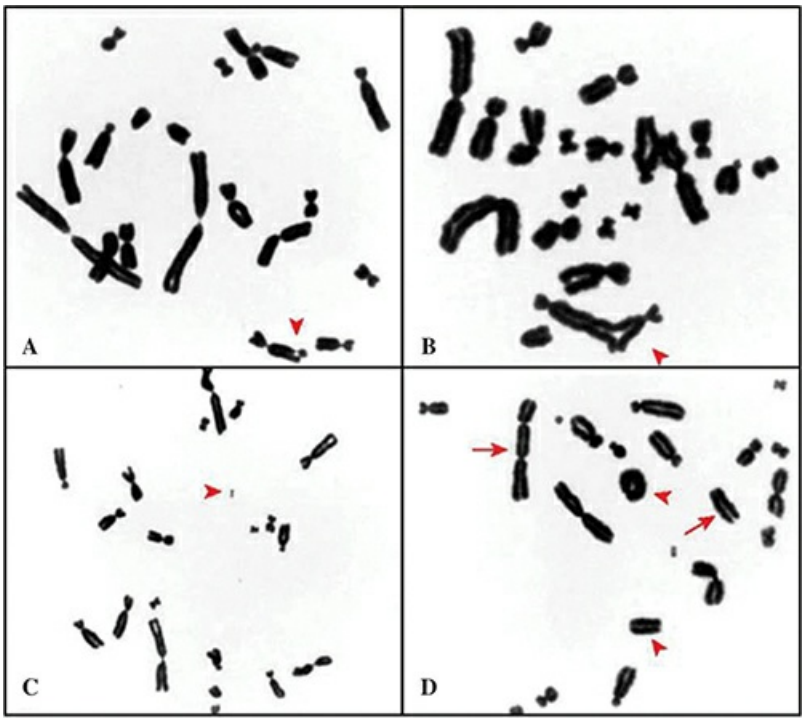

FIGURE 9–3 *Chromosome aberrations induced by x-rays in Chinese hamster ovary (CHO) cells.* (**A**) A chromatid deletion (►). (**B**) A chromatid exchange called a triradial (►). (**C**) A small interstitial deletion (►) that resulted from chromosome breakage. (**D**) A metaphase with more than one aberration: a centric ring plus an acentric fragment (►) and a dicentric chromosome plus an acentric fragment (→).

Cytogenetic assays require careful attention to growth conditions, controls, dose levels, treatment conditions, and time intervals between treatment and the sampling of cells for analysis. It is essential that sufficient cells be analyzed because a negative result in a small sample is inconclusive. Results should be recorded for specific classes of aberrations, not just an overall index of aberrations per cell. In interpreting results on the induction of chromosome aberrations in cell cultures, artifacts associated with extreme assay conditions may not be a reflection of a chemical-specific genotoxicity. Excessively high concentrations may lead to false positive responses. Therefore, testing should be extended to a concentration at which there is some cytotoxicity, such as a reduction in a replicative index or the mitotic index.

In vivo assays for chromosome aberrations involve treating intact animals and later collecting cells for cytogenetic analysis. The main advantage of in vivo assays is that they include mammalian metabolism, DNA repair, and pharmacodynamics. The target is typically a tissue from which large numbers of dividing cells are easily prepared for analysis. Effective testing requires dosages and routes of administration that ensure adequate exposure of the target cells,

proper intervals between treatment and collecting cells, and sufficient numbers of animals and cells analyzed.

Fluorescent in situ hybridization (FISH) (Fig. 9–4), involves a nucleic acid probe labeled with a fluorescent dye is hybridized to complementary sequences in chromosomal DNA. The chromosomal location to which it binds is visible by fluorescence microscopy, and the signal can be increased in strength by computer-enhanced processes. The use of whole-chromosome probes during metaphase or interphase is commonly called “chromosome painting.”

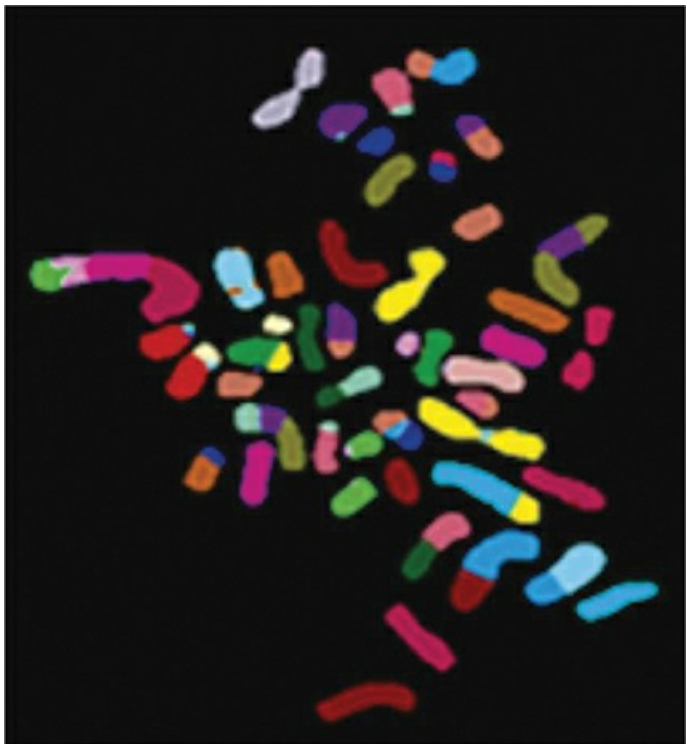

FIGURE 9–4 *Chromosome aberrations identified by FISH.* Human breast cancer cell with aneuploidy for some chromosomes and with reciprocal translocations (identified by color switches along a chromosome).

Regions of hybridization can be determined by using fluorescent antibodies that detect modified DNA bases incorporated during amplification or by incorporating fluorescent bases themselves during amplification.

Micronuclei— Micronuclei are membrane-bound structures that contain chromosomal fragments, or sometimes whole chromosomes, that were not incorporated into a daughter nucleus at mitosis. Micronuclei usually represent acentric chromosomal fragments and are commonly used as simple indicators of chromosomal damage. Micronuclei in a binucleate human lymphocyte are shown in Fig. 9–5.

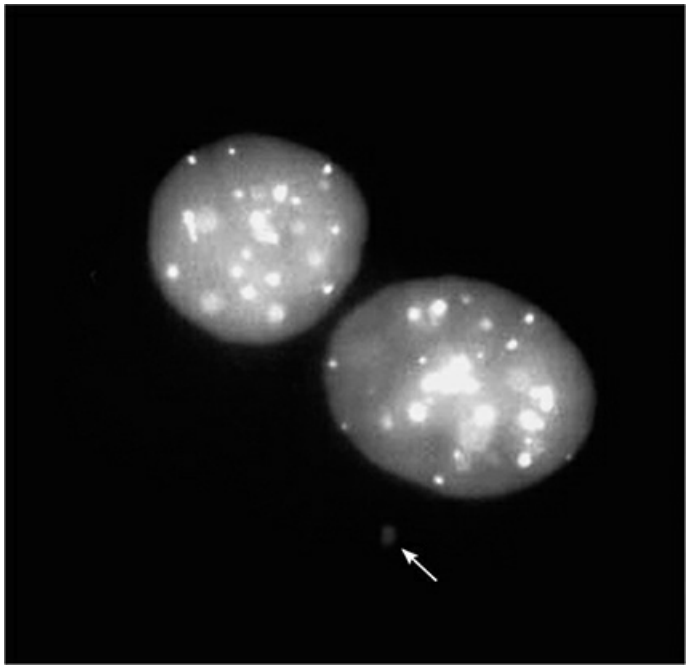

FIGURE 9–5 *Micronucleus in a human lymphocyte.* The cytochalasin B method was used to inhibit cytokinesis that resulted in a binucleate nucleus. The micronucleus (arrow) resulted from failure of an acentric chromosome fragment or a whole chromosome being included in a daughter nucleus following cell division. (Image used with permission from James Allen, Jill Barnes, and Barbara Collins.)

Sister Chromatid Exchange (SCE)—SCE, in which there has been an apparently reciprocal exchange of segments between the two chromatids of a chromosome, are visible cytologically through differential staining of chromatids. Figure 9–6 shows SCE in human cells. Many mutagens induce SCE in cultured cells and in mammals in vivo. SCE assays are best regarded as general indicators of mutagen exposure, analogous to DNA damage and repair assays, rather than measures of a mutagenic effect.

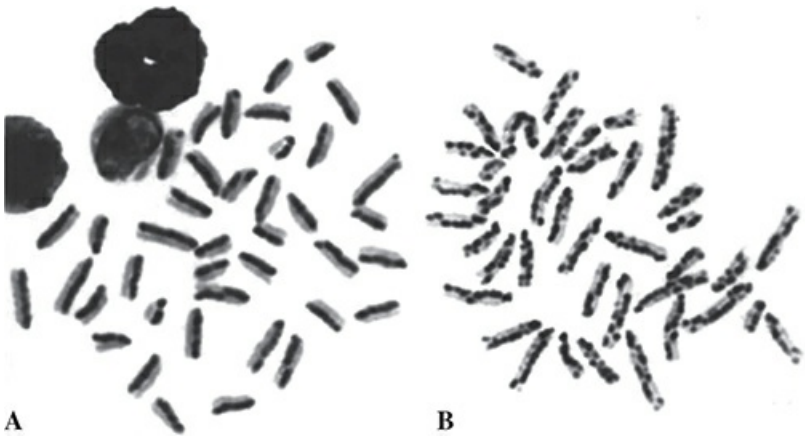

FIGURE 9–6 *Sister chromatid exchanges (SCEs) in human lymphocytes.* (**A**) SCE in untreated cell. (**B**) SCE in cell exposed to ethyl carbamate. The treatment results in a large increase in the number of SCE. (Image used with permission from James Allen and Barbara

Collins.)

Aneuploidy—Means for determining aneuploidy include chromosome counting, the detection of micronuclei that contain kinetochores, and the observation of abnormal spindles or spindle–chromosome associations in cells in which spindles and chromosomes have been differentially stained. FISH-based assays have also been developed for the assessment of aneuploidy in interphase somatic cells. A complication in chromosome counting is that a metaphase may lack chromosomes because they were lost during cell preparation for analysis, rather than having been absent from the living cell. To avoid this artifact, cytogeneticists generally use extra chromosomes (i.e., hyperploidy) rather than missing chromosomes (i.e., hypoploidy) as an indicator of aneuploidy in chromosome preparations.

Aneuploidy may be detected by means of antikinetochore antibodies with a fluorescent label or FISH with a probe for centromere-specific DNA. Micronuclei containing kinetochores or centromeric DNA may be detected in cultured cells and in mouse bone marrow. Frequencies of micronuclei ascribable to aneuploidy and to clastogenic effects may therefore be determined concurrently by tabulating micronuclei with and without kinetochores.

Germ Cell Mutagenesis

Gene Mutations—Mammalian germ cell assays provide the best basis for assessing risks to human germ cells at different germ cell stages. Late stages of spermatogenesis are often found to be sensitive to mutagenesis, but effects in spermatocytes, spermatids, and spermatozoa are transitory. Mutagenesis in stem-cell spermatogonia and resting oocytes is of special interest in genetic risk assessment because of the persistence of these stages throughout reproductive life. Many chemical mutagens show specificity with respect to germ cell stages.

Chromosomal Alterations Knowledge of chromosome aberration induction in germ cells is important for assessing risks to future generations. Metaphase analysis of germ cells is feasible in rodent spermatogonia, spermatocytes, or oocyte. A germ cell micronucleus assay measures chromosomal damage induced in meiosis by examining rodent spermatids. Aneuploidy originating in mammalian germ cells may be detected cytologically through chromosome counting for hyperploidy or genetically in the mouse sex-chromosome loss test.

Indirect evidence for chromosome aberrations is obtained in the mouse heritable translocation assay, which measures reduced fertility in the offspring of treated males. This presumptive evidence of chromosomal rearrangements can be confirmed through cytogenetic analysis.

Dominant Lethal Mutations—An extensive database on genetic damage in mammalian germ cells was obtained with the mouse or rat dominant lethal assay. Commonly, males are treated on an acute or subchronic basis with the chemical of interest and then mated with virgin females at appropriate intervals. The females are necropsied during pregnancy so that embryonic mortality, assumed to arise from chromosomal anomalies, may be characterized and quantified.

Development of Testing Strategies

Concern about adverse effects of mutation on human health has provided the impetus to identify environmental mutagens. Genetic toxicology assays screen chemicals to detect mutagens, but information on mutagenic mechanisms and dose–responses contributes to an evaluation of

hazards and risk. Besides testing pure chemicals, environmental samples are tested because many mutagens exist in complex mixtures. The analysis of complex mixtures often requires a combination of mutagenicity assays and refined analytical methods. In predicting carcinogenicity, one should consider both the sensitivity and the specificity of an assay. Sensitivity refers to the proportion of carcinogens that are positive in the assay, whereas specificity is the proportion of non-carcinogens that are negative. Sensitivity and specificity both contribute to the predictive reliability of an assay.

HUMAN POPULATION MONITORING

For cancer risk assessment considerations, the human data utilized most frequently, in the absence of epidemiologic data, are those collected from genotoxicity/mutagenicity assessments in human populations. The studies conducted most frequently are for chromosome aberrations, micronuclei, mutations (for several loci), and SCEs in peripheral lymphocytes.

The size of each study group should be sufficiently large to avoid confounders. Certain characteristics should be matched among exposed and unexposed groups, including age, sex, smoking status, and general dietary features. Study groups of 20 or more individuals can be used as a reasonable substitute for exact matching because confounders will be less influential on chromosome alteration or mutation frequency in larger groups. In some instances, it might be informative to compare exposed groups with a historical control, as well as to a concurrent control.

NEW APPROACHES FOR GENETIC TOXICOLOGY

Genetic toxicology has moved into the molecular and computational era to advance our understanding of basic cellular processes and how they can be perturbed. The ability to manipulate and characterize DNA, RNA, and proteins are important methods in knowledge. New understanding in biological mechanism and mode of action (MOA), along with new high-content and high-throughput approaches, and increasingly sensitive analytical methods bring new information into the field of genetic toxicology and the quantitative risk assessment processes for DNA-reactive chemicals. Complex interactions within a particular DNA repair pathway and interplay between different DNA repair and DDR pathways counteract the effects of ever-present background DNA damage. The examination of key cellular, mechanistic responses should be integrated into the designs of the mutagenic MOA studies and risk characterization processes for DNA-reactive chemicals.

Advances in Cytogenetics

Conventional chromosome staining with DNA stains such as Giemsa or the process of chromosome banding require considerable expenditure of time and a rather high level of expertise. Chromosome banding can assess transmissible aberrations such as reciprocal

translocations and inversions with considerable accuracy. Stable aberrations are transmissible from parent to daughter cell, they represent accumulated effects of chronic exposures. The more readily analyzed, but cell-lethal, nontransmissible aberrations such as dicentrics and deletions reflect only recent exposures and then only when analyzed at the first division after exposure.

The types of data collected will affect our understanding of how tumors develop. Data on the dose–response characteristics for a specific chromosomal alteration as a proximate marker of cancer can enhance the cancer risk assessment process by describing effects of low exposures that are below those for which tumor incidence can be reliably assessed. Cytogenetic data can improve extrapolation from data generated with laboratory animals to humans.

Molecular Analysis of Mutations and Gene Expression

Molecular biology techniques allow establishment of the exact basis of a mutation at the level of the DNA sequence. In many cases, the genetic basis of human disease can be determined even though human genes have long DNA sequences and a complex genomic arrangement. Molecular biology techniques have also enabled a distinguishing between background mutations and those induced by specific agents. The genes analyzed for mutations are ones for which mutated forms can be selected. The confounding factor of many normal cells, which far outnumber a few mutant cells in an exposed cellular population, can be removed by mutant selection approaches. Methods to overcome the drawback of only being able to study selectable genes are being developed.

The ability to detect and characterize mutations at both the DNA and RNA levels has been enhanced by the development of chip technology, array-based assay systems, and next-generation high-throughput DNA sequencing technologies at the whole genome level. With hybridization of test DNAs to oligonucleotide arrays, specific genetic alterations or their cellular consequences can be determined rapidly and automatically. Genome-wide gene expression microarray technologies now allow measurement of changes in expression of thousands of genes at one time. The sensitivity of next-generation sequencing protocols is rapidly increasing. Overall, the current potential for assessing specific cellular changes following chemical exposure is enormous.

CONCLUSION

Ionizing radiation and chemical exposures can induce mutations and chromosome alterations in plant, insect, and mammalian cells. A broad range of short-term assays for genotoxicity have identified many mutagens and demonstrated the relationship between mutagens and cancer-causing agents, or carcinogens. Failure of the assays to be completely predictive resulted in the identification of nongenotoxic carcinogens.

Next-generation sequencing technologies have allowed whole genome mutation analysis to be accomplished quickly. Key cellular processes related to mutagenesis have been identified, including multiple pathways of DNA repair, cell cycle controls, and the role of checkpoints in ensuring that the cell cycle does not proceed until the DNA and specific cellular structures are checked for fidelity. Improvements in the qualitative assessment of mutation in somatic cells and germ cells have paralleled the advances to assess genetic alterations quantitatively. These new

approaches will reduce overall uncertainty in the process of risk characterization surrounding exposures at environmentally relevant concentrations.

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QUESTIONS

1. Oncogenes:
   - **a.** maintain normal cellular growth and development.
   - **b.** exert their action in a genetically recessive fashion.
   - **c.** are often formed via translocation to a location with a more active promoter.
   - **d.** can be mutated to form proto-oncogenes.
   - **e.** include growth factors and GTPases, but not transcription factors.

2. Which of the following is NOT one of the more common sources of DNA damage?
   - **a.** ionizing radiation.
   - **b.** UV light.
   - **c.** electrophilic chemicals.
   - **d.** DNA polymerase error.
   - **e.** x-rays.

3. Which of the following pairs of DNA repair mechanisms is most likely to introduce mutations into the genetic composition of an organism?
   - **a.** nonhomologous end-joining (NHEJ) and base excision repair.
   - **b.** nonhomologous end-joining and homologous recombination.
   - **c.** homologous recombination and nucleotide excision repair.
   - **d.** nucleotide excision repair and base excision repair.
   - **e.** homologous recombination and mismatch repair.

4. Which of the following DNA mutations would NOT be considered a frameshift mutation?
   - **a.** insertion of 5 nucleotides.
   - **b.** insertion of 7 nucleotides.

c. deletion of 18 nucleotides.
d. deletion of 13 nucleotides.
e. deletion of 1 nucleotide.

5. Which of the following base pair mutations is properly characterized as a transversion mutation?
   a. T → C.
   b. A → G.
   c. G → A.
   d. T → U.
   e. A → C.

6. All of the following statements regarding nondisjunction during meiosis are true EXCEPT:
   a. Nondisjunction events can happen during meiosis I or meiosis II.
   b. All gametes from nondisjunction events have an abnormal chromosome number.
   c. Trisomy 21 (Down syndrome) is a common example of nondisjunction.
   d. In a nondisjunction event in meiosis I, homologous chromosomes fail to separate.
   e. The incorrect formation of spindle fibers is a common cause of nondisjunction during meiosis.

7. Which of the following diseases does NOT have a recessive inheritance pattern?
   a. phenylketonuria.
   b. cystic fibrosis.
   c. Tay–Sachs disease.
   d. sickle cell anemia.
   e. Huntington's disease.

8. What is the purpose of the Ames assay?
   a. to determine the threshold of UV light bacteria can receive before having mutations in their DNA.
   b. to measure the frequency of aneuploidy in bacterial colonies treated with various chemicals.
   c. to determine the frequency of a reversion mutation that allows bacterial colonies to grow in the absence of vital nutrients.
   d. to measure the rate of induced recombination in mutagen-treated fungi.
   e. to measure induction of phenotypic changes in *Drosophila*.

9. In mammalian cytogenic assays, chromosomal aberrations are measured after treatment of the cells at which sensitive phase of the cell cycle?
   a. interphase.
   b. M phase.
   c. S phase.
   d. G1.
   e. G2.

10. Which of the following molecules is used to gauge the amount of a specific gene being transcribed to mRNA?
    a. protein.
    b. mRNA.
    c. DNA.
    d. cDNA.
    e. CGH.

CHAPTER 10

Developmental Toxicology

John M. Rogers

KEY POINTS

- Developmental toxicology encompasses the study of pharmacokinetics, mechanisms, pathogenesis, and outcomes following exposure to agents or conditions leading to abnormal development.
- Developmental toxicology includes teratology, or the study of structural birth defects.
- *Gametogenesis* is the process of forming the haploid germ cells: the egg and the sperm.
- *Organogenesis* is the period during which most bodily structures are established. This period of heightened susceptibility to malformations extends from the third to the eighth week of gestation in humans.
- *Teratology* is the study of abnormal structural development.

HUMAN TERATOGENS AND DEVELOPMENTAL TOXICANTS

With the development of highly sensitive tests that can detect pregnancies shortly after fertilization, recognized pregnancy loss increased to as much as 60% to 70%. Estimates of adverse outcomes include postimplantation pregnancy loss, 31%; major birth defects, 2% to 3% at birth and increasing to 6% to 7% at 1 year with continuing diagnoses; minor birth defects, 14%; low birth weight, 7%; infant mortality (prior to 1 year of age), 1.4%; and abnormal neurological function, 16% to 17%. Of over 4100 chemicals tested for teratogenicity, a much smaller number, about 35 to 40 chemicals, chemical classes, or conditions (Table 10–1), have been documented to be developmentally toxic in humans.

TABLE 10–1 Human Developmental Toxicants

Radiation	Busulfan
Atomic fallout	Carbon monoxide
Radioiodine	Chlorambucil
Therapeutic radiation	Cocaine
Infections	Coumarins
Cytomegalovirus	Cyclophosphamide
Herpes simplex virus I and II	Cytarabine
Parvovirus B-19 (erythema infectiosum)	Diethylstilbestrol
Rubella virus	Danazol
Syphilis	Ergotamine
Toxoplasmosis	Ethanol
Varicella virus	Ethylene oxide
Venezuelan equine encephalitis virus	Fluconazole
Maternal trauma and metabolic imbalances	Folate antagonists: aminopterin, methotrexate
Alcoholism	Iodides
Amniocentesis, early	Lead
Chorionic villus sampling (before day 60)	Lithium
Cretinism	Mercury, organic
Diabetes	Methimazole
Folic acid deficiency	Methylene blue
Hyperthermia	Misoprostal
Phenylketonuria	Penicillamine
Rheumatic disease and congenital heart block	Polychlorobiphenyls
Sjögren syndrome	Quinine (high dose)
Virilizing tumors	Retinoids: isotretinoin, etretinate, acitretin
Drugs and chemicals	Tetracyclines
Aminoglycosides	Thalidomide
Androgenic hormones	Tobacco smoke
Angiotensin converting enzyme inhibitors: captopril, enalapril	Toluene
Angiotensin receptor antagonists: sartans	Vitamin A (high dose)
Anticonvulsants: diphenylhydantoin, trimethadione, valproic acid, carbamazepine	

Data from Shepard TH, Lemire RJ. *Catalog of Teratogenic Agents*. 11th ed. Baltimore, MD: Johns Hopkins University Press; 2004; Schardein JL, Macina OT. *Human Developmental Toxicants: Aspects of Toxicology and Chemistry*. Boca Raton, FL: CRC Press; 2007:427.

Maternal Rubella Infection

In the 1940s, an epidemic of rubella virus infection in Australia was linked to congenital malformations including eye, heart, and ear defects, and mental retardation. Heart and eye defects were associated with maternal infection in the first 2 months of pregnancy, while hearing and speech defects and mental retardation occurred with infection in the third month. Rubella is now uncommon in developed countries due to widespread vaccination, but recent outbreaks have been reported.

Thalidomide

In 1960, a striking increase in newborns with rare limb malformations was recorded in West Germany. The affected individuals had amelia (absence of the limbs) or various degrees of phocomelia (reduction of the long bones of the limbs). Congenital cardiac, ocular, otic, intestinal, and renal anomalies were also reported. The rarity of the malformations contributed to recognizing the epidemic caused by thalidomide, which was withdrawn in November 1961, and case reports ended in mid-1962.

Thalidomide presents a variable pattern of developmental toxicity in at least 19 species. Twenty-four putative mechanisms include biochemical alterations involving vitamin B, glutamic acid, acylation, nucleic acids, and oxidative phosphorylation; cellular mechanisms including cell death and cell–cell interactions; and inhibition of nerve and blood vessel outgrowth. More recent hypotheses include effects on angiogenesis, integrins, oxidative DNA damage, and inhibition of ubiquitin-mediated protein degradation. In the sensitive rabbit, thalidomide is activated to a free-radical intermediate that is key to its teratogenicity. Limb defects are due to altered expression of genes critical for limb outgrowth. Differences in redox regulation and consequent effects on redox-sensitive NF-κB may underlie species differences in thalidomide teratogenicity.

Ethanol/Alcoholism

The fetal alcohol syndrome (FAS) is a broad array of effects of alcohol on development. FAS includes craniofacial dysmorphology, growth retardation, and psychomotor and intellectual deficiencies. The molecular mechanisms underlying FAS are still poorly understood, and the possible molecular and cellular targets are many. Epigenetic effects of alcohol exposure during prenatal development include DNA methylation and histone modification. FAS has been observed primarily in children born to alcoholic mothers, with an incidence estimated at 25 per 1000 pregnancies. The level of maternal ethanol consumption associated with FAS is estimated at 3 to 4 oz of alcohol per day. FAS represents the extreme end of a wide range of effects of developmental alcohol exposure termed fetal alcohol spectrum disorders.

Diethylstilbestrol

A synthetic nonsteroidal estrogen, diethylstilbestrol (DES), used in the United States from the 1940s to the 1970s to prevent miscarriage was linked with clear cell adenocarcinoma of the vagina between 1966 and 1969. Maternal exposure to DES during the first trimester caused anomalies in the developing female reproductive tract. Male offspring reported epididymal cysts, hypotrophic testes, capsular induration, low ejaculated semen volume, and poor semen quality. The latent and devastating manifestations of prenatal DES exposure broadened our concept of the potential adverse outcomes of intrauterine exposures.

Tobacco Smoke

Tobacco smoke and its constituents may be the leading cause of environmentally induced developmental disease today. The adverse consequences of developmental tobacco smoke exposure include spontaneous abortion, perinatal death, sudden infant death syndrome (SIDS),

disorders of learning, behavior and attention, and lower birth weight. Offspring of smoking mothers have a higher risk of obesity, hypertension, and type 2 diabetes than offspring of nonsmokers.

A meta-analysis of observational studies reported significant associations with maternal smoking for: cardiovascular, musculoskeletal, limb reduction, missing/extra digits, clubfoot, craniosynostosis, facial, eye, orofacial clefts, gastrointestinal, gastroschisis, anal atresia, hernia, and undescended testes. Perinatal exposure to tobacco smoke can also affect branching morphogenesis and maturation of the lung, altering lung function. Nicotine in tobacco smoke is a known neuroteratogen in experimental animals producing such adverse developmental effects as impaired fertility, neurobehavioral effects, respiratory dysfunction, obesity, hypertension, and type 2 diabetes. Exposure to secondhand tobacco smoke represents a significant risk to the pregnant nonsmoker and her baby, causing a 22% increased risk of being of low birth, a 23% higher risk of stillbirth, and a 13% higher risk of having a child with a congenital malformation.

Maternal smoking has been associated with changes in DNA methylation in cord blood and other offspring cells. One gene consistently affected by CpG methylation changes is the aryl-hydrocarbon receptor repressor (*AHRR*) gene that participates in AhR signaling involved in dioxin toxicity as well as cell growth and differentiation. This CpG in *AHRR* is currently considered a biomarker of smoking.

Retinoids

Developmental effects of retinol, retinoic acid, and structurally related chemicals bind to and activate specific nuclear receptors producing characteristic malformations in infants involving the ears, heart, brain, and thymus. There has been concern about the use of topical retinoids during pregnancy. A recent meta-analysis ruled out a major increase in adverse pregnancy outcomes associated with use of topical retinoids, but the authors concluded that the statistical power of the analysis was inadequate to justify the use of topical retinoids during pregnancy.

Antiepileptic Drugs

Most current antiepileptic drugs (AEDs) have been shown to cause birth defects, cognitive impairment, and fetal death. AEDs including phenytoin, carbamazepine, topiramate, and valproic acid are considered human teratogens. Other AEDs including gabapentin, lamotrigine, oxcarbazone, levetiracetam, and zonizamide may be safer alternatives to the older AEDs or topiramate.

Fetal hydantoin syndrome comprises craniofacial abnormalities, limb defects, growth deficiency, and mental retardation. This syndrome has been reported to occur in 5% to 10% of exposed offspring. Putative mechanisms underlying the teratogenicity of phenytoin include folic acid deficiency and oxidative damage of tissue macromolecules.

Spina bifida aperta occurred in offspring of mothers who had taken valproic acid (2-propylpentanoic acid) during the first trimester of pregnancy. Valproic acid may alter expression of several genes that confer sensitivity. Inhibition of histone deacetylase and interference with folate metabolism may play a role in its teratogenicity.

Epidemiology of Human Developmental Toxicity

Reproductive epidemiology studies associations between specific exposures of the father or pregnant woman and her conceptus and the outcome of pregnancy. Associations are sought through either a case–control or a cohort approach. Accurate ascertainment of abnormal outcomes and exposures and a large enough effect and study population to detect an elevated risk are required. Another challenge is the fact that pregnancy failures related to a particular exposure may go undetected in the general population. Furthermore, availability of prenatal diagnostic procedures leads to abortion of some pregnancies with malformed embryos (particularly neural tube defects). Thus, the incidence of abnormal outcomes at birth may not reflect the true rate of abnormalities, and the term prevalence, rather than incidence, is preferred when the denominator is the number of live births rather than total pregnancies.

Epidemiologic studies of abnormal reproductive outcomes usually have three objectives: the first is scientific research into the causes of abnormal birth outcomes and usually involves analysis of case reports or clusters, a second aim is prevention and is targeted at broader surveillance of trends by birth defect registries around the world, and the last objective is informing the public and providing assurance.

PRINCIPLES OF DEVELOPMENTAL TOXICOLOGY

Some basic principles of teratology suggested in 1959 are listed in Table 10–2. Despite significant advances in our knowledge of and approaches to the study of teratology, Wilson's principles remain basic to developmental toxicology.

TABLE 10–2 Wilson's General Principles of Teratology

I.	Susceptibility to teratogenesis depends on the genotype of the conceptus and the manner in which this interacts with adverse environmental factors.
II.	Susceptibility to teratogenesis varies with the developmental stage at the time of exposure to an adverse influence.
III.	Teratogenic agents act in specific ways (mechanisms) on developing cells and tissues to initiate sequences of abnormal developmental events (pathogenesis).
IV.	The access of adverse influences to developing tissues depends on the nature of the influence (agent).
V.	The four manifestations of deviant development are death, malformation, growth retardation, and functional deficit.
VI.	Manifestations of deviant development increase in frequency and degree as dosage increases, from the no effect to the totally lethal level.

Data from Wilson JG. Experimental studies on congenital malformations. *J Chronic Dis*. 1959;10:111–130; Wilson JG. *Environment and Birth Defects*. New York: Academic Press; 1973.

Critical Periods of Development and Susceptibility to Toxicity

Development is characterized by rapid changes in size, shape, biochemistry, physiology, and functionality that are the consequence of sequential patterns of gene expression under the control of a cascade of epigenetic factors, the first of which are present in the egg prior to fertilization. Maternal factors active in the zygote and very early embryo activate the embryonic genome, and sequential gene activation continues throughout development. The embryo/fetus as a target for toxicity is continually changing as development proceeds. Comparative timing of developmental events in humans and some common laboratory species is presented in Table 10–3.

TABLE 10–3 Timing of Key Developmental Events in Some Mammalian Species*

	Rat	Rabbit	Monkey	Human
Blastocyst formation	3–5	2.6–6	4–9	4–6
Implantation	5–6	6	9	6–7
Organogenesis	6–17	6–18	20–45	21–56
Primitive streak	9	6.5	18–20	16–18
Neural plate	9.5	—	19–21	18–20
First somite	10	—	—	20–21
First branchial arch	10	—	—	20
First heartbeat	10.2	—	—	22
10 Somites	10–11	9	23–24	25–26
Upper limb buds	10.5	10.5	25–26	29–30
Lower limb buds	11.2	11	26–27	31–32
Testes differentiation	14.5	20	—	43
Heart septation	15.5	—	—	46–47
Palate closure	16–17	19–20	45–47	56–58
Urethral groove closed in male	—	—	—	90
Length of gestation	21–22	31–34	166	267

*Developmental ages are days of gestation.

Gametogenesis is the process culminating in formation of the haploid germ cells or gametes, the egg, and sperm. Male and female gametes fuse during fertilization to form the diploid *zygote*, or one-celled embryo. The maternal and paternal genomes are not functionally equivalent in their contributions to the genome of the zygote. The process of *imprinting* during gametogenesis confers on some genes a differential expressivity based on whether they are of maternal or paternal origin. Imprinting, which involves cytosine methylation and changes in chromatin conformation, may be susceptible to toxicants that affect these targets.

Epigenetics refers to the biochemical changes that control chromatin conformation and gene expression. Epigenetic factors include DNA methylation, histone modifications, and expression of noncoding RNAs. While epigenetics provides the basis for imprinting of genes, epigenetic marks may be erased and reestablished primarily during two periods of development. One is during migration and proliferation of the primordial germ cells when imprinted genes are

demethylated, followed by gender-specific remethylation during gametogenesis in offspring. Widespread epigenetic reprogramming also occurs after formation of the zygote and in the early embryo, with total genomic methylation being lowest at the early blastocyst stage.

The *preimplantation* period comprises mainly an increase in cell number through a rapid series of cell divisions (*cleavage* of the zygote) with little growth in size. A stage termed the *blastocyst* follows, consisting of about a thousand cells surrounding a fluid-filled central cavity. Some cells from the blastocyst give rise to the embryo proper, whereas other cells give rise to membranes, trophoblast, and placenta. The fates of cells in the early embryo are not rigidly determined, and the preimplantation embryo has great restorative (regulative) growth potential.

Toxicity during the preimplantation period results in either no or slight effect on growth (because of regulative growth recovery) or death (through insurmountable damage or failure to implant). Following implantation, g*astrulation* is the process by which the trilaminar embryo is formed. *Ectoderm*, *mesoderm*, and *endoderm* are the resultant three germ layers. During gastrulation, cells migrate through a midline groove called the *primitive streak*, and this cell migration sets up morphogenetic fields in the embryo. Gastrulation is highly susceptible to teratogenesis. Teratogen exposure during gastrulation may produce anterior malformations of the eye, brain, and face, indicative of damage to the anterior *neural plate*, one of the regions defined by cellular migration during gastrulation.

The formation of the neural plate in the midline ectoderm marks the onset of *organogenesis*. This period extends from about the third to the eighth weeks of gestation in humans and is highly susceptible to teratogenesis. The embryo undergoes dramatic changes during this brief period, proceeding in the human from a few cell types in a trilaminar arrangement indistinguishable from most other vertebrate embryos, to a fetus clearly recognizable as human. The rapid changes during organogenesis require cell proliferation and migration, cell–cell interactions, and morphogenetic tissue remodeling. The *neural crest* cells originate at the border of the neural plate and migrate to form diverse structures throughout the embryo. There are periods of peak susceptibility for each forming structure as each malformation coincides with the timing of key developmental events in the affected structure. In general, morphogenesis proceeds from anterior to posterior in the embryo.

The *fetal period*, from about day 57 to birth in humans, comprises tissue differentiation, growth, and maturation. Most organs are present and grossly recognizable, with further development/maturation during the fetal period. Toxic exposure during the fetal period is most likely to result in effects on growth and function. Functional anomalies of the central nervous system and reproductive organs—including behavioral, mental, and motor deficits as well as decreases in fertility—are among the possible adverse outcomes. Functional deficits may not be apparent prenatally and may require postnatal observation and testing. Major structural alterations can occur during the fetal period, usually *deformations* of previously normal structures.

Dose–Response Patterns and Thresholds for Induction of Developmental Toxicity

The relationships between growth retardation, malformations, and mortality in developmental toxicity studies are complex. These endpoints may represent a continuum of toxicity, with growth retardation, malformations, and death occurring with increasing dosage.

The shape of the dose–response curve at low exposures is also important. The mammalian embryo possesses high restorative growth potential and cellular homeostatic mechanisms, which, along with maternal metabolic defenses, contribute to the conventional wisdom that there is a maternal dosage below which no increase in an adverse outcome will occur. Lack of a threshold implies that exposure to any amount of a toxic chemical, even one molecule, can cause developmental toxicity.

For risk assessment, it is important to consider the distinction between individual and population thresholds. There is variability in sensitivity among humans, and the threshold for a population can be defined as the threshold for the most sensitive individual in the population. Although the biological target of a developmental toxicant may exhibit a threshold, background factors such as preexisting conditions or other exposures may leave individuals already at or even beyond the threshold for failure of that biological process.

MECHANISMS AND PATHOGENESIS

Mechanisms of teratogenesis include, but are not limited to, mutations, chromosomal breaks, altered mitosis, altered nucleic acid integrity or function, diminished supplies of precursors or substrates, decreased energy supplies, altered membrane characteristics, osmolar imbalance, and enzyme inhibition. Such cellular insults may quickly trigger pathogenetic responses in the embryo, including reduced cell proliferation, cell death, altered cell–cell interactions and signaling, reduced biosynthesis, inhibition of morphogenesis, or mechanical disruption of development.

The Molecular Basis of Dysmorphogenesis

Pathways of normal and abnormal development are being elucidated using molecular biology, genomics, proteomics, bioinformatics, tissue engineering, and computer modeling. Targeted gene disruption by homologous recombination (gene “knockout”) has been used to study the loss-of-function of many gene products in developing and adult animals.

In addition to directly changing the DNA sequence using homologous recombination, it is also possible to modulate gene expression using synthetic oligonucleotides. Antisense oligonucleotides, with 15 to 25 nucleotides that are complementary to the mRNA to be disrupted can be synthesized. These probes can enter embryonal cells, hybridize with cellular mRNA, and disrupt the native message, such that gene function can be turned off at specific times. RNA interference is a gene knockdown technique, whereby small interference (si)RNA and plasmid- and virus-encoded small RNAs can be used to downregulate the expression of specific genes posttranscriptionally. Off-target binding can occur, and appropriate controls of scrambled sequences or an unmodified vector are essential. Determining the level of mRNA change is critical because it is possible to decrease (knockdown) the level of mRNA without knocking it out. Additionally, the protein is usually the functional molecule, so it is essential to understand the relationship between changes in mRNA and protein when interpreting results. A transient decrease in mRNA may not change the level of functional protein.

Gain of gene function can be studied by engineering genetic constructs with an appropriate promoter attached to the gene of interest. Ectopic gene expression can be ubiquitous or tissue-

specific depending on the promoter selected to drive expression. Transient overexpression of specific genes can be accomplished using adenoviral transduction.

Advances in gene targeting and transgenic strategies now allow modification of gene expression at specific points in development and in specific cell types. Conditional knockouts, knockdowns or knockins, inducible gene expression, and other techniques are being used to study the effects of specific gene products on development.

Understanding the developmental consequences of altered gene expression requires understanding the temporal and spatial regulation of expression. Early studies of gene expression using in situ mRNA hybridization in tissue slices were supplanted by those using whole-mount techniques. In another approach, reporter transgenes containing a gene with a readily detectable product fused downstream of a selected regulatory region have been used to temporally map gene expression. The *E. coli lacZ* (β-galactosidase) gene is commonly used for this purpose. The expression pattern of a gene can be localized by fusing the gene's upstream regulatory elements to *lacZ*, which will then be transcribed under control of those upstream elements. Cell lineage studies can be carried out by fusing *lacZ* to a constitutive regulatory sequence and introducing the construct into a somatic cell early in development. The reporter gene will then be expressed in and mark all progeny of the transfected cell. This method has been used to study postimplantation development in the mouse embryo.

MATERNAL ADAPTATIONS, METABOLISM, AND TOXICOKINETICS IN PREGNANCY

In determining the impact of chemical exposure on development, the route by which the mother is exposed, the timing of the exposure, and the extent to and from in which the chemical reaches the conceptus are important determinants. The maternal, placental, and embryonic compartments are distinct but interacting systems that undergo profound changes during pregnancy.

Maternal Adaptations to Pregnancy

Maternal adaptations to pregnancy include changes in water and lipid content, hepatic metabolism, the gastrointestinal tract, cardiovascular system, excretory system, and the respiratory system. These changes to maintain adequate nutrition and eliminate waste products from the conceptus significantly impact the uptake, distribution, metabolism, and elimination of xenobiotics. For example, decreases in intestinal motility and increases in gastric emptying time result in longer retention of ingested chemicals in the upper gastrointestinal tract. Cardiac output in humans increases by 30% to 40% by the 27th week of pregnancy, while blood volume increases and plasma protein concentration and peripheral vascular resistance decrease. The relative increase in blood volume over red cell volume leads to borderline anemia and a generalized edema with about 70% elevation of extracellular space. Thus, the volume of distribution of a chemical and the amount bound by plasma proteins may change considerably during pregnancy. Renal blood flow and glomerular filtration rate also increase during

pregnancy. Increases in tidal volume, minute ventilation, and minute O_2 uptake can result in increased pulmonary distribution of gases and decreased time to reach alveolar steady state. All of these maternal factors determine the amount of chemical and metabolites available to the embryo and potential embryotoxicity.

Role of the Placenta

The placenta plays a central role in transferring nutrients and waste products between the conceptus and mother. The extent of transfer depends on three major factors: the type of placentation, the physicochemical properties of the chemical, and rates of placental metabolism. The passage of most drugs across the placenta seems to occur by simple passive diffusion. Important modifying factors to the rate and extent of transfer include lipid solubility, molecular weight, protein binding, the type of transfer (passive diffusion, facilitated, or active transport), the degree of ionization, and placental metabolism. Placental cells express mdr1a also known as P-glycoprotein (P-gp).

Maternal and Conceptus Xenobiotic Metabolism

The amount of xenobiotic, its form (parent or metabolite), and timing of delivery to the embryo are important components of understanding species differences in sensitivity. Hepatic drug metabolizing systems in humans can be categorized into three classes based on the developmental trajectory of their expression. Class I enzymes appear during the first trimester, remain elevated or decrease during gestation, and drop to low or no expression postnatally; Class II enzymes are expressed at a consistent level throughout gestation through adulthood; and Class III enzymes are nondetectable or low in the fetus, with expression increasing during 1 to 2 years postnatally and full expression not occurring until puberty for some Class III enzymes.

Physiologically Based Pharmacokinetic Models of Pregnancy

Physiologically based pharmacokinetic (PBPK) models provide the framework to integrate what is known about xenobiotic metabolism with physiology during pregnancy and embryo/fetal development into a quantitative description. A life-stage PBPK model was developed and integrated with adverse outcome pathways (AOPs) for developmental toxicity related to vasculogenesis, in vitro assays relevant to AOPs, and in vitro to in vivo extrapolation models as an approach to screening and prioritizing chemicals for potential risk. The PBPK model described chemical disposition during pregnancy, fetal, neonatal, infant, and adult stages to estimate a maternal exposure that would produce fetal blood concentrations similar to those producing toxicity in the in vitro assays. This estimate could be compared to maternal environmental exposure estimates to derive a margin of exposure.

RELATIONSHIPS BETWEEN MATERNAL AND DEVELOPMENTAL TOXICITY

All developmental toxicity ultimately results from insult to the conceptus at the cellular level, but in mammalian embryos the insult may occur through a direct effect on the embryo/fetus, indirectly through toxicity of the chemical to the mother or the placenta, or a combination of direct and indirect toxicity. Maternal physiological conditions with the potential to adversely affect the conceptus include decreased uterine blood flow, anemia, malnutrition, toxemia, altered organ function, autoimmune states, diabetes, electrolyte or acid–base disturbances, and elevated stress hormones. Some conditions that contribute to abnormal development are depicted in Fig. 10–1.

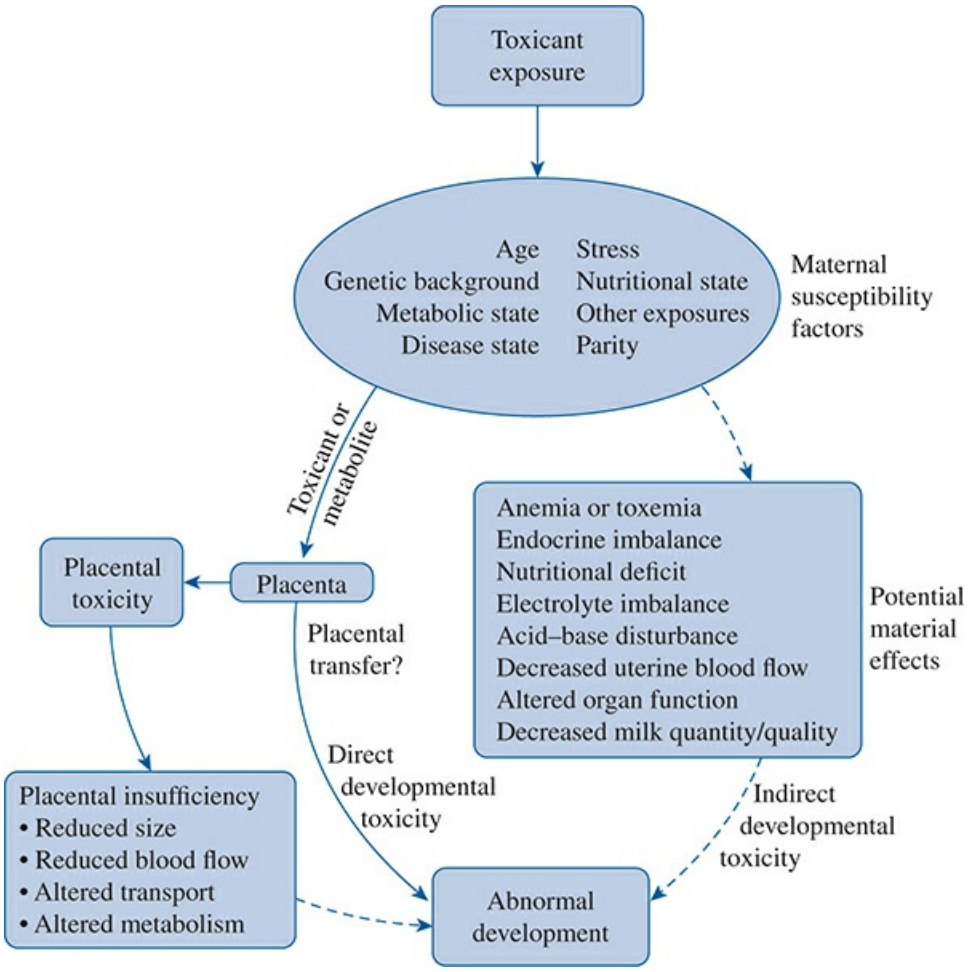

FIGURE 10–1 *Interrelationships between maternal susceptibility factors, metabolism, induction of maternal physiologic or functional alterations, placental transfer and toxicity, and developmental toxicity.* A developmental toxicant can cause abnormal development through any one or a combination of these pathways. Maternal susceptibility factors determine the predisposition of the mother to respond to a toxic insult, and the maternal effects listed can adversely affect the developing conceptus. Most chemicals traverse the placenta in some form, and the placenta can also be a target for toxicity. In most cases, developmental toxicity is probably mediated through a combination of these pathways.

Distinguishing between direct and indirect developmental toxicity in standard developmental toxicity studies can be critical for interpreting results, as measurable maternal toxicity (e.g.,

decreased food or water intake, weight loss, clinical signs) is expected by design at the highest dosage level used. An understanding of the physiology underlying observed maternal toxicity is needed before one can begin to address the relevance of the observations for human risk assessment.

Maternal Factors Affecting Development

Genetic Background—The genetic makeup of the pregnant female has been well documented as a determinant of developmental outcome in both humans and animals. The incidence of cleft lip and/or palate (CL(P)), which occurs more frequently in Caucasians than in African Americans, has been investigated in offspring of interracial couples in the United States. Offspring of white mothers had a higher incidence of CL(P) than offspring of black mothers after correcting for paternal race. The incidence was similar in fathers of both races after correcting for maternal race. Thus, maternal race (and presumable genotype) was the factor associated with differential risk.

Disease—Several maternal conditions and diseases can exert untoward effects during pregnancy. Chronic hypertension, uncontrolled maternal diabetes mellitus, and certain maternal infections can adversely affect the conceptus (e.g., rubella virus, cytomegalovirus, *Toxoplasma gondii,* and the Zika virus). Maternal hyperthermia with febrile illness during the first trimester of pregnancy is associated with birth defects in humans, most notably neural tube defects, heart defects, and oral clefts.

Nutrition—Dietary insufficiencies ranging from protein-calorie malnutrition to deficiencies of vitamins, trace elements, and/or enzyme cofactors adversely affect pregnancy. Women at risk of having infants with neural tube defects (NTDs) were supplemented with folate, which may reduce neural tube defects by functioning as a methyl donor for DNA methylation.

Stress—Diverse forms of maternal toxicity likely have in common the induction of a physiologic stress response. Subjecting pregnant rats or mice to noise stress throughout gestation can produce developmental toxicity. Restraint stress produces increased fetal death in rats and cleft palate, fused and supernumerary ribs, and encephaloceles in mice.

Maternal and Placental Toxicity

The mother or the placenta may be the primary target for some developmental toxicants, with effects on the developing embryo/fetus occurring secondarily due to changes in the intrauterine environment or the nutrient/gas/waste exchange capabilities of the placenta.

Maternal Toxicity—Several studies relate specific elements of maternal toxicity to developmental toxicity, including those in which the test chemical caused maternal effects that exacerbated the chemical’s developmental toxicity, and instances in which developmental toxicity was thought to be secondary to maternal effects. Clearly delineating the relative role(s) of indirect maternal and direct embryo/fetal toxicity is difficult.

Diflunisal, an analgesic and anti-inflammatory drug, causes axial skeletal defects in rabbits. Developmentally toxic dosages resulted in severe maternal anemia and depletion of erythrocyte

ATP. Teratogenicity, anemia, and ATP depletion were unique to the rabbit among the species studied. The teratogenicity of diflunisal seen in the rabbit was probably due to persistent hypoxia resulting from maternal anemia.

The anticonvulsant phenytoin can affect maternal folate metabolism in laboratory animals, which may play a role in its teratogenicity. A mechanism of teratogenesis relating depressed maternal heart rate to embryonic hypoxia was proposed. Supporting studies have demonstrated that hyperoxia reduced the teratogenicity of phenytoin in mice.

Placental Toxicity—The placenta produces peptide and steroid hormones critical to the maintenance of pregnancy, the regulation of fetal and maternal metabolism, and metabolism or storage of xenobiotics. The placenta can undergo adaptive responses to the intrauterine milieu, and that such responses can influence the metabolic "programming" of the fetus. Adverse conditions including hypoxia and nutritional deficiencies can lead to alterations in placental structure and function including altered expression or activity of transporters and receptors.

DEVELOPMENTAL TOXICITY OF ENDOCRINE-DISRUPTING CHEMICALS

An "endocrine disruptor" has been broadly defined as "an exogenous agent that interferes with the production, release, transport, metabolism, binding, action, or elimination of natural hormones responsible for the maintenance of homeostasis and the regulation of developmental processes." Given the critical role of hormones in directing differentiation of many tissues, the developing organism is particularly vulnerable to exposure to chemicals with hormonal or antihormonal activity. Numerous chemical classes (e.g., pesticides, herbicides, fungicides, plasticizers, surfactants, organometals, halogenated polyaromatic hydrocarbons, and phytoestrogens) have been shown to cause developmental toxicity by (1) acting as hormone receptor ligands, (2) altering hormone synthetic and metabolic enzymes, or (3) perturbing hypothalamic-pituitary release of tropic hormones. Interactions with the functions of estrogens, androgens, and thyroid hormones have been the most studied.

Laboratory Animal Evidence

Chemicals with estrogenic activity induce pleiotropic effects, acting on diverse cell types with estrogen receptors, and can display cell- and organ-specific agonist and antagonist actions. The pattern of outcomes is generally similar across different estrogenic chemicals. Female offspring are generally more sensitive than males and altered pubertal development, reduced fertility, and reproductive tract anomalies are common findings.

Effects of developmental exposure to antiandrogenic chemicals are generally restricted to males, and include hypospadias, retained nipples, reduced testes and accessory sex gland weights, and decreased sperm production.

Hypothyroidism during pregnancy and early postnatal development causes growth retardation, cognitive deficits, delayed eye opening, hyperactivity, and auditory defects in rodents. Mild thyroid deficiency during critical periods can have permanent adverse effects on brain development. Mild developmental thyroid insufficiency induces cortical heterotopia in rats

and compromises activity-dependent neuroplasticity in the hippocampus of male rat offspring as adults.

Human Evidence

The extent to which human health is being adversely impacted from exposures to environmental endocrine disruptors remains unclear. Reports in humans, which are or may be relevant, are of two types. The first type of report includes observations of adverse effects on reproductive system development and function following exposure to chemicals with known endocrine activities that are present in medicines, contaminated food, or the workplace. These have tended to involve relatively high exposures to chemicals with known endocrine effects. The second type of report relates to epidemiologic evidence of increasing incidence of adverse reproductive and developmental outcomes having an endocrine basis. Increasing trends have been reported for cryptorchidism, hypospadias, semen quality, and testicular cancer. Connections between environmental exposures and early onset of puberty in girls have been suggested. A systematic review and meta-analysis of 185 studies across 40 years, 50 different countries, and 42,935 men reported that sperm counts declined significantly between 1973 and 2011 in men from North America, Europe, Australia, and New Zealand.

Considerations of Endocrine Disruption in Screening and Testing

The findings of altered reproductive development following early-life exposures to endocrine disruptors prompted revisions of safety evaluation tests. These include assessments of female estrous cyclicity, sperm parameters (total number, percent progressively motile, and sperm morphology in both the parental and F1 generations), the age at puberty in the F1 (vaginal opening in the female, preputial separation in the males); an expanded list of organs for pathology and/or histopathology to identify and characterize effects; as well as some triggered endpoints including anogenital distance in the F2 and primordial follicle counts in the parental and F1 generations. An important modification of prenatal developmental toxicity test guidelines is the extension of the period of dosing from the period of organogenesis (i.e. palatal closure) to near the end of pregnancy in rodents in order to encompass the developmental period of urogenital tract differentiation.

REGULATORY ASSESSMENT OF RISK OF DEVELOPMENTAL TOXICITY

Experience with chemicals that have the potential to induce developmental toxicity in humans indicates that laboratory animal testing, surveillance of the human population (i.e., epidemiologic studies), and alert clinical awareness are all necessary to provide adequate public health protection. Laboratory animal investigations are guided by both regulatory requirements for drug or chemical marketing and the need to understand mechanisms of toxicity.

Regulatory Guidelines for In Vivo Testing

The International Conference of Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) allows considerable flexibility in implementation depending on particular circumstances of the chemical under evaluation. Key elements of the FDA Segment I, II, and III studies, the ICH protocols, the OECD equivalent of the FDA Segment II test, and the OECD-extended one-generation reproductive toxicity study (EOGRTS) are provided in Table 10–4. Variations in these protocols include extensions of exposure to earlier or later time points in development and observations to postnatal ages with more sophisticated endpoints. Standard regulatory studies identify a No Observed Adverse Effect Level (NOAEL), the highest dosage level that does not produce a significant increase in adverse effects in offspring or juvenile animals.

TABLE 10–4 Summary of In Vivo Regulatory Protocol Guidelines for Evaluation of Developmental Toxicity

Study	Exposure	Endpoints Covered	Comments
Segment I: Fertility and general reproduction study	♂: 10 weeks prior to mating ♀: 2 weeks prior to mating	Gamete development, fertility, pre- and postimplantation viability, parturition, lactation	Assesses reproductive capabilities of ♂ and ♀ following exposure over one spermatogenic cycle or several estrous cycles
Segment II: Teratogenicity test	Implantation (or mating) through end of organogenesis (or term)	Viability and anatomy (external, visceral, skeletal) of fetuses prior to birth	Shorter exposure prevents metabolic adaptation and provides high exposure during gastrulation and organogenesis. Earlier dosing option for bioaccumulative agents. Later dosing to cover reproductive tract development
Segment III: Perinatal study	Last trimester of pregnancy through lactation	Postnatal survival, growth, and external morphology	To observe effects on development of major organ function during the perinatal period; may be more sensitive to adverse effects at this time
ICH 4.1.1: Fertility protocol	♂: 4 weeks prior to mating ♀: 2 weeks prior to mating	♂: Reproductive organ weights and histology, sperm counts, and motility ♀: Viability of conceptuses at mid-pregnancy or later	Improved assessment of ♂ reproductive endpoints; shorter treatment duration than Segment I
ICH 4.1.2: Prenatal and postnatal development, including maternal function	Implantation through end of lactation	Toxicity to pregnant vs. nonpregnant; postnatal viability, growth, and function (behavior, maturation, reproduction)	Similar to Segment I study
ICH 4.1.3: Embryo/fetal development	Implantation through end of organogenesis	Viability and morphology (external, visceral, skeletal) of fetuses prior to birth	Similar to Segment II study. Usually conducted in two species (rodent and nonrodent)
OECD 414: Prenatal developmental	Implantation (or mating) through day prior to cesarean section	Viability and morphology (external, visceral, skeletal) of fetuses prior to birth	Similar to Segment II study. Usually conducted in two species (rodent and nonrodent)
OECD 443: Extended one-generation reproductive toxicity study	♂, ♀: 2 weeks or more prior to mating ♀: Through gestation and lactation ♂; Continue for 10 weeks	Clinical pathology and histopathology of parents and F_1; reproductive organ integrity, DNT, DIT on F_1 cohorts; triggers for F_2 production and testing	Preferred exposure route is dietary; replacement for two-generation study, using more endpoints and reducing animal use; technically challenging; flexible. See citations in text for details

DIT, developmental immunotoxicity tests; DNT, developmental neurotoxicity tests.

Multigeneration Tests

Information pertaining to developmental toxicity can also be obtained from studies in which animals are exposed to the test substance continuously over one or more generations.

Concordance of Data Between Laboratory Animals and Humans

Several reviews of the similarity of responses of laboratory mammals and humans to developmental toxicants support the assumption that results from laboratory tests are predictive of potential human effects. Concordance is strongest when there are positive data from more than one test species. In a quantitative sense, the few comparisons that have been made suggest that humans tend to be more sensitive to developmental toxicants than the most sensitive test species.

Children's Health and the Food Quality Protection Act

Infants and children differ both qualitatively and quantitatively from adults in their exposure to pesticide residues in food because of different dietary composition and intake patterns. Children have activity patterns that change their exposure profile compared to adults, such as crawling on the floor or ground, putting their hands and foreign objects in their mouths, and raising dust and dirt during play. Even the level of their activity (i.e., closer to the ground) can affect their exposure to some toxicants. Growing and developing makes children more susceptible to some types of insults. Effects of early childhood exposure, including neurobehavioral effects, obesity, diabetes, cardiovascular disease, and cancer, may not be apparent until later in life.

Alternative Testing Strategies

Various alternative test systems have been proposed to refine, reduce, and replace whole animal regulatory tests for developmental toxicity. Alternative assays use cultures of cells, developing organs/structures, and whole embryos in vitro, including those of worms, flies, frogs, fish, and mammals and short term in vivo tests. Considering the complexity of embryogenesis and the multiple cellular and molecular mechanisms and target of potential teratogens, it may be unrealistic to expect a single test, or even a small battery of tests, to accurately predict the potential developmental activity of chemicals.

The In Vivo Chernoff–Kavlock Assay—Rodents are used for prescreening chemical-induced developmental toxicity, which streamlines risk assessment. Pregnant females are exposed to a limited number of test compound dosages during the period of major organogenesis. Dosages are selected to be near those inducing maternal toxicity. Assessment focuses on evaluating the offspring over a brief neonatal period for external malformations, growth, and viability. This test has proven reliable to identify developmental toxicants of many chemical agents and classes.

Rodent and Rabbit Whole Embryo Culture—The WEC model for assessing developmental toxicity directly exposes mammalian embryos to the compound of interest and then distributes embryos in the same litter to different treatment groups. Disadvantages include the technically difficult and time-consuming process of excising the embryos and scoring them at the end of culture, and the cost in animals for obtaining the embryos and the rat serum used as culture medium. As an alternative or in addition to morphological scoring, effects of chemicals on gene expression in the embryos have been studied to discern common patterns ("signatures") of transcriptomic effects. There are key signaling pathways and intermediates in embryological development that hold promise as potential biomarkers of developmental toxicity.

Mouse Embryonic Stem Cell Tests—Mouse embryonic stem cell tests (mESTs) examine effects of test chemicals on viability and differentiation of mouse embryonic stem cell lines. These mESTs use established cell lines, and not live animals. They are also fairly rapid and inexpensive. One disadvantage is that these stem cell models evaluate embryonic cells during an early window of development and do not represent the full spectrum of cellular and molecular mechanisms and targets throughout embryogenesis. Human embryonic stem cell lines and induced pluripotent stem cells are also being developed for toxicity testing.

The Zebrafish—Submammalian species have been used for many years in the study of normal

developmental biology, and the African clawed frog, *Xenopus laevis* or *X. tropicalis*, and the zebrafish, *Danio rerio*, have been used for developmental toxicology. Advantages include the rapid external development of the embryos, a large extant literature on their normal development, and the availability of genetic mutants and molecular biological tools for studying these embryos. They can be bred to produce large numbers of embryos in a relatively short period and are easy and inexpensive to maintain.

In recent years, the zebrafish has emerged as the prominent submammalian species in use for developmental toxicity testing. These small fish exhibit a great deal of homology to higher vertebrates in their development, anatomy, physiology, and behavior. Their rapidly developing embryos are transparent, allowing visualization of internal anatomy.

ToxCast—The Toxicity Forecaster (ToxCast) program at the EPA web-site, https://www.epa.gov/chemical-research/toxicity-forecasting, includes hundreds of high-throughput assays directed at molecular and cellular targets of toxicity, a number of which are relevant to developmental toxicity.

Validation of Alternative Approaches to Testing for Developmental Toxicity—Continued development of in vitro platforms for developmental toxicity screening requires proper validation of such assays. One approach is to develop lists of "positive" and "negative" reference chemicals and then test the ability of assays to properly categorize these. However, this approach does not consider how concentrations of the chemicals in vitro compare to toxic blood or tissue concentrations in vivo. To address this gap, an approach that classifies chemical *exposures* (including concentrations) rather than chemicals per se as positive or negative uses available data on toxic internal (e.g., blood or tissue) levels in vivo (human or test species) to characterize the chemical exposure as positive or negative, such that a given chemical can be a positive at one concentration and a negative at a lower concentration. Further, physiologically based chemical kinetic models can be added to predict in vivo target dose with in vitro toxic concentrations, thereby permitting in vitro to in vivo extrapolation.

Structure Activity and Read Across for Developmental Toxicity—Relationships between specific chemical structures or structural components and their effects on developing organisms are being elucidated, increasing confidence in our ability to predict developmental toxicity based on chemical structure alone. Read-across methods for predicting developmental toxicity of related chemicals can be employed using shared chemical structural components, or commonalities in their effects on the transcriptome.

Contemporary and Emerging Elements of Developmental Toxicity Risk Assessment

The development of the benchmark-dose approach has provided a clear improvement to the NOAEL, and approaches such as the development of biologically based dose–response models and adverse outcome pathways begin to piece together the early events in the etiology of abnormal development, in the hope that such knowledge will facilitate both more scientifically plausible risk assessments and relevant high-throughput assays based on key events leading to elucidated pathways.

The Benchmark Dose versus the No Observed Adverse Effect Level (NOAEL)—The use of safety or uncertainty factors applied to an experimentally derived NOAEL to arrive at a presumed safe level of human exposure is predicated on the risk assessment assumption that a threshold for developmental toxicity exists. A threshold should not be confused with the NOAEL, as the NOAEL is dependent entirely on the power and design of the study. Nor should the value obtained by the application of uncertainty factors to the NOAEL be confused with a threshold, as this exposure is only assumed to be without appreciable added risk.

Use of the NOAEL in risk assessment has been criticized for several reasons. Because it is dependent on statistical power to detect pair-wise differences between a treated and a control group, the use of larger sample sizes and more dose groups (which might better characterize the dose–response relationship) can only yield lower NOAELs, and thus better experimental designs are actually penalized by this approach. In addition, the NOAEL is limited to an experimental dose level, and an experiment might need to be repeated to develop a NOAEL for risk assessment. Given varying experimental designs and variability of control values, NOAELs actually represent different levels of risk in different studies.

A mathematical model to estimate the lower confidence bounds on a predetermined level of risk, the “benchmark dose” (BMD), avoids many disadvantages of the NOAEL. Discrepancies between the benchmark dose and the NOAEL were most pronounced when one or more of the following conditions were present: a shallow dose–response, small sample sizes, wide spacing of experimental dosage levels, or more than the typical number of dose levels. These features tend to make determination of the NOAEL more problematic (usually higher) and the confidence limits around the maximum likelihood estimate broader (resulting in lower BMDs).

Biologically Based Dose–Response Models and Adverse Outcome Pathways—Statistical dose–response models for noncancer endpoints help reduce uncertainties in high-to-low dose and species-to-species extrapolation of experimental data. Biologically based dose–response models integrate pharmacokinetic information on target tissue dosimetry with molecular/biochemical responses, cellular/tissue responses, and developmental toxicity. Understanding the relative importance of various pathways of abnormal development provides a basis for models that incorporate species-specific response parameters.

Adverse outcome pathways (AOPs) are chemical-agnostic descriptions of the etiology of an adverse apical outcome such as a birth defect. The chemical or agent that causes the adverse effect is not stipulated. An AOP begins with the molecular initiating event, which occurs at the first interaction of the toxic moiety with the cell or organism. With adequate initiation, a pathogenic series of key events leads to an adverse outcome. The BBDR model for 5-FU in essence is an AOP, in which inhibition of thymidylate synthetase is the initiating event, digit agenesis is the adverse outcome, and reduced DNA synthesis and cell cycle blockage are key events.

PATHWAYS TO THE FUTURE

Many animals have been used extensively in developmental biology and genetics, including the fruit fly, roundworm, zebrafish, frog, chick, and mouse. These organisms are advantageous for developmental toxicity studies owing to their well-known genetics and embryology, their rapid generation time, and the ability to manipulate their genetics to probe specific developmental

pathways or to incorporate human genes, such as those of drug metabolizing enzymes, to answer questions of interspecies extrapolation.

Advances in Genomics, Proteomics, Metabolomics, and Bioinformatics

As our knowledge of the genetic and epigenetic control of normal development has progressed, so has the technology to examine gene expression and its control, networks of interrelated gene products, and changes in gene expression induced by alterations in the developmental environment. Advances in genomics, proteomics, metabolomics, and bioinformatics are being used to enhance our understanding of normal and abnormal development. The ability to gather and analyze large amounts of biological data using databases and models of biological systems allows a more comprehensive approach to understanding pathways to developmental toxicity. The model of a proposed AOP for embryonic vascular disruption includes potentially responsive gene networks that may be involved in responses to hypoxia, the angiogenic switch, extracellular matrix interactions, and vessel remodeling, as well as predicted downstream consequences of disrupting these networks (Fig. 10–2).

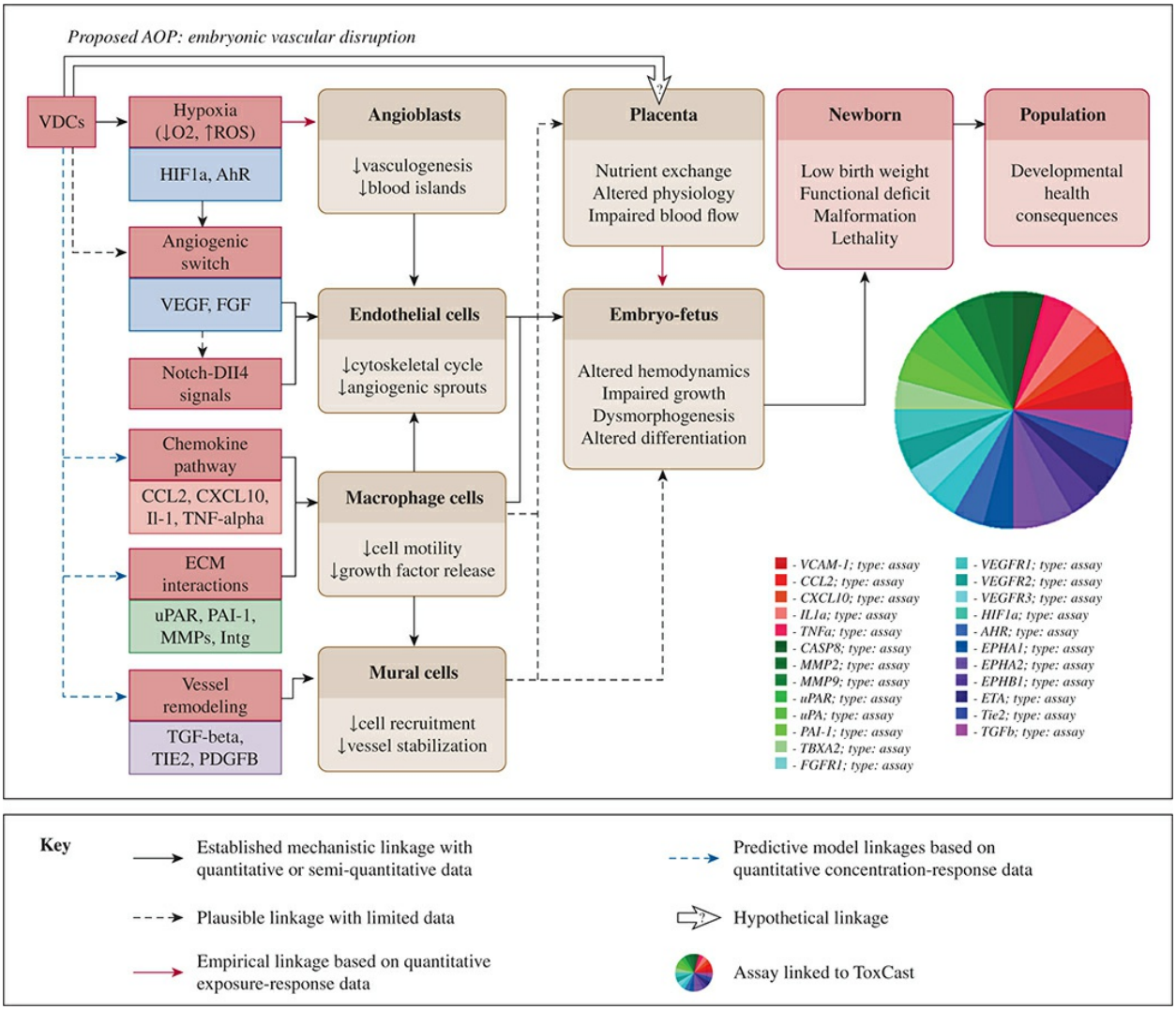

FIGURE 10–2 *Adverse outcome pathway (AOP) for embryonic vascular disruption*. This AOP for embryonic vascular disruption was built using high-throughput screening data from the EPA's ToxCast database. Anchor 1 (red boxes on the left side) addresses macromolecular interactions of the toxicant with elements of the pathway. Anchor 2 (red boxes on the right) refers to relevant organismal and population responses. The middle columns address intervening cellular and organ responses. The color wheel indicates sectors for 25 ToxCast assays that had evidence of abnormal embryonic vascular development based on genetic mouse models and mapped to previously identified critical pathways. VDC, vascular disrupting chemical. (Adapted from Knudsen TB, Kleinstreuer NC. Disruption of embryonic vascular development in predictive toxicology. *Birth Defects Res C Embryo Today*. 2011;93:312–323.)

Computational and Organotypic Models of Developing Systems

Software is being developed that can recapitulate development of tissues and organs based on the properties and relationships of cells. These so-called agent-based models (the cell is the agent) allow dynamic interactions among cells that through simulated morphogenesis can build good

facsimiles of embryological structures. These models contain signaling pathways, receptors, and extracellular matrices that invoke specific cell behaviors such as adhesion, migration, mitosis, apoptosis, and differentiation. The computational model of secondary palate fusion and cleft palate pathogenesis is illustrated in Fig. 10–3. Signaling pathways underlying palate development include TGF-β, BMP, FGF, EGF, SHH, noggin, and ephB. This signaling network drives the virtual palate model as diagrammed in Fig. 10–3A. The model begins at the prefusion stage at which the palatal shelves have elevated to the horizontal position, and the shelves consist of mesenchyme, basal epithelium and periderm, basement membrane, and extracellular matrix. The signaling pathways and gradients drive growth and fusion through contact of the medial edge epithelia and dissolution of the medial edge seam (Fig. 10–3B). Computational gene knockouts of signaling molecules can be executed to observe effects on phenotype (Fig. 10–3C), and predictions of the effects of chemical exposure during palatogenesis can be made based on knowledge of the underlying mechanisms of toxicity (Fig. 10–3D).

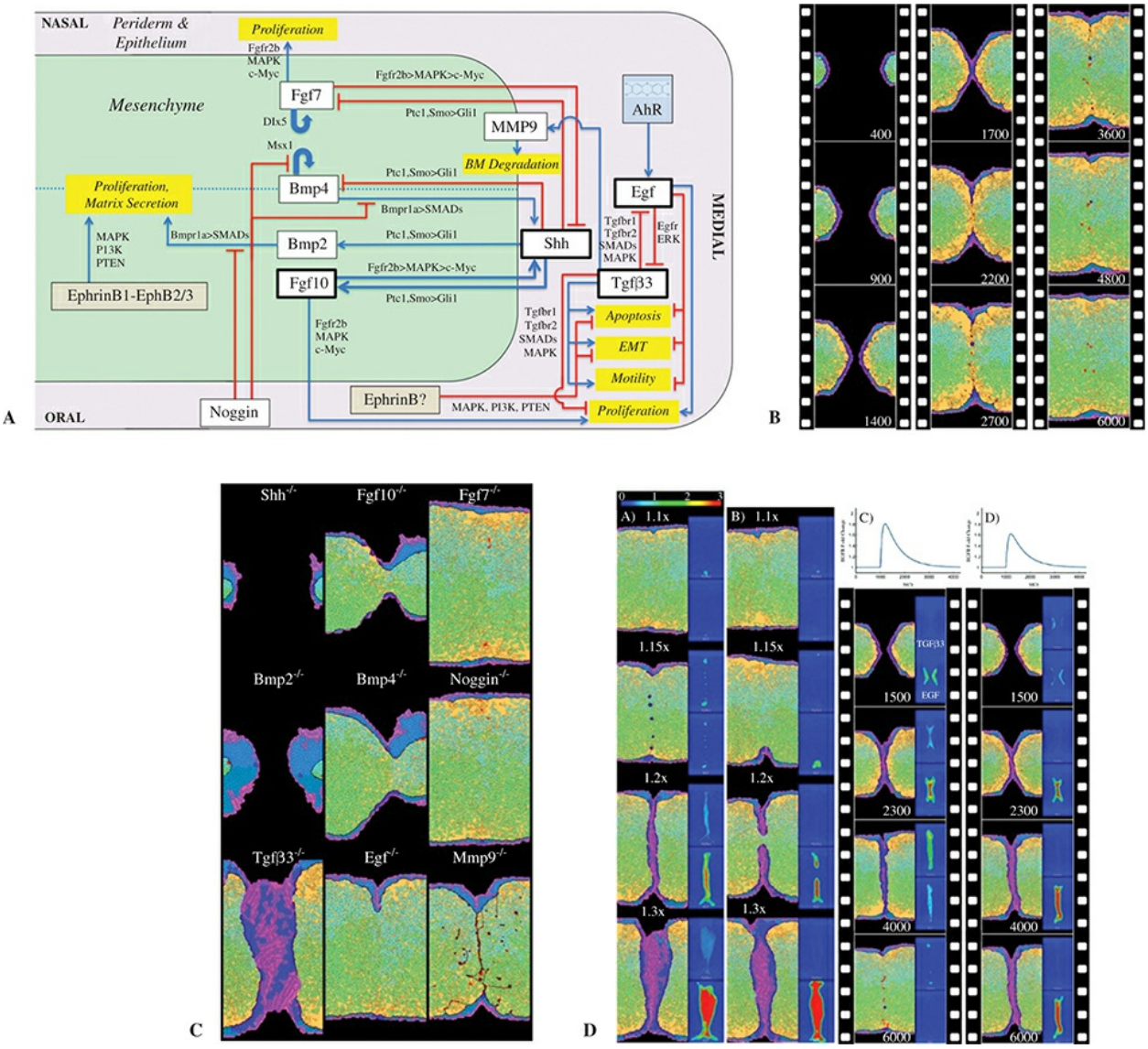

FIGURE 10–3 *Computational agent–based model of normal palate development and effects*

of virtual gene knockout or chemical toxicity. Temporally, the model begins with the palatal shelves elevated to the horizontal position. The model includes mesenchymal, epithelial, and periderm cells, as well as basement membrane and extracellular matrix. **A.** Signaling pathways and molecules incorporated into the model and driving cellular (agent) behavior and interactions. **B.** Model simulation of normal palatogenesis, exhibiting growth (*left column top to bottom*), medial contact of the palatal shelves (*middle column top to bottom*), and dissolution of the medial edge epithelium and fusion of the shelves (*right column top to bottom*). **C.** Effects of virtual (computational) knockout of the various signaling molecules shown in panel A. Effects range from lack of growth to lack of fusion to incomplete fusion to normal fusion. **D.** Simulated fusion defects induced by TCDD exposure via AhR-mediated fold changes in EGFR (fold changes indicated in columns A and B) and by all-trans retinoic acid (columns C and D) displayed for time-dependent changes in EGFR. (Reprinted with permission from Hutson MS, et al. Computational model of secondary palate fusion and disruption. *Chem Res Toxicol*. 2017;30:965–979).

In vitro techniques for developmental toxicology and other disease studies are progressing from two-dimensional cell culture systems to complex three-dimensional “organoid” cultures. This approach may provide systems having adequate complexity to recapitulate facets of morphogenesis from precursor cells not possible in current two-dimensional cell cultures. Several groups are currently developing “organ-on-a-chip” models of organogenesis.

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QUESTIONS

1. Diethylstilbestrol (DES):
   - **a.** was used to treat morning sickness from the 1940s to the 1970s.
   - **b.** was found to affect only female offspring in exposed pregnancies.
   - **c.** greatly affects the development of the fetal brain.
   - **d.** exposure increases the risk of clear cell adenocarcinoma of the vagina.
   - **e.** is now used to treat leprosy patients.

2. Early (prenatal) exposure to which of the following teratogens is most often characterized by craniofacial dysmorphism?

a. thalidomide.
b. retinol.
c. ethanol.
d. tobacco smoke.
e. diethylstilbestrol (DES).

3. The nervous system is derived from which of the following germ layers?
a. ectoderm.
b. mesoderm.
c. epidermal placodes.
d. paraxial mesoderm.
e. endoderm.

4. Toxicant exposure during which of the following periods is likely to have the LEAST toxic effect on the developing fetus?
a. gastrulation.
b. organogenesis.
c. preimplantation.
d. third trimester.
e. first trimester.

5. Regarding prenatal teratogen exposure, which of the following statements is FALSE?
a. Major effects include growth retardation and malformations.
b. Exposure to teratogens during critical developmental periods will have more severe effects on the fetus.
c. There is considered to be a toxicant level threshold below which the fetus is capable of repairing itself.
d. The immune system of the fetus is primitive, so the fetus has little to no ability to fight off chemicals and repair itself.
e. Embryo lethality becomes more likely as the toxic dose is increased.

6. Which of the following stages of the cell cycle are important in monitoring DNA damage and inhibiting progression of the cell cycle?
a. G_1–S, anaphase, M–G_1.
b. G_1–S, S, G_2–M.
c. S, prophase, G_1.
d. G_2–M, prophase.
e. M–G_1, anaphase.

7. Which of the following molecules is NOT important in determining the ultimate outcome of embryonal DNA damage?
a. p53.
b. Bax.

c. Bcl-2.
d. c-Myc.
e. NF-κB.

8. Which of the following is NOT a physiologic response to pregnancy?
a. increased cardiac output.
b. increased blood volume.
c. increased peripheral vascular resistance.
d. decreased plasma proteins.
e. increased extracellular space.

9. All of the following statements are true EXCEPT:
a. Offspring of white mothers have a higher incidence of cleft lip or palate than do black mothers, after adjusting for paternal race.
b. Cytomegalovirus (CMV) is a common viral cause of birth defects.
c. Folate supplementation during pregnancy decreases the risk of neural tube defects.
d. Cigarette smoke and ethanol are both toxic to the placenta.
e. In humans, there is a negative correlation between stress and low birth weight.

10. Which of the following is NOT a mechanism involving the endocrine system by which chemicals induce developmental toxicity?
a. acting as steroid hormone receptor ligands.
b. disrupting normal function of steroid hormone metabolizing enzymes.
c. disturbing the release of hormones from the hypothalamus.
d. disturbing the release of hormones from the pituitary gland.
e. elimination of natural hormones.

UNIT 4 TARGET ORGAN TOXICITY

CHAPTER 11

Toxic Responses of the Blood

Martyn T. Smith and Cliona M. McHale

KEY POINTS

- Hematotoxicology is the study of adverse effects of exogenous chemicals on blood and blood-forming tissues.
- Direct or indirect damage to blood cells and their precursors includes tissue hypoxia, hemorrhage, and infection.
- Xenobiotic-induced aplastic anemia is a life-threatening disorder characterized by peripheral blood pancytopenia, reticulocytopenia, and bone marrow hypoplasia.
- Idiosyncratic xenobiotic-induced agranulocytosis may involve a sudden depletion of circulating neutrophils concomitant with exposure that persists as long as the agent or its metabolites are in the circulation.
- Leukemias are proliferative disorders of hematopoietic tissue that originate from individual bone marrow cells.
- Xenobiotic-induced thrombocytopenia may result from increased platelet destruction or decreased platelet production, which lead to decreased platelet aggregation and bleeding disorders.
- Blood coagulation is a complex process involving a number of proteins whose synthesis and function can be altered by many xenobiotics.

BLOOD AS A TARGET ORGAN

Hematotoxicology is the study of adverse effects of chemicals on the blood and blood-forming tissues. The vital functions that blood cells perform, together with the susceptibility of this highly proliferative tissue to intoxication, make the hematopoietic system unique as a target organ.

The blood comprises around 7% of the body weight of a typical adult who has 4.7 to 5.5 L of blood. The delivery of oxygen to tissues throughout the body, maintenance of vascular integrity, and provision of the many affector and effector immune functions necessary for host defense require a prodigious proliferative and regenerative capacity. Each of the various blood cells (erythrocytes, granulocytes, and platelets) is produced at a rate of approximately 1 to 3 million/s in a healthy adult, making hematopoietic tissue particularly sensitive to cytoreductive or antimitotic drugs, such as those used to treat cancer, infection, and immune-mediated disorders. Toxic chemicals may affect the supply of nutrients, such as iron; the clearance of toxicants and metabolites, such as urea; or the production of vital growth factors, such as erythropoietin and

granulocyte colony-stimulating factor (G-CSF).

Hematotoxicity may be regarded as *primary*, where one or more blood components are directly affected, or *secondary*, where the toxic effect is a consequence of other tissue injury or systemic disturbances. Primary toxicity is regarded as among the more common serious effects of xenobiotics, particularly drugs. Secondary toxicity is exceedingly common, due to the propensity of blood cells to reflect a wide range of local and systemic effects of toxicants on other tissues.

HEMATOPOIESIS: THE FORMATION OF THE BLOOD CELLULAR COMPONENTS

The production of blood cells, or hematopoiesis, is a highly regulated sequence of events by which blood cell precursors proliferate and differentiate to meet the relentless needs of oxygen transport, host defense and repair, hemostasis, and other vital functions. The bone marrow is the principal site of hematopoiesis in humans and most laboratory and domestic animals. However, the lung harbors blood stem cells that can repopulate the bone marrow. In the human fetus, hematopoiesis can be found in the liver, spleen, bone marrow, thymus, and lymph nodes. The bone marrow is the dominant hematopoietic organ in the latter half of gestation and the major blood-cell-producing organ at birth. All marrow is active, or “red marrow,” at birth. During early childhood, hematopoiesis recedes in long bones and, in adults, is confined to the axial skeleton and proximal humerus and femur, and the marrow becomes “yellow” or fatty. When demand for blood cell production is great, fatty marrow can be reactivated as sites of hematopoiesis. This can be useful in toxicology studies as a marker of sustained hematopoietic stress. Whereas the central function of bone marrow is hematopoiesis and lymphopoiesis, bone marrow is also one of the sites of the mononuclear phagocyte system (MPS), contributing monocytes that differentiate into a variety of MPS cells located in liver (Kupffer cells), spleen (littoral cells), lymph nodes, and other tissues.

All cellular components of blood are derived from pluripotent HSCs (Fig. 11–1). HSCs differentiate into common myeloid progenitors (CMPs) and common lymphoid progenitors (CLP) that ultimately generate the entire range of mature blood cells. CLPs give rise to B cells, T cells, and natural killer (NK) cells. CMPs differentiate into platelets (also known as thrombocytes), erythrocytes, and mast cells and into myeloblast cells that give rise to monocytes (which ultimately become macrophages), neutrophils, basophils, and eosinophils.

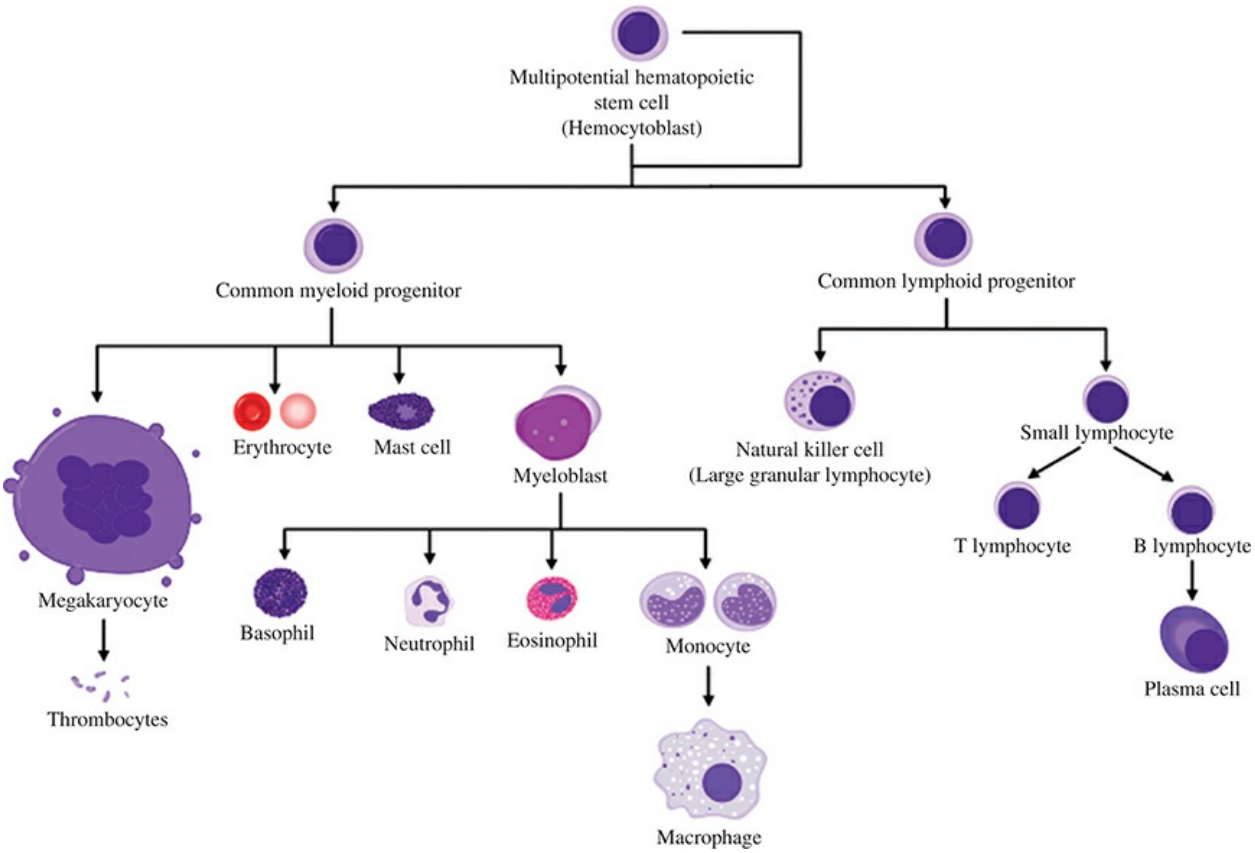

FIGURE 11–1 *Schematic of hematopoiesis.* All mature blood cells are derived from pluripotent HSCs in a hierarchical process. HSCs differentiate into common myeloid progenitors (CMPs) and common lymphoid progenitors (CLP). CLPs give rise to B cells, T cells, and natural killer (NK) cells. CMPs differentiate into thrombocytes, erythrocytes, and mast cells, and into myeloblast cells that give rise to monocytes (which ultimately become macrophages), neutrophils, basophils, and eosinophils. (Reproduced from Wikimedia Commons.)

Suffixes are commonly used in describing altered hematopoiesis. These include -emia, with reference to the blood (e.g., uremia, septicemia, anemia); -penia, decreased number of blood cells (e.g., leukopenia, pancytopenia); -cytosis, increased number of blood cells (e.g., granulocytosis, thrombocytosis, erythrocytosis).

TOXICOLOGY OF THE ERYTHRON

The Erythrocyte

Erythrocytes make up 40% to 45% of the circulating blood volume and transport of oxygen from the lungs to the peripheral tissues and carbon dioxide from tissues to the lung. Erythrocytes can also act as a carrier and/or reservoir for drugs and toxicants and are being explored as delivery systems for drugs, biological, and nanoparticles.

Xenobiotics may affect the production, function, and/or survival of erythrocytes. These

effects are most frequently manifest as a change in the circulating red cell mass, usually resulting in a decrease (anemia). Occasionally, chemicals that increase oxygen affinity lead to an increase in red cell mass (erythrocytosis). Shifts in plasma volume can alter the relative concentration of erythrocytes/hemoglobin and can be easily confused with true anemia or erythrocytosis.

Two general mechanisms that lead to true anemia are either decreased production or increased destruction of erythrocytes. The usual parameters of a complete blood count (CBC), including the RBC count, hemoglobin concentration (Hbg), hematocrit (also referred to as packed cell volume), mean corpuscular volume (MCV) and reticulocyte count, can establish the presence of anemia. Increased destruction is usually accompanied by an increase in reticulocytes (young erythrocytes containing residual RNA). Other parameters helpful in the evaluation of the human erythron include erythrocyte morphology (e.g., megaloblastic changes, erythrocyte fragmentation, sickled RBCs); serum concentration of haptoglobin, lactate dehydrogenase, free hemoglobin, vitamin B_{12}, folate, iron, and ferritin; direct and indirect red cell antiglobulin tests; bone marrow morphology, time-lapse interferometry to quantify membrane fluctuations at the nanometer scale and metabolomics.

Alterations in Red Cell Production

Erythrocyte production is a continuous process that depends on frequent cell division and a high rate of hemoglobin synthesis. Human adult hemoglobin (hemoglobin A) is a tetramer composed of two α-globin and two β-globin chains, each with a heme residue. Abnormalities that lead to decreased hemoglobin synthesis are relatively common (e.g., iron deficiency). An imbalance between α- and β-chain production is the basis of congenital thalassemias and results in decreased hemoglobin production and microcytosis. Xenobiotics can affect globin chain synthesis and alter the composition of hemoglobin within erythrocytes.

Heme synthesis requires incorporation of iron into a porphyrin ring (Fig. 11–2). Iron deficiency is usually the result of dietary deficiency or increased blood loss. Drugs that contribute to blood loss may potentiate the risk of developing *iron deficiency anemia*. Defects in the synthesis of porphyrin ring of heme can lead to *sideroblastic anemia*, with its characteristic accumulation of iron in bone marrow erythroblasts. Xenobiotics that can interfere with one or more of the steps in erythroblast heme synthesis and result in sideroblastic anemia are listed in Table 11–1.

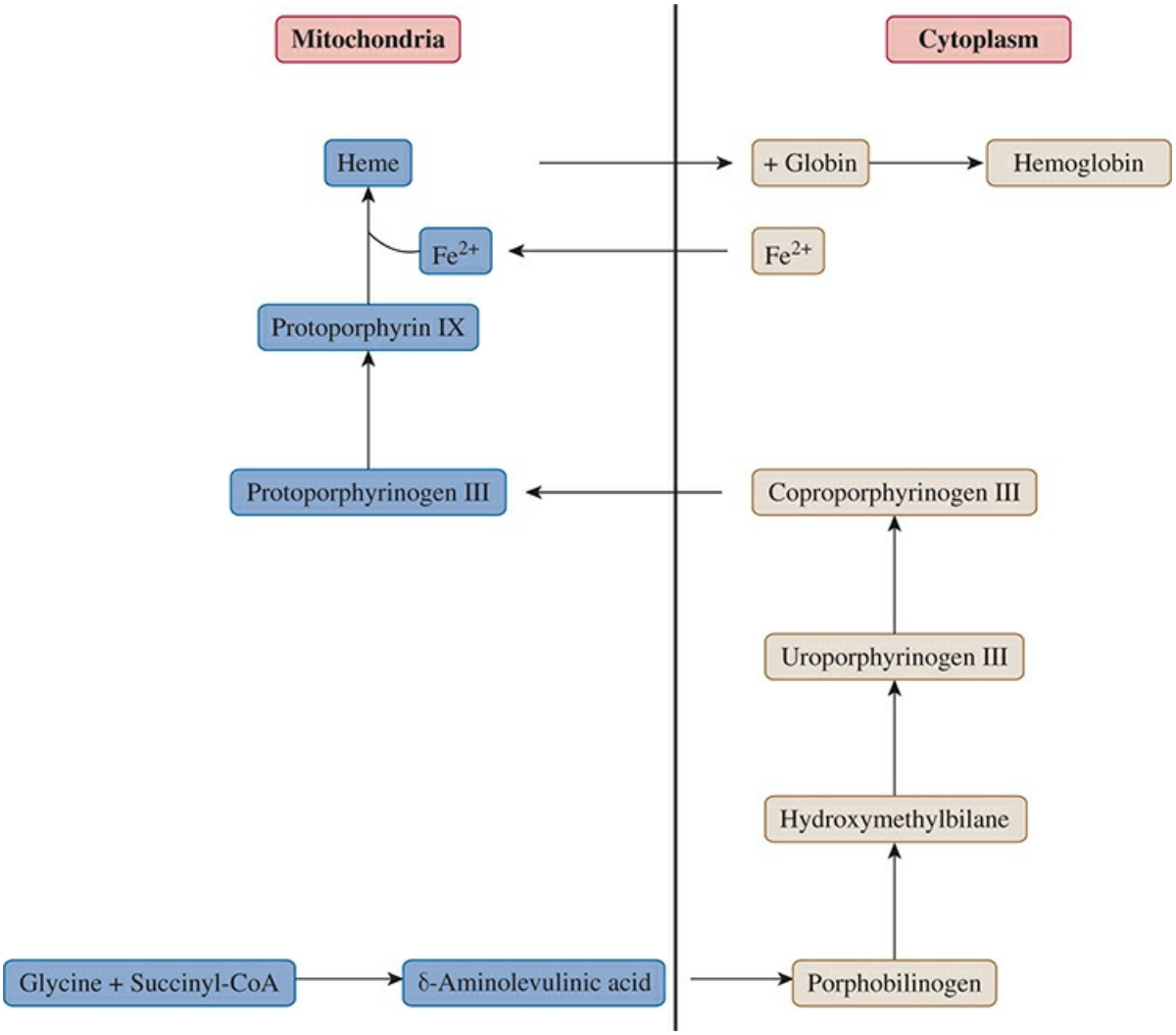

FIGURE 11–2 *The synthesis of heme involves a series of reactions that occur in the cytoplasm and mitochondria of erythroblasts.* The initial step in the pathway is the mitochondrial synthesis of δ-aminolevulinic acid, a step that is commonly affected by xenobiotics, including lead. Ferrochelatase catalyzes the incorporation of ferrous iron into the tetrapyrrole protoporphyrin IX. Inhibition of the synthetic pathway leading to protoporphyrin IX, as occurs in the sideroblastic anemias, can cause an imbalance between iron concentration and ferrochelatase activity, resulting in iron deposition within mitochondria. Mitochondrial accumulation of iron is the hallmark lesion of the sideroblastic anemias.

TABLE 11–1 Xenobiotics Associated with Sideroblastic Anemia

Ethanol	Chloramphenicol
Isoniazid	Copper chelation/deficiency
Pyrazinamide	Zinc intoxication
Cycloserine	Lead intoxication

Hematopoiesis requires active DNA synthesis and frequent mitoses. Folate and vitamin B_{12} are necessary to maintain synthesis of thymidine for incorporation into DNA. Deficiency of folate and/or vitamin B_{12} results in *megaloblastic anemia.* Xenobiotics may contribute to a deficiency of vitamin B_{12} and/or folate (Table 11–2), leading to megaloblastic anemia.

TABLE 11–2 Xenobiotics Associated with Megaloblastic Anemia

B_{12} Deficiency	Folate Deficiency
Colchicine	Ampicillin
Cycloserine	Antimetabolites
Ethanol	Chloramphenicol
Isoniazid	Cholestyramine
Metformin	Carbamazepine
Neomycin	Erythromycin
Omeprazole	Ethanol
Paraaminosalicylic acid	Phenobarbital
Sodium nitroprusside	Phenytoin
Zidovudine	Primidone
	Quinine
	Tetracyclines
	Triamterene
Purine Metabolism	**Pyrimidine Synthesis**
Azathioprine	Gemcitabine
Thioguanine	Hydroxyurea
Mercaptopurine	Methotrexate
Fludarabine	Mercaptopurine
Pentostatin	Fluorouracil
Methotrexate	Nitrous Oxide

Many antiproliferative drugs used in the treatment of malignancy inhibit hematopoiesis. The resulting bone marrow toxicity may be dose-limiting. Drugs, such as amifostine, may help protect against the marrow toxicity of these drugs by quenching free radicals, promoting DNA repair by donating hydrogen, and inducing cellular hypoxia.

Drug-induced *aplastic anemia* may represent either a predictable or idiosyncratic reaction to a xenobiotic. This life-threatening disorder is characterized by peripheral blood pancytopenia, reticulocytopenia, and bone marrow hypoplasia. Agents associated with the development of aplastic anemia are included in Table 11–3.

TABLE 11–3 Drugs and Other Chemicals Associated with the Development of Aplastic Anemia

Chloramphenicol	Organic arsenicals	Quinacrine
Methylphenylethylhydantoin	Trimethadione	Phenylbutazone
Gold	Streptomycin	Benzene
Penicillin	Allopurinol	Tetracycline
Methicillin	Sulfonamides	Chlortetracycline
Sulfisoxazole	Sulfamethoxypyridazine	Amphotericin B
Mefloquine	Ethosuximide	Felbamate
Carbimazole	Methylmercaptoimidazole	Potassium perchlorate
Propylthiouracil	Tolbutamide	Pyrimethamine
Chlorpropamide	Carbutamide	Tripelennamine
Indomethacin	Carbamazepine	Diclofenac
Meprobamate	Chlorpromazine	Chlordiazepoxide
Mepazine	Chlorophenothane	Parathion
Thiocyanate	Methazolamide	Dinitrophenol
Bismuth	Mercury	Chlordane
Carbon tetrachloride	Cimetidine	Metolazone
Azidothymidine	Ticlopidine	Isoniazid
Trifluoperazine	D-Penicillamine	

Alterations in the Respiratory Function of Hemoglobin

Hemoglobin is necessary for effective transport of oxygen and carbon dioxide between the lungs and tissues. Electrostatic charges hold the globin chains of deoxyhemoglobin in a “tense” (T) conformation characterized by a relatively low affinity for oxygen. Binding of oxygen produces a “relaxed” (R) conformation that is associated with a 500-fold increase in oxygen affinity. The individual globin units show cooperativity in the binding of oxygen, resulting in the familiar sigmoid shape to the oxygen dissociation curve (Fig. 11–3). Release of the first oxygen molecule facilitates the release of the second, third, and fourth oxygen molecules.

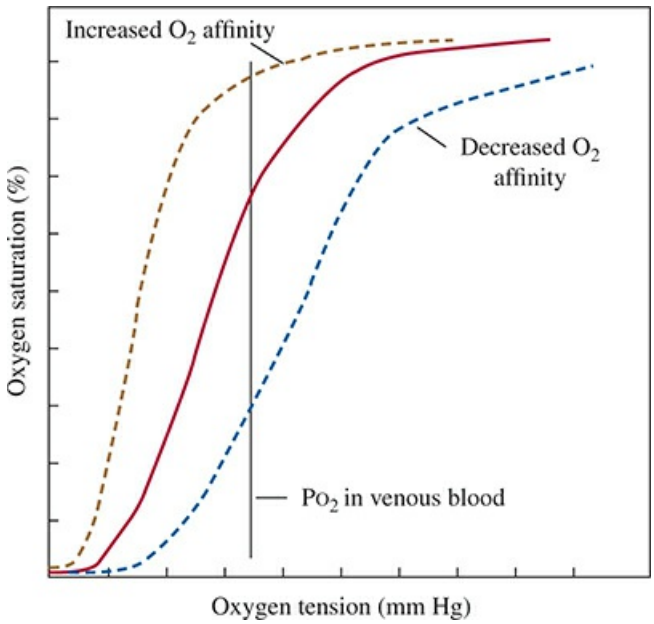

FIGURE 11–3 *The normal oxygen dissociation curve (solid line) has a sigmoid shape due to the cooperative interaction between the four globin chains in the hemoglobin molecule*. Fully deoxygenated hemoglobin has a relatively low affinity for oxygen. Interaction of oxygen with one heme-iron moiety induces a conformational change in that globin chain. Through surface interactions, that conformational change affects the other globin chains, causing a conformational change in all the globin chains increasing their affinity for oxygen. Homotropic and heterotropic parameters also affect the affinity of hemoglobin for oxygen. An increase in oxygen affinity results in a shift to the left in the oxygen dissociation curve. Such a shift may decrease oxygen delivery to the tissues. A decrease in oxygen affinity results in a shift to the right in the oxygen dissociation curve, facilitating oxygen delivery to the tissues. In healthy resting adults, the extraction of oxygen from capillary blood is about 25%.

Homotropic Effects Leading to Methemoglobinemia— An important homotropic (intrinsic) property of oxyhemoglobin is the slow but consistent oxidation of heme iron from the ferrous (Fe^{2+}) to the ferric (Fe^{3+}) state to form methemoglobin, which is unable to transport oxygen. The presence of methemoglobin in a hemoglobin tetramer has allosteric effects that increase the affinity of oxyhemoglobin for oxygen, resulting in a leftward shift of the oxygen dissociation curve (Fig. 11–4). The combination of decreased oxygen content and increased affinity may significantly impair oxygen delivery to tissues.

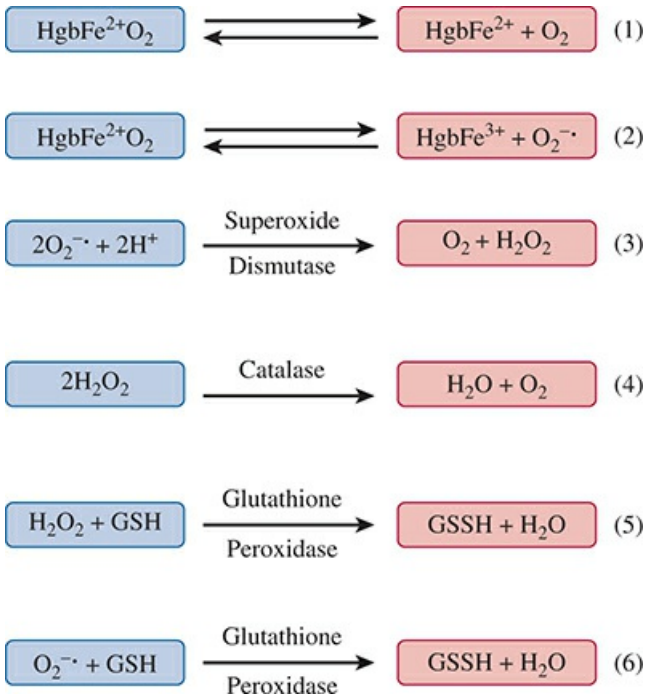

FIGURE 11–4 *Oxygen normally exchanges with the ferrous iron of deoxyhemoglobin (Eq. (1)).* Oxygen can "capture" one of the iron electrons, resulting in the generation of methemoglobin ($HgbFe^{3+}$) and superoxide (O_2^-.) (Eq. (2)). Superoxide must be detoxified or it can lead to oxidative injury within the cell. The pathways involved include superoxide dismutase (Eq. (3)), catalase (Eq. (4)), and glutathione peroxidase (Eqs. (5) and (6)). A supply of reduced glutathione (GSH) is necessary to prevent excessive oxidative injury.

The normal erythrocyte has metabolic mechanisms for reducing heme iron back to the ferrous state. Failure of these control mechanisms leads to *methemoglobinemia*, in which arterial blood becomes a characteristic chocolate-brown color. A common cause of methemoglobinemia is exposure to an oxidizing xenobiotic that overwhelms the NADH-methemoglobin reductase system. Chemicals that may cause methemoglobinemia are listed in Table 11–4.

TABLE 11–4 Xenobiotics Associated with Methemoglobinemia

Therapeutic Agents	Environmental Agents
Benzocaine	Nitrites
Lidocaine	Nitrates
Prilocaine	Nitrobenzenes
Dapsone	Aniline and aniline derivatives
Amyl nitrate	Butyl nitrite
Isobutyl nitrite	Potassium chlorate
Nitroglycerine	Gasoline additives
Primaquine	Aminobenzenes
Sulfonamide	Nitrotoluenes
Phenacetin	Trinitrotoluene
Nitric oxide	Nitroethane
Phenazopyridine	*ortho*-Toluidine
Metoclopramide	*para*-Toluidine
Flutamide	β-Naphthol disulfonate
Silver nitrate	
Quinones	
Methylene blue	

Heterotropic Effects—Three major heterotropic (extrinsic) effectors of hemoglobin function are pH, erythrocyte 2,3-bisphosphoglycerate (2,3-BPG) concentration, and temperature. A decrease in pH (e.g., lactic acid, carbon dioxide) lowers the affinity of hemoglobin for oxygen, causing a right shift in the oxygen dissociation curve, facilitating delivery of oxygen to tissues (Fig. 11–3). As bicarbonate and carbon dioxide equilibrate in the lung, the hydrogen ion concentration decreases, increasing the affinity of hemoglobin for oxygen and facilitating oxygen uptake.

Binding of 2,3-BPG to deoxyhemoglobin results in stabilization of the "T" conformation, with reduced oxygen affinity (a shift to the right of the oxygen dissociation curve). The conformational change induced by binding of oxygen alters the binding site for 2,3-BPG and results in release of 2,3-BPG from hemoglobin. This facilitates uptake of more oxygen for delivery to tissues. The concentration of 2,3-BPG increases whenever there is tissue hypoxemia but may decrease in the presence of acidosis or hypophosphatemia.

The oxygen affinity of hemoglobin decreases as the body temperature increases. This facilitates delivery of oxygen to tissues during periods of extreme exercise and febrile illnesses associated with increased temperature. Correspondingly, oxygen affinity increases during hypothermia, which may lead to decreased oxygen delivery.

Carboxyhemoglobinemia—The respiratory function of hemoglobin may be impaired by blockade of the ligand binding site following interaction with other substances, most notably

carbon monoxide (CO). CO has a relatively low rate of association with deoxyhemoglobin but has high affinity of about 200 times that of oxygen. Persistent exposure to a low level of CO (e.g., 0.1%) may lead to 50% saturation of hemoglobin. Binding of carbon monoxide causes the oxygen dissociation curve to shift to the left, further compromising oxygen delivery to the tissues.

Alterations in Erythrocyte Survival

Erythrocytes, during their circulation of about 120 days, are exposed to oxidative injuries and must negotiate the tortuous passages of the microcirculation and spleen. This requires a deformable cell membrane and energy to maintain the sodium–potassium gradients and repair mechanisms. Very little protein synthesis occurs, as erythrocytes are anucleate when they enter the circulation and residual mRNA is rapidly lost over 1 to 2 days. As senescence occurs, aged erythrocytes are removed by the spleen, and the iron is recovered for reutilization in heme synthesis. Any insult that increases oxidative injury, decreases metabolism, or alters the membrane may cause a decrease in erythrocyte concentration and a corresponding anemia.

Nonimmune Hemolytic Anemia

***Microangiopathic Anemias*—**Intravascular fragmentation of erythrocytes gives rise to *microangiopathic hemolytic anemia* with the hallmark presence of schistocytes (fragmented RBCs) in the peripheral blood. The formation of fibrin strands in the microcirculation is a common mechanism for RBC fragmentation, and this may occur in disseminated intravascular coagulation, sepsis, the hemolytic-uremic syndrome (HUS), and thrombotic thrombocytopenic purpura (TTP). The erythrocytes are essentially sliced into fragments by the fibrin strands that extend across the vascular lumen and impede the flow of erythrocytes through the vasculature. The high shear associated with malignant hypertension may also lead to RBC fragmentation.

***Oxidative Hemolysis*—**Molecular oxygen is reactive and potentially toxic. Consequently, the normal respiratory function of erythrocytes generates oxidative stress on a continuous basis. The major mechanisms that protect against oxidative injury in erythrocytes include NADH-methemoglobin reductase, superoxide dismutase, catalase, and the glutathione pathway.

Xenobiotics capable of inducing oxidative injury in erythrocytes are listed in Table 11–5. These chemicals appear to potentiate the normal redox reactions and to overwhelm the usual protective mechanisms. The interaction between these xenobiotics and hemoglobin leads to the formation of free radicals that denature critical proteins, including hemoglobin, thiol-dependent enzymes, and components of the erythrocyte membrane.

TABLE 11–5 Xenobiotics Associated with Oxidative Injury

Arsine	Copper
Acetanilide	Phenylhydrazine
Naphthalene	Nitrobenzene
Nitrofurantoin	Phenacetin
Sulfamethoxypyridazine	Phenol
Aminosalicylic acid	Hydroxylamine
Sodium sulfoxone	Methylene blue
Dapsone	Toluidine blue
Phenazopyridine	Furazolidone
Primaquine	Nalidixic acid
Chlorates	Sulfanilamide
Sulfasalazine	

Oxidative injury decreases the viability of erythrocytes. Protection against free radical–induced modifications is mediated mostly by reduced glutathione, along with antioxidant defense enzymes including superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase (see Chapter 3). Significant oxidative injury usually occurs when the concentration of the xenobiotic is high enough (due to either high exposure or decreased metabolism) to overcome normal protective mechanisms, or when an underlying defect exists in the protective mechanisms. The most common enzyme defect associated with oxidative hemolysis is G-6-PD deficiency, a relatively common X-linked disorder characterized by alterations in G-6-PD structure that diminish its functional activity. It is often clinically asymptomatic until the erythrocytes are exposed to oxidative stress.

Nonoxidative Chemical-Induced Hemolysis— Some xenobiotics are associated with hemolysis without significant oxidative injury. Arsenic hydride inhalation can result in severe hemolysis, with anemia, jaundice, and hemoglobinuria. Stibine, a hydride of antimony, lead, copper, and chromium may result in hemolytic anemia and thrombocytopenia. Significant hemolysis may also occur with biologic toxicants found in insect and snake venoms.

Immune Hemolytic Anemia—Immunologic destruction of erythrocytes is mediated by the interaction of IgG or IgM antibodies with surface antigens on the erythrocyte. In autoimmune hemolytic anemia, the antigens are intrinsic components of the patient's own erythrocytes.

Several mechanisms have been implicated in xenobiotic-mediated antibody binding to erythrocytes. Some drugs, of which penicillin is a prototype, appear to bind to the surface of the cell, with the "foreign" drug acting as a *hapten* to elicit an immune response. The antibodies that arise in this response only bind drug-coated erythrocytes. Other drugs, of which quinidine is a prototype, bind to components of the erythrocyte surface and induce a conformational change in one or more components of the membrane. An additional mechanism, for which α-methyldopa is

a prototype, results in production of a *drug-induced autoantibody* that cannot be distinguished from the antibodies arising in idiopathic autoimmune hemolytic anemia.

TOXICOLOGY OF THE LEUKON

Components of Blood Leukocytes

The leukon consists of leukocytes, or white blood cells, including granulocytes (neutrophils, eosinophils, and basophils), monocytes, and lymphocytes. Granulocytes and monocytes are nucleated ameboid cells that are phagocytic and play a central role in the inflammatory response and host defense. Unlike RBCs, which reside exclusively within blood, granulocytes and monocytes generally pass through the blood on their way to extravascular tissues, where they reside in large numbers.

Granulocytes are defined by the characteristics of their cytoplasmic granules as they appear on a blood smear. Neutrophils, the largest component of blood leukocytes, specialize in the mediation of inflammation and the ingestion and destruction of pathogenic microorganisms. Eosinophils and basophils modulate inflammation through the release of various mediators and play an important role in other homeostatic functions.

Evaluation of Granulocytes

In the blood, neutrophils are distributed between *circulating* and *marginated* pools, which are of equal size in humans and in constant equilibrium. A blood neutrophil count assesses only the circulating pool, which remains between 1800/μL and 7500/μL in a healthy adult human. During inflammation, an increased number of immature (non-segmented) granulocytes may be seen in peripheral blood. In certain conditions, neutrophils may show morphological changes indicative of toxicity.

Toxic Effects on Granulocytes

Xenobiotics can affect granulocyte proliferation (granulopoiesis) and kinetics, the extent to which a chemical can impair the vital functions these cells perform, and how neutrophils mediate or exacerbate inflammatory disease or other target organ toxicity.

Effects on Proliferation and Kinetics—The high rate of neutrophil proliferation makes their progenitor and precursor granulocyte pool particularly susceptible to inhibitors of mitosis. Such effects by cytotoxic drugs are generally nonspecific, as they similarly affect cells of the dermis, gastrointestinal tract, and other rapidly dividing tissues. Agents that affect both neutrophils and monocytes pose a greater risk for toxic sequelae, such as infection. Such effects tend to be dose-related, with mononuclear phagocyte recovery preceding neutrophil recovery.

Myelotoxicity is commonly seen with cytoreductive cancer chemotherapeutics. However, this is changing, as more cancer cell–targeted, normal-tissue-sparing anticancer agents are being developed. These drugs vary in their mechanism, the kinetics of the cytopenias they induce, and how individual patients or animals respond. Most inhibit DNA synthesis or directly attack its

integrity through the formation of DNA adducts or enzyme-mediated breaks. The cytoreductive alkylating agents, cisplatin, and nitrosoureas can be toxic to both resting and actively dividing cells; nonproliferating cells such as metamyelocytes, bands, and mature neutrophils are relatively resistant. Generally, stem cells cycle slowly and are minimally affected by a single administration of a cytotoxic drug. Sustained exposure to drugs affect slowly cycling stem cells and cause more prolonged myelosuppression.

Two innovations have had a dramatic impact on the dose-limiting myelotoxicity associated with cancer chemotherapeutics: (1) development of drugs with cancer cell–specific molecular targets that are relatively bone marrow sparing and target aberrant growth factor receptor signaling, apoptosis, angiogenesis, and other metabolic, immune, inflammatory, and mutation-promoting pathways that selectively advantage tumor cells; and (2) cotreatment with hematopoietic growth factors, which mitigates or successfully rescues patients from the effects of myelosuppression.

Effects on Function—Demonstrable in vivo effects associated with drugs and nontherapeutic chemicals are surprisingly few. Examples include ethanol and glucocorticoids, which impair phagocytosis and microbe ingestion in vitro and in vivo. Iohexol and ioxaglate, components of radiographic contrast media, reportedly inhibit phagocytosis.

Several chemicals inhibit neutrophil chemotaxis, including macrolide antibiotics, zinc salts, chlordane, and mercuric chloride/methylmercuric chloride. Activation of neutrophils with the potential for proinflammatory consequences, specifically through increased phagocytosis, O_2^- production, or both, may occur with sodium sulfite, mercuric chloride, chlordane, and toxaphene.

Idiosyncratic Toxic Neutropenia—Chemicals that unexpectedly damage neutrophils and granulocyte precursors to the extent of inducing *agranulocytosis* (depletion of blood neutrophils to less than 500/μL) are particularly concerning. Idiosyncratic drug-induced neutropenia may be dose-related and involve a nonselective disruption of protein synthesis or cell replication resulting in agranulocytosis. Alternatively, it may not be dose-dependent, but allergic or immunologic in origin. Idiosyncratic xenobiotic-induced agranulocytosis may involve a sudden depletion of circulating neutrophils concomitant with exposure. Hematopoietic function is usually restored when the chemical is detoxified or excreted. Toxicants affecting uncommitted stem cells induce total marrow failure, as seen in aplastic anemia. Surviving uncommitted stem cells eventually produce recovery, provided the risk of infection is managed during the leukopenic episodes.

Mechanisms of Toxic Neutropenia—*Immune-mediated* or *nonimmune-mediated* toxic neutropenia may be due to the *hapten hypothesis* or the *danger hypothesis*. The former involves a reactive metabolite binding to a protein making it “foreign,” which in turn induces an immune response that leads to toxicity. The latter has a reactive metabolite damaging a cell, which elicits an immune response against the drug or an autoimmune response. The “perfect storm” in the rare individual in which these reactions occur is thought to be caused by individual-specific circumstances that drive both metabolism of the drug and the immune reactions to altered proteins. Some agents associated with immune and nonimmune neutropenia/agranulocytosis are listed in Table 11–6.

TABLE 11–6 Examples of Toxicants that Cause Immune and Nonimmune Idiopathic

Neutropenia

Drugs Associated With WBC Antibodies	Drugs Not Associated With WBC Antibodies
Aminopyrine	Isoniazid
Propylthiouracil	Rifampicin
Ampicillin	Ethambutol
Metiamide	Allopurinol
Dicloxacillin	Phenothiazines/Chlorpromazine
Phenytoin	Flurazepam
Aprindine	Hydrochlorothiazide
Azulfidine	
Chlorpropamide	
Chlorpromazine/phenothiazines	
Procainamide	
Nafcillin	
Tolbutamide	
Lidocaine	
Methimazole	
Levamisole	
Gold	
Quinidine	
Clozapine	

Adapted from Pisciotta AV. Response of granulocytes to toxic Injury. In: Sipes IG, McQueen AC, Gandolfi AJ, eds. *Comprehensive Toxicology*. Oxford: Pergamon Press; 1997:145–158.

LEUKEMOGENESIS AS A TOXIC RESPONSE

Human Leukemia and Other Hematopoietic Malignancies

Leukemias arise when hematopoietic stem or progenitor cells (HSC/HPC) in bone marrow

undergo uncontrolled proliferation and cannot differentiate normally into mature blood cells. Leukemias are broadly characterized as myeloid or lymphoid depending on the lineage of origin. Based on the stage of differentiation and rate of clonal expansion, they are characterized as acute (rapid onset, immature blast cells) or chronic (gradual onset over months or years, more mature cells). The four major types of leukemia are commonly referred to—acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML) also known as acute nonlymphocytic leukemia (ANLL), chronic lymphoblastic leukemia (CLL), and chronic myeloid leukemia (CML).

Therapy-Related Leukemia—More than half of all leukemias occur in people with no obvious prior exposures of relevance and are termed de novo leukemias. *Secondary leukemias* are those that develop in individuals who have previously been exposed to radiation, industrial chemicals, or chemotherapeutic drugs or who previously had myelodysplastic syndrome (MDS). *Therapy-related MDS* and *AML* (t-MDS and t-AML) arise following cytotoxic therapy for an unrelated disease. They are distinct from their de novo counterparts with regard to onset, prognosis, and response to therapy.

Childhood Leukemia—Adult leukemias are predominantly myeloid and originate in pluripotent stem or progenitor cells. In contrast, 30% of pediatric acute leukemias are AML and 70% are ALL. The underlying aberrations often occur in two distinct stages, with oncogenic fusion proteins generated in utero, and subsequent cooperating hits of a genetic, epigenetic, or immune nature occurring after birth.

General Mechanisms of Leukemogenesis

Figure 11–5 illustrates the multistep process of leukemogenesis involving the acquisition of various combinations of chromosomal, genetic, and epigenetic aberrations that transform a normal stem cell into a leukemic stem cell (LSC). The resulting altered gene expression patterns disrupt critical hematopoietic processes such as signal transduction, alternative splicing, and epigenetics, and give rise to increased proliferation or survival, block of differentiation, and immortalization.

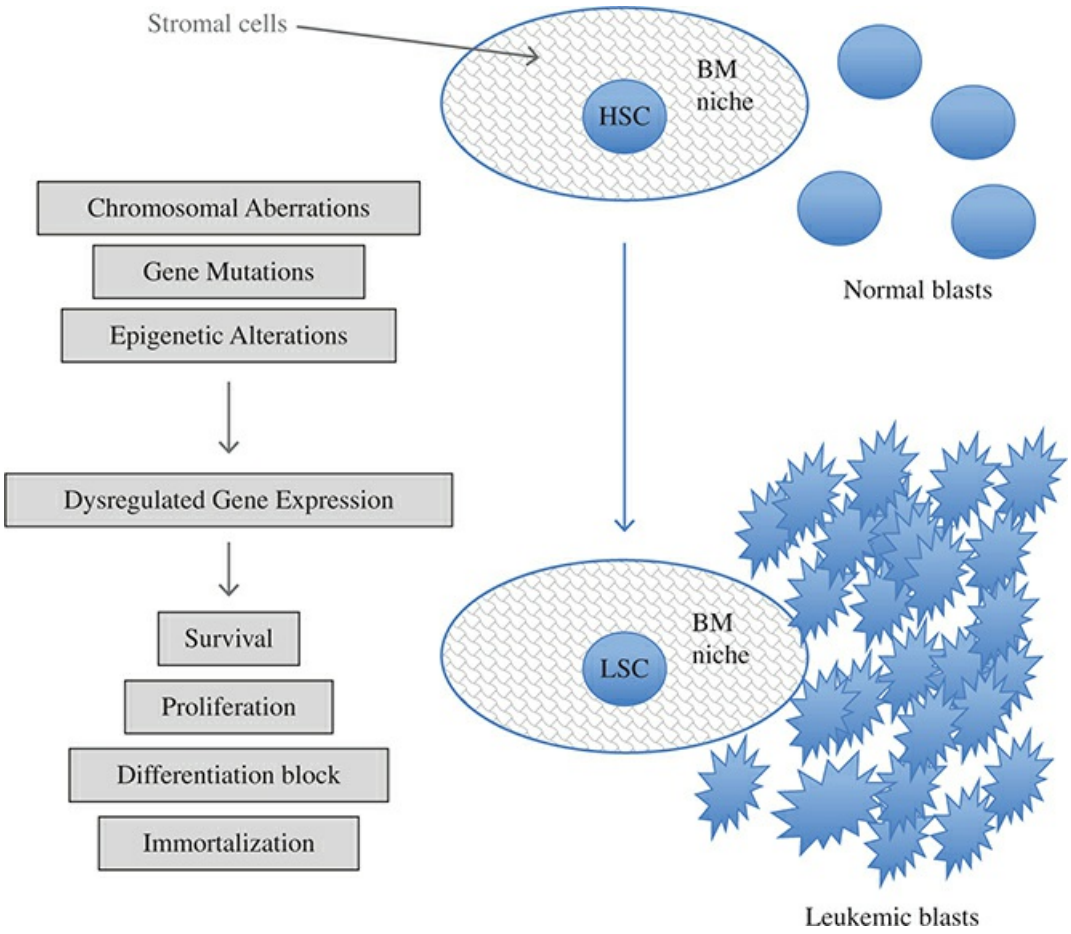

FIGURE 11–5 *Pathogenesis of leukemia.* Leukemogenesis is a multistep process involving the acquisition of various combinations of chromosomal, genetic, and epigenetic aberrations that transform a normal HSC into an LSC. The resulting altered gene expression patterns disrupt critical hematopoietic processes such as signal transduction, alternative splicing, and epigenetics, and give rise to cellular phenotypes such as increased proliferation or survival, block of differentiation, and immortalization. Thus, the bone marrow becomes replete with leukemic blasts and LSCs that replenish them.

Chromosomal Aberrations—Several kinds of chromosomal aberation can occur and alter the function of genes in the affected region. A translocation is an abnormality in which chromosome regions are swapped between chromosomes. Translocations are designated as t(a;b), where part of chromosome a is translocated to chromosome b and vice versa. Chromosomal deletion, designated as del(#p or #q), involves the loss of a chromosomal region and an inversion, designated as inv(#), involves the end-to-end reversal of a region within a chromosome.

Many leukemia patients exhibit chromosomal alterations in their tumor cells. Common aberrations in AML include loss or gain of one or more chromosomes (e.g., monosomy 7 and trisomy 8, respectively), recurrent chromosomal translocations such as t(8;21) and t(15;17), deletions, and inversions such as inv(16). In ALL, t(9;22) frequently occurs in adults, whereas t(12;21) is more common in children.

Gene Mutations—Mutations in a recurring set of genes in HSC/HPC that alter cell growth,

apoptosis, differentiation, epigenetic processes, and tumor suppression characterize AML. Next generation sequencing technologies have shown that over 70% of AML cases have mutations in genes encoding DNA methylation or histone modifiers.

Epigenetic Modifications—Altered epigenetic signatures such as DNA methylation and miRNA expression have been seen in many types of leukemia. Long noncoding RNAs, important regulators of gene expression, may also contribute to the initiation and development of AML.

Leukemic Stem Cells and the Bone Marrow Niche— Leukemia-related aberrations arise in HSC/HPC and in other cellular components of the bone marrow niches where HSC/HPC reside. The acquisition of increased self-renewal capability in HSC/HPC leads to the generation of leukemic stem cells (LSCs) resembling multilineage progenitors that initiate and maintain AML. LSCs can evade programmed cell death and removal. Toxicity-induced aberrations in stromal cells may lead to disruption of signals from the bone marrow niche microenvironment, which enables the survival and proliferation of AML blasts.

Leukemogenic Agents and Mechanisms of Leukemogenesis

Around 25% of the agents identified as human carcinogens in humans by IARC are known to induce lymphohematopoietic cancers. This includes antineoplastic drugs, industrial chemicals, various forms of radiation, immunosuppressive drugs, and infectious agents. The established nongenetic causes of AML and MDS are ionizing radiation, cytotoxic chemotherapeutic drugs, tobacco smoking, and occupational exposure to benzene or high levels of formaldehyde. Pesticide exposure and obesity have also been associated with AML in some studies.

Cytotoxic Chemotherapeutic Agents—Approximately 10% of AMLs in the United States that are therapy-related are associated alkylating agents, topoisomerase II inhibitors, antimetabolite, and antitubulin drugs. The risk of t-MDS/t-AML varies considerably with the therapeutic regimen and ranges from 0.8% to 24%. Alkylating agents, such as nitrogen mustards, and antimetabolites induce bone marrow damage primarily characterized by pancytopenia, as does ionizing radiation. The main dose-limiting toxicity associated with the majority of anticancer drugs is therapy-related myelosuppression.

Most chemotherapeutic *alkylating agents* (e.g., melphalan, cyclophosphamide, nitrogen mustard, busulfan, chlorambucil, carboplatin, cisplatin, dacarbazine, procarbazine, carmustine, mitomycin C, thiotepa, lomustine) can cause MDS and/or AML. AMLs are preceded by a myelodysplastic phase and are often characterized by loss of all or part of chromosome 5 or 7, complex karyotypes, and mutations in p53. Monofunctional alkylating agents, such as nitrosoureas, dacarbazine, and temozolomide, transfer an alkyl group to DNA bases to form lesions that result in mutations, secondary DNA double-stranded breaks, and eventual cytotoxicity. Bifunctional alkylators, such as cyclophosphamide, melphalan, and chlorambucil, induce the formation of cross-links within and between DNA lesions in addition to alkylated base lesions. Resulting double-stranded DNA breaks can lead to chromosomal translocations, insertions, inversions, and loss-of-heterozygosity. Regimens that include etoposide or doxorubicin are linked to AMLs that feature rearrangements at chromosomal band 11q23. Regimens that include mitoxantrone and epirubicin are linked to acute promyelocytic leukemias

that feature t(15;17) rearrangements. Patients treated with the *antimetabolite drugs* azathioprine, 6-thioguanine, and 6-mercaptopurine have an increased risk of developing AML. Many patients treated with antimetabolite drugs have loss of chromosome 7 or partial loss of chromosome 5 or 7 ($5q^-$ or $7q^-$).

As t-AML is the dominant hematopoietic disorder associated with drug or chemical exposure followed by t-MDS, these diseases may represent a continuum of toxic response. More t-AML and t-MDS cases (up to 50%) have a *complex karyotype* (more than three aberrations). The direct induction of oncogenic aberrations, genetic instability, and accumulation of complex aberrations represent one mechanism of t-AML/MDS.

Radiation—Exposure to ionizing radiation has been associated with several types of leukemia. Leukemogenesis associated with radiation involves myelotoxicity, immune suppression, chromosomal aberrations, and genetic mutations. Increased risk of leukemia has also been reported following the use of radiation for diagnostic tests, for the treatment of benign diseases, and following radiotherapy for cancer.

Occupational and Environmental Exposures

Benzene— Exposure to benzene may cause as much as 8% to 16% of the AML and MDS cases in the general U.S. population. Reactive metabolites include benzene oxide, benzene dihydrodiol epoxide, and the phenols: catechol, benzenetriol, and hydroquinone, which are subsequently metabolized in the bone marrow to produce benzoquinone metabolites, including benzene epoxide, benzene dihydrodiol epoxide, *t,t*-muconaldehyde, 1,2- and 1,4-benzoquinone. Genotoxicity and myelotoxicity are induced by benzene metabolites and by reactive oxygen species (ROS) formation via redox cycling of reactive intermediates.

Benzene targets pluripotent bone marrow HSC and causes pancytopenia and, in severe cases, aplastic anemia. Hematotoxicity is manifest as reduced numbers of total white blood cells, granulocytes, lymphocytes, B cells, and platelets. The number of circulating hematopoietic myeloid progenitor cells is also suppressed.

Benzene exposure has been associated with higher levels of chromosomal changes commonly observed in AML, including 5q/5 or 7q/7, þ8, and t(8;21) in the peripheral blood lymphocytes of highly exposed workers. Benzene-induced AMLs share cytogenetic aberrations with t-AML induced by alkylating agents and topoisomerase II inhibitors. Additional mechanisms of benzene-induced hematotoxicity include altered proliferation and differentiation of HSCs, oxidative stress, altered epigenetics and transcriptomics, bone marrow niche dysregulation, altered AhR signaling, and reduced immunosurveillance.

Formaldehyde— Globally, millions of people are occupationally and environmentally exposed to formaldehyde. Occupational exposure to formaldehyde in healthy workers decreased counts of lymphocytes, granulocytes, and platelets, and caused aneuploidy of chromosome 5 and 7, and structural aberrations in chromosome 5 in myeloid progenitor cells. Formaldehyde is toxic to mature blood cells, bone marrow cells, and hematopoietic stem/progenitor cells (HSCs/HPCs). It induces DNA adducts, protein-adducts, and DNA-protein cross-links (DPCs) as well as chromosomal aberrations (CA), sister chromatid exchanges (SCEs), and micronuclei (MN).

Due to its highly reactive nature, formaldehyde is unlikely to exert hematotoxic/genotoxic damage directly in the bone marrow. Inhaled formaldehyde is thought to induce mutations or pre-mutagenic lesions in (1) HSCs and HPCs circulating in peripheral blood proximal to the

lungs; or (2) primitive pluripotent stem cells present within the nasal turbinates and/or olfactory mucosa. The damaged stem cells could travel to the bone marrow to initiate leukemogenesis.

Causes of Childhood Leukemia—In utero exposure to low-dose radiation from medical x-rays, pesticides through home use or paternal occupational exposure, paternal preconception smoking, solvents such as benezene, and traffic emissions have consistently been associated with disease risk. Infant AML with chromosome 11q23 rearrangements is associated with maternal diets high in naturally occurring topoisomerase II-active agents, including soy. The most common translocation in ALL, ETV6-RUNX1, appears to be associated with parental smoking and home paint use. Prenatal and early-life tobacco smoke exposure was shown to increase the frequency of ALL-associated gene deletions in children who develop ALL.

Factors That Modulate Leukemia Risk

Modifiers of leukemia risk by leukemia-inducing agents include metabolism, pharmacokinetics, DNA-adduct type and repair, as well as individual and age-related susceptibility factors. In multiple candidate gene association studies, susceptibility to benzene-induced hematotoxicity is mediated by polymorphisms in genes involved in benzene metabolism, oxidative stress, DNA repair and genomic maintenance, innate immunity, cytokines, and cellular adhesion molecules. One of these polymorphisms in NAD(P)H:quinone oxidoreductase (NQO1) lowers or eliminates its activity and increases susceptibility to benzene-induced hematotoxicity, as well as overall risk of de novo AML and therapy-induced myeloid neoplasms.

TOXICOLOGY OF PLATELETS AND HEMOSTASIS

A multicomponent system is responsible for preventing the loss of blood from sites of vascular injury and maintaining circulating blood in a fluid state. Loss of blood is prevented by formation of stable hemostatic plugs. This procoagulant response is normally limited to sites of vascular injury by the regulatory arm of hemostasis. The dynamically modulated balance between procoagulant and regulatory pathways permits a rapid, localized response to injury. The major constituents of the hemostatic system include circulating platelets, a variety of plasma proteins, vascular endothelial cells, and other cells. Alterations in these components or systemic activation of this system can derange hemostasis, causing excessive bleeding and thrombosis.

Toxic Effects on Platelets

The Thrombocyte—Platelets initially adhere to the damaged wall through binding of von Willebrand factor (vWF) with the platelet glycoprotein Ib/IX/V (GP Ib/IX/V) receptor complex. Ligand binding to GP Ib/IX/V or interaction of other platelet agonists (e.g., thrombin, collagen, ADP, thromboxane A_2) with their specific receptors initiates biochemical response pathways that lead to shape change, platelet contraction, platelet secretion of granule contents, activation of the GP IIb/IIIa receptor, and externalization of phosphatidylserine. Activation of the GP IIb/IIIa

receptor permits fibrinogen and other multivalent adhesive molecules to form cross-links between nearby platelets, resulting in platelet aggregation. Xenobiotics may interfere with the platelet response by causing thrombocytopenia or interfering with platelet function; some agents are capable of affecting both platelet number and function.

Thrombocytopenia—Thrombocytopenia may be due to decreased production or increased destruction of platelets. Immune thrombocytopenia was thought to be an antibody-mediated disease with the responding antibody binding resulting in removal of the platelet from the circulation by the mononuclear phagocytic system. Other lymphocyte abnormalities that are found in patients with immune thrombocytopenia include skewing of T helper cells to an autoimmune phenotype, poor activity of regulatory T cells or regulatory B cells, and direct killing of platelets by cytotoxic T cells. Antibodies and T cells may also attack megakaryocytes, causing reduced platelet production. More than 100 drugs have been associated with causing immune thrombocytopenia, but carbamazepine, eptifibatide, ibuprofen, quinidine, quinine, oxaliplatin, rifampicin, sulfamethoxazole, trimethoprim, and vancomycin are the most frequently implicated.

Heparin-induced thrombocytopenia (HIT) is due to the development of antibodies that react with a multimolecular complex formed by the interaction between heparin and a protein, usually platelet factor 4 (PF 4). When the relative concentration of heparin to PF 4 is appropriate, a neoepitope on PF 4 (or another target protein) is exposed and an IgG response develops. IgG bindung to the PF 4–heparin complex activates biochemical signaling pathways that result in platelet activation and aggregation, plus release of platelet microparticles that promote thrombin generation. Consequently, HIT is associated with both thrombocytopenia and an increased risk of arterial and venous thrombosis. Streptokinase–IgG may trigger platelet activation and thrombocytopenia through a similar mechanism.

Thrombotic thrombocytopenic purpura (TTP) is characterized by microangiopathic hemolytic anemia, severe thrombocytopenia, and organ ischemia linked to disseminated microvascular platelet-rich thrombi. TTP is specifically related to deficiency in ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type 1 repeats, member 13), the specific von Willebrand (vWF) factor-cleaving protease. The organ failure and hemolysis in TTP is due to the formation of platelet-rich microthrombi throughout the circulation.

Hemolytic uremic syndrome (HUS) has clinical features of microangiopathic hemolytic anemia, thrombocytopenia, and renal failure. Typical HUS is caused by Shiga toxin-producing *Escherichia coli* (STEC) infection, atypical HUS (aHUS), usually caused by uncontrolled complement activation, or secondary HUS with a coexisting disease. The common features in STEC-HUS, aHUS, and secondary HUS are simultaneous damage to endothelial cells, intravascular hemolysis, and activation of platelets leading to a procoagulative state, formation of microthrombi, and tissue damage.

Toxic Effects on Platelet Function—Platelet function is dependent on the coordinated interaction of several biochemical response pathways. Nonsteroidal anti-inflammatory agents inhibit the phospholipase A_2/cyclooxygenase pathway and synthesis of thromboxane A_2. Other drugs (e.g., antibiotics, ticlopidine, clopidogrel) appear to interfere with the interaction between platelet agonists and their receptors. Any chemical that interferes with translocation of calcium may inhibit platelet function (e.g., calcium channel blockers). Occasionally, drug-induced antibodies will bind to a critical platelet receptor and inhibit its function, thereby potentiating the

bleeding risk associated with the xenobiotic-induced thrombocytopenia.

Toxic Effects on Fibrin Clot Formation

Coagulation—Fibrin clot formation results from sequential activation of a series of serine proteases that culminates in thrombin formation. Thrombin is a multifunctional enzyme that converts fibrinogen to fibrin; activates factors V, VIII, XI, XIII, protein C, and platelets; and interacts with a variety of cells (e.g., leukocytes and endothelial cells) activating cellular signaling pathways. The most common toxic effect of xenobiotics on fibrin clot formation are related to a decreased level of one or more of the critical proteins necessary for this process.

Decreased Synthesis of Coagulation Proteins—Most proteins involved in coagulation are synthesized in the liver. Therefore, any chemical that impairs liver function may cause a decrease in coagulation factors production. The common tests of the coagulation cascade, prothrombin time (PT) and activated partial thromboplastin time (aPTT), may be used to screen for liver dysfunction and a decrease in clotting factors.

Factors II, VII, IX, and X are dependent on vitamin K for their complete synthesis. Anything that interferes with vitamin K metabolism may lead to a deficiency of these factors and a bleeding tendency. This may occur with xenobiotics that interfere with the intestinal absorption of vitamin K or with the reduction of vitamin K epoxide (Table 11–7).

TABLE 11–7 Conditions Associated with Abnormal Synthesis of Vitamin K–Dependent Coagulation Factors

Warfarin and analogs	Intravenous α-tocopherol
Rodenticides (e.g., brodifacoum)	Dietary deficiency
	Cholestyramine resin
Broad-spectrum antibiotics	Malabsorption syndromes
N-Methyl-thiotetrazole cephalosporins	

Increased Clearance of Coagulation Factors— Idiosyncratic reactions to xenobiotics include the formation of antibodies that react with coagulation proteins, forming an immune complex that is rapidly cleared from the circulation and resulting in deficiency of the factor. The factors that are most often affected include factors V, VIII, and XIII, vWF, prothrombin, and thrombin (Table 11–8). Many of these antibodies inhibit the functional activity of the coagulation factor in addition to increasing the rate of clearance. Other antibodies have catalytic activity, resulting in proteolysis of the target coagulation factor.

TABLE 11–8 Relationship Between Xenobiotics and the Development of Specific Coagulation Factor Inhibitors

Coagulation Factor	Xenobiotic
Thrombin	Topical bovine thrombin
	Fibrin glue
Factor V	Streptomycin
	Penicillin
	Gentamicin
	Cephalosporins
	Topical bovine thrombin
Factor VIII	Penicillin
	Ampicillin
	Chloramphenicol
	Phenytoin
	Methyldopa
	Nitrofurazone
	Phenylbutazone
Factor XIII	Isoniazid
	Procainamide
	Penicillin
	Phenytoin
	Practolol
von Willebrand factor	Ciprofloxacin
	Hydroxyethyl starch
	Valproic acid
	Griseofulvin
	Tetracycline
	Pesticides

Lupus anticoagulants are antibodies that interfere with in vitro phospholipid-dependent coagulation reactions. These antibodies are directed against phospholipid-binding proteins, including prothrombin and β_2-glycoprotein 1. In vivo, these antibodies can potentiate

procoagulant mechanisms and interfere with the protein C system, increasing the risk of thrombosis. The development of lupus anticoagulants has been associated with medications including procainamide, chlorpromazine, and hydralazine.

Toxicology of Chemicals Used to Modulate Hemostasis

Oral Anticoagulants—Oral anticoagulants (warfarin) interfere with the reduction of vitamin K epoxide, resulting in a functional deficiency of reduced vitamin K. These drugs are widely used for prophylaxis and therapy of venous and arterial thrombosis. The therapeutic window for oral anticoagulants is relatively narrow, and there is considerable interindividual variation in the response to a given dose. Several factors, including concurrent medications and genetics, affect the individual response to oral anticoagulants. Therapy with these drugs must be routinely monitored to maximize both safety and efficacy. This is routinely performed with the PT, with results expressed in terms of the international normalized ratio (INR).

Many xenobiotics and foods affect the response to oral anticoagulants. Mechanisms for interference with oral anticoagulants include inhibition or induction of CYP2C9; interference with absorption of warfarin from the gastrointestinal tract; displacement of warfarin from albumin in plasma; diminished vitamin K availability, due to either dietary deficiency or interference with the absorption of this lipid-soluble vitamin; and inhibition of the reduction of vitamin K epoxide, which potentiates the effect of oral anticoagulants. Administration of oral anticoagulants may affect the activity of other medications, particularly those that are metabolized by CYP2C9.

Warfarin-induced skin necrosis is an uncommon toxic effect that is thought to be related to a rapid drop in protein C following administration of the drug, resulting in impaired protein C function.

Vitamin K is necessary for the synthesis of proteins other than the coagulation-related factors, including osteocalcin, a major component of bone. Long-term administration of warfarin has been associated with bone demineralization. This effect can be important in patients with borderline bone density.

Administration of warfarin during pregnancy, particularly the first 12 weeks of pregnancy, is associated with congenital anomalies in 25% to 30% of exposed infants. Many of the anomalies are related to abnormal bone formation. It is thought that warfarin may interfere with synthesis of proteins critical for normal structural development.

Heparin—Heparin is a widely used anticoagulant for both prophylaxis and therapy of acute venous thromboembolism. The major complication associated with heparin therapy is bleeding, a direct manifestation of its anticoagulant activity. The risk of bleeding is related to the intensity of therapy, the patient’s body mass and underlying condition, and the presence of other hemostatic defects (e.g., thrombocytopenia). Long term administration of heparin is associated with an increased risk of osteoporosis.

Fibrinolytic Drugs—Fibrinolytic drugs dissolve the pathogenic thrombi by converting plasminogen, an inactive zymogen, to plasmin, an active proteolytic enzyme. Plasmin is normally tightly regulated and not freely present in the circulation. Administration of fibrinolytic drugs can generate free plasmin leading to systemic fibrin(ogen)olysis, which is characterized by a decrease in fibrinogen, factors V and VIII, and α_2-antiplasmin; an increase in circulating fibrin

split products; degradation of platelet GP Ib/IX/V and IIb/IIIa; degradation of endothelial cell glycoproteins; degradation of fibronectin and thrombospondin; and prolongation of the PT, aPTT, and thrombin time. All these effects potentiate the risk of bleeding.

Streptokinase is a protein derived from group C β-hemolytic streptococci that is antigenic in humans. Antibody formation to streptokinase occurs commonly in association with streptococcal infections as well as exposure to streptokinase. Acute allergic reactions may occur in 1% to 5% of patients exposed to streptokinase.

Inhibitors of Fibrinolysis—Antifibrinolytic drugs are commonly used to control bleeding in patients with congenital abnormalities of hemostasis, such as von Willebrand disease. Tranexamic acid and ε-aminocaproic acid are small molecules that block the binding of plasminogen and plasmin to fibrin and other substrate proteins through interaction with lysine binding sites on plasmin(ogen). These chemicals may increase the risk of thrombosis due to the inhibition of the fibrinolytic system. Aprotinin is a naturally occurring polypeptide inhibitor of serine proteases. It is usually derived from bovine material and consequently is immunogenic when administered to humans. Allergic reactions in response to aprotinin range from minor cutaneous manifestations to anaphylactic reactions.

HEMATOTOXICITY TESTS

Assessing the risk that exposure to new drugs, chemical products, and other agents poses to humans—in terms of significant toxic effects on hematopoiesis and the functional integrity of blood cells and hemostatic mechanisms—can be logistically and intellectually challenging owing to the complexity of hematopoiesis and the wide-ranging tasks that these components perform.

Animal Studies

Conventional hematotoxicity testing in animals should provide information on the effects of single- and multiple-dose exposure on erythrocyte parameters (RBC, Hbg, PCV, MCV, MCHC), leukocyte parameters (WBC and absolute differential counts), thrombocyte counts, coagulation tests (PT, aPTT), peripheral blood cell morphology, and bone marrow cytologic and histologic features. Cytological or flow cytometric evaluation of bone marrow is useful to characterize effects on different hematopoietic lineages or stem or progenitor cells. Additional tests to better characterize findings of hematotoxicologic potential are listed in Table 11–9.

TABLE 11–9 Examples of Problem-Driven Tests Used to Characterize Hematologic Observations in Preclinical Toxicology

Flow cytometry (see text)
Heinz body preparation
Cell-associated antibody assays (erythrocyte, platelet, neutrophil)
Erythrocyte osmotic fragility test
Erythrokinetic/ferrokinetic analyses
Cytochemical/histochemical staining
Electron microscopy
In vitro hematopoietic clonogenic assays
Platelet aggregation
Plasma fibrinogen concentration
Clotting factor assays
Thrombin time
Bleeding time

Human Population Studies

Treatment with drugs and therapeutics can lead to *idiosyncratic reactions* that cause toxicity in a small number of susceptible individuals and may include aplastic anemia, thrombocytopenia, hemolysis, and leukopenia.

In Vitro Bone Marrow Assays

Suppressive effects on HSC can be assessed using short-term clonogenic assays including burst-forming-unit erythroid (BFU-E), colony-forming-unit erythroid (CFU-E), colony-forming-unit granulocyte/monocyte (CFU-GM), colony-forming-unit megakaryocyte (CFU-MK), and colony-forming-unit granulocyte, erythroid, megakaryocyte, monocyte (CFU-GEMM), which have been developed for several laboratory animal species. These assays examine tightly controlled exposure concentrations and durations effects on myeloid, erythroid, and megakaryocytic lineages. Advantages of in vitro clonogenic assays include the testing of human hematopoietic cells directly in a preclinical setting, thus obviating extrapolation considerations. In vitro assays can be used to test combinations of chemicals as well as their metabolites and effects of serum and other cell components, such as lymphocytes. In vitro clonogenic assays in risk assessment have been useful for making interspecies comparisons regarding sensitivity to a particular agent or group of chemicals.

One of the limitations of the assays is the low-throughput capacity. Some newer techniques use liquid culture. In the automated non-clonogenic fluorometric microculture cytotoxicity assay (FMCA), human CD34 cells are incubated in 384-well microplates with drugs in liquid culture,

supplemented with GM-stimulating cytokines, and cell survival is assessed. HALO® (Hemotoxicity Assays via Luminescence Output) is an ATP-based bioluminescence assay that quantitates fourteen different lymphohematopoietic populations from three different tissues (peripheral blood, bone marrow, and cord blood) and from five different species (human, nonhuman primate, dog, rat, and mouse). Platforms that enable the liquid culture of HSC/HPC can be used in conjunction with multiple other assays to understand mechanism of action, including mitochondrial dysfunction, genotoxicity (micronucleus assay), proliferation, differentiation, oxidative stress, cytokine release, chemotaxis/migration, oxidative stress, apoptosis, etc.

Emerging Technologies

Toxicogenomics—Toxicogenomics involves the assessment by omic technologies of the genome, transcriptome, epigenome, proteome, and metabolome, of animals, people, or in vitro models that have been exposed to toxic agents. Toxicogenomics in exposed human populations can elucidate mechanisms of action underlying exposure-related disease, identify susceptible individuals, and reveal responses at low doses. Integrative omic analysis of various data types can provide more detailed pathway and network information, identify more robust biomarkers, and elucidate mechanisms of toxicity and disease, thus aiding risk characterization.

3D Bone Marrow Niche Models—Research groups have attempted to develop 3D models of the bone marrow niche using biomimetic materials, such as collagen microspheres, hydrogels, decellularized extracellular matrix extracts, and synthetic polyacrylates, as scaffolds. Although progress has been made, key challenges remain in replicating the complex interactions between HSCs and marrow microenvironments and precisely controlling the biochemical and physical niches that are critical in supporting hematopoiesis.

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QUESTIONS

1. Which of the following statements is FALSE regarding true anemia?
   a. Alterations of the mean corpuscular volume are characteristic of anemia.

b. Increased destruction of erythrocytes can lead to anemia.
c. Decreased production of erythrocytes is not a common cause of anemia because the bone marrow is continuously renewing the red blood cell pool.
d. Reticulocytes will live for a longer period of time in the peripheral blood when a person is anemic.
e. The main parameters in diagnosing anemia are RBC count, hemoglobin concentration, and hematocrit.

2. Which of the following types of anemia is properly paired with its cause?
a. iron deficiency anemia—blood loss.
b. sideroblastic anemia—vitamin B_{12} deficiency.
c. megaloblastic anemia—folate supplementation.
d. aplastic anemia—ethanol.
e. megaloblastic anemia—lead poisoning.

3. The inability to synthesize the porphyrin ring of hemoglobin will most likely result in which of the following?
a. iron deficiency anemia.
b. improper RBC mitosis.
c. inability to synthesize thymidine.
d. accumulation of iron within erythroblasts.
e. bone marrow hypoplasia.

4. Which of the following will cause a right shift in the oxygen dissociation curve?
a. increased pH.
b. decreased carbon dioxide concentration.
c. decreased body temperature.
d. increased 2,3-BPG concentration.
e. fetal hemoglobin.

5. All of the following statements regarding erythrocytes are true EXCEPT:
a. Aged erythrocytes are removed by the liver, where the iron is recycled.
b. Erythrocytes have a life span of approximately 120 days.
c. Red blood cells generally lose their nuclei before entering the circulation.
d. Reticulocytes are immature RBCs that still have a little RNA.
e. Persons with anemia have a higher than normal reticulocyte:erythrocyte ratio.

6. All of the following statements regarding oxidative hemolysis are true EXCEPT:
a. Reactive oxygen species are commonly generated by RBC metabolism.
b. Superoxide dismutase and catalase are enzymes that protect against oxidative damage.
c. Reduced glutathione (GSH) increases the likelihood of oxidative injuries to RBCs.
d. Glucose-6-phosphate dehydrogenase deficiency is commonly associated with oxidative hemolysis.
e. Xenobiotics can cause oxidative injury to RBCs by overcoming the protective

mechanisms of the cell.

7. Which of the following sets of leukocytes is properly characterized as granulocytes because of the appearance of cytoplasmic granules on a blood smear?
   a. neutrophils, basophils, and monocytes.
   b. basophils, eosinophils, and lymphocytes.
   c. eosinophils, neutrophils, and lymphocytes.
   d. basophils, eosinophils, and neutrophils.
   e. lymphocytes, neutrophils, and basophils.

8. All of the following statements are true EXCEPT:
   a. Xenobiotics can greatly slow down the proliferation of neutrophils and monocytes, increasing the risk of infection.
   b. Ethanol and cortisol decrease phagocytosis and microbe ingestion by the immune system.
   c. Agranulocytosis is predictable and can be caused by exposure to a number of environmental toxicants.
   d. Heroin and methadone abusers have reduced ability to kill microorganisms due to drug-induced reduction in superoxide production.
   e. Toxic neutropenia may be mediated by the immune system.

9. Leukemias:
   a. are often due to cytogenic abnormalities, particularly damage to or loss of chromosomes 8 and 11.
   b. are rarely caused by agents used in cancer chemotherapy.
   c. originate in circulating blood cells.
   d. are characterized as “acute” if their effects are short-lived and severe.
   e. have long been associated with exposure to x-ray radiation.

10. Regarding platelets and thrombocytopenia, which of the following statements is FALSE?
   a. Platelets can be removed from the circulation through a hapten-mediated pathway that is induced by drugs or chemicals.
   b. Cortisol decreases platelet activity by inhibiting thromboxane prostaglandin synthesis.
   c. Toxicants can induce a change in a platelet membrane glycoprotein, leading to recognition and removal of the platelet by phagocytes.
   d. Heparin administration can result in platelet aggregation and cause thrombocytopenia.
   e. Thrombotic thrombocytopenic purpura is most commonly caused by infectious disease, but can also be associated with administration of pharmacologic agents.

CHAPTER 12

Toxic Responses of the Immune System

Barbara L.F. Kaplan, Courtney E.W. Sulentic, Helen G. Haggerty, Michael P. Holsapple, and Norbert E. Kaminski

KEY POINTS

- Immunity is a series of delicately balanced, complex, multicellular, and physiologic mechanisms that allow an individual to distinguish foreign material from "self" and to neutralize and/or eliminate that foreign matter.
- Innate immunity, which eliminates most potential pathogens before significant infection occurs, includes physical and biochemical barriers both inside and outside of the body as well as immune cells designed for specific responses.
- Acquired immunity involves producing a specific immune response to each infectious agent (*specificity*) and remembering that agent so as to mount a faster response to a future infection by the same agent (*memory*).
- Autoimmunity occurs when the reactions of the immune system are directed against the body's own tissues, resulting in tissue damage and disease.
- Hypersensitivity reactions require prior exposure leading to sensitization in order to elicit a reaction on subsequent challenge.
- Immunotoxicology is the study of adverse effects of drugs, environmental chemicals, and, in some instances, biological materials on the immune system resulting from occupational, inadvertent, or therapeutic exposure.
- Xenobiotics that alter the immune system can upset the balance between immune recognition and destruction of foreign invaders and the proliferation of these microbes and/or cancer cells.

INTRODUCTION

Immunotoxicology can be defined as the study of adverse effects on the immune system resulting from occupational, inadvertent, or therapeutic exposure to drugs, environmental chemicals, and, in some instances, biological materials.

The immune system is a series of delicately balanced, complex, multicellular, and physiologic mechanisms that allow an individual to distinguish foreign material (i.e., "nonself") from "self," and to neutralize, eliminate, and/or coexist with the foreign matter. Examples of self are all the tissues, organs, and cells of the body. Examples of nonself include opportunistic bacteria and viruses, transformed cells or tissues (i.e., tumors), drugs, xenografts, or allergens.

Immunotoxicology should be considered as a continuum (Fig. 12–1). Due to the potentially profound effects resulting from disruption of the delicately balanced immune system, there is a need to understand the cellular, biochemical, and molecular mechanisms of xenobiotic-induced immune modulation.

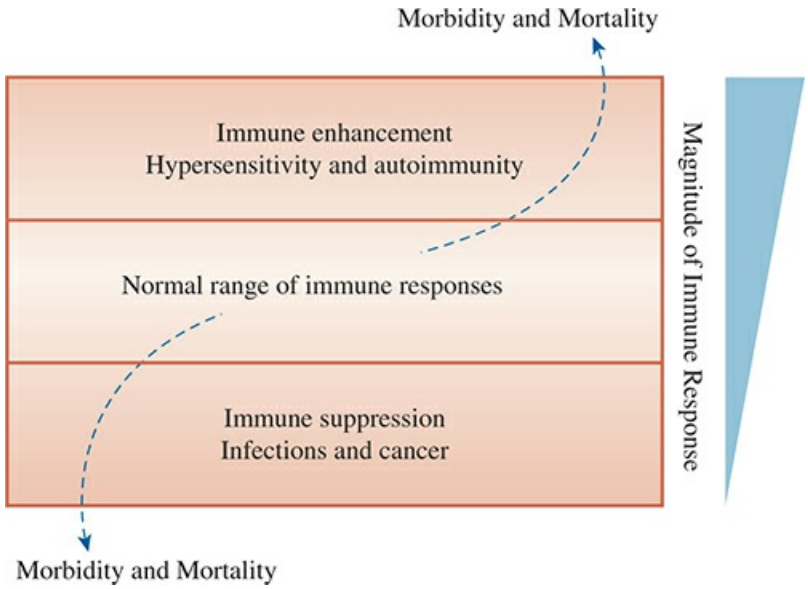

FIGURE 12–1 *The continuum of immunotoxicology.* Immune toxicity results from xenobiotic-induced suppression or enhancement of immune function.

The reader might also find the list of abbreviations in Table 12–1 helpful.

TABLE 12–1 Abbreviations

Term	Abbreviation
Antibody-dependent cell-mediated cytotoxicity	ADCC
Antibody forming cell	AFC
Antigen presenting cell	APC
Antibody secreting cell	ASC
Aryl hydrocarbon receptor	AhR
B-cell receptor	BCR
B-regulatory cell	Breg
Carboxyfluorescein succinimidyl ester	CFSE
CD40 ligand	CD40L
Cell-mediated immunity	CMI
Central nervous system	CNS
Cluster of differentiation	CD
Colony stimulating factor	CSF
Common lymphoid progenitor	CLP
Common myeloid progenitor	CMP
Concanavalin-A	Con-A
Conventional dendritic cell	cDC
Cyclooxygenase	COX
Cytotoxic T lymphocyte	CTL
Danger-associated molecular patterns	DAMPs
Delayed-type hypersensitivity	DTH
Dendritic cell	DC
Developmental and reproductive toxicology	DART
Developmental immunotoxicology	DIT
Embryo fetal development	EFD
Experimental autoimmune encephalomyelitis	EAE
Fc receptor	FcR
Hematopoietic stem cell	HSC
Human lymphocyte activation	HuLa
Human peripheral blood	HPB
Immunoglobulin	Ig
Interferon	IFN
Inducible T regulatory cell	iTreg
Innate lymphoid cell	ILC

Interleukin	IL
Keyhole limpet hemocyanin	KLH
Lipopolysaccharide	LPS
Local lymph node assay	LLNA
Major histocompatibility complex	MHC
Membrane attack complex	MAC
Mixed lymphocyte response	MLR
Neutrophil extracellular trap	NET
National Toxicology Program	NTP
Natural killer cell	NK
Natural T regulatory cell	nTreg
NOD-like receptors	NLR
Non-human primates	NHP
Nuclear factor of activated T cells	NFAT
Nuclear factor κB	NF-κB
Pathogen-associated molecular patterns	PAMPs
Pattern recognition receptors	PRR
Peripheral blood mononuclear cell	PBMC
Phytohemagglutinin	PHA
Plaque forming cell	PFC
Plasmacytoid dendritic cell	pDC
Polymerase chain reaction	PCR
Pre/postnatal development	PPND
Reactive oxygen species	ROS
RIG-like receptors	RLR
Reverse transcriptase	RT
Sheep red blood cells	sRBCs
Single nucleotide polymorphism	SNP
Systemic lupus erythematosus	SLE
T-cell-dependent antibody response	TDAR
T-cell-independent antibody response	TIAR
T-cell receptor	TCR
T-helper cell	Th
Toll-like receptor	TLR
T-regulatory cell	Treg
Transforming growth factor	TGF
Tumor necrosis factor	TNF

THE IMMUNE SYSTEM

The immune system comprises lymphoid organs, many different cellular populations, and soluble factors with a variety of functions focused on providing immunity against nonself or exogenous substances (i.e., antigens). Immunity can be classified into two functional divisions, innate immunity and acquired (adaptive) immunity. Innate immunity is a first-line defense response with little immunological memory. By contrast, acquired (adaptive) immunity is characterized by antigen specificity and immunological memory. The speed and magnitude of the acquired immune response to a foreign organism is greater for a secondary challenge than it is for the primary challenge.

All immune cells originate from the bone marrow, which contains the pluripotent and self-renewing hematopoietic stem cell (HSC) from which all other hematopoietic cells are derived. Figure 12–2 shows one model of hematopoiesis. During gestation, the HSC is found in the embryonic yolk sac and fetal liver; eventually, it migrates to the bone marrow and developmentally commits to either the common lymphoid progenitor (CLP) or the common myeloid progenitor (CMP) lineages.

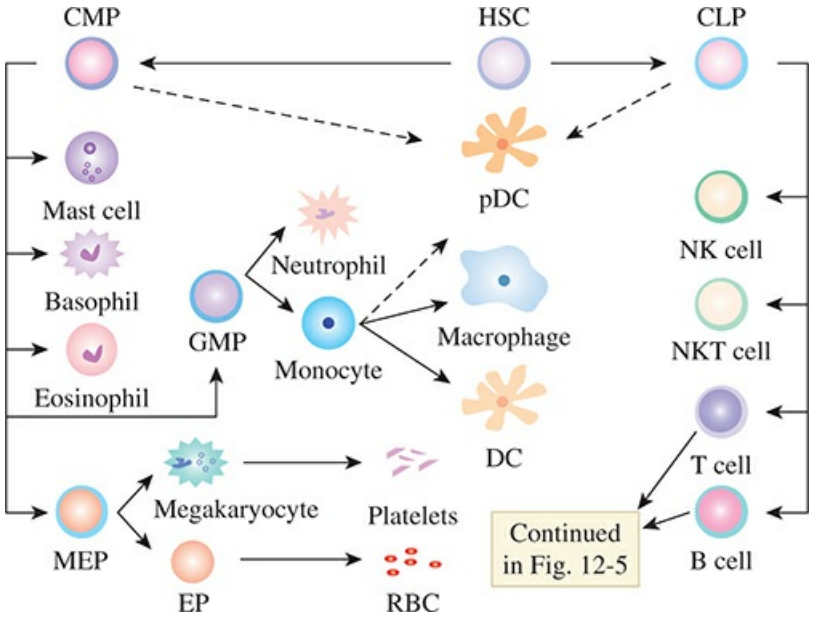

FIGURE 12–2 *Development of the cellular components of the immune system.* All immune cells initially develop in the bone marrow. The HSC differentiates into two main progenitors from which all other cells develop. The progenitor for pDCs is still unclear (dashed lines). CLP, common lymphoid progenitor; CMP, common myeloid progenitor; DC, dendritic cell; EP, erythrocyte precursor; GMP, granulocyte-macrophage progenitor; HSC, hematopoietic stem cell; MEP, megakaryocyte-erythrocyte progenitor; pDC, plasmacytoid dendritic cell; RBC, red blood cells.

Effective immunity results from the interaction and communication of the cells that comprise innate and acquired immunity in a coordinated multifunctional system. Cells interact with antigens and each other via receptors, and often produce soluble factors termed cytokines and chemokines as a means of cell–cell communication. The resulting actions by cells receiving

messages include cellular activation, initiation or termination of intracellular signaling events, proliferation, differentiation, migration, trafficking, or effector functions. While some of these molecules might be constitutively expressed, most are inducible in response to antigens, cellular stressors, or other cytokines. Many cytokines and chemokines are not stored in the cell, but are tightly regulated, often at the transcriptional level, so that they are quickly generated on demand. A partial list of cytokines and a brief description of the cell types that release and are acted upon by these various mediators is provided in Table 12–2.

TABLE 12–2 Cytokines: Sources and Functions in Immune Regulation

Cytokine	Source	Physiologic Actions
IL-1	Macrophages Epithelial cells	Activation and proliferation of T cells Pro-inflammatory Induces fever and acute-phase proteins Induces synthesis of pro-inflammatory cytokines
IL-2	T cells	Primary T-cell growth factor Growth factor for B cells and NK cells
IL-4	Th2 cells Mast cells	Proliferation of activated Th2 and B cells B-cell differentiation and IgE isotype switching Antagonizes IFN-γ Inhibits Th1 responses
IL-5	Th2 cells Mast cells	Proliferation and differentiation of eosinophils
IL-6	Macrophages Th2 cells B cells Endothelial cells	Enhances B-cell differentiation and Ig secretion Induction of acute-phase proteins by liver Pro-inflammatory Proliferation of T cells and increased IL-2 receptor expression
IL-10	Tregs Bregs Macrophages	Inhibits T-cell and macrophage responses
IL-12	DCs Macrophages	Activates NK cells Induces Th1 responses
IL-13	Th2 cells	Stimulates B-cell growth Inhibits Th1 responses
IL-17	Th17 cells NK cells γδ T cells Neutrophils	Pro-inflammatory Inhibits Tregs
IL-22	Th17 cells ILCs	Activates neutrophils Mucosal protection
IFN-α/β (Type I IFN)	Leukocytes DCs Fibroblasts	Induction of MHCI expression Antiviral activity Stimulation of NK cells
IFN-γ	T cells NK cells	Induction of MHCI and MHCII Activates macrophages
TGF-β	Macrophages Megakaryocytes T cells	Enhances monocyte/macrophage chemotaxis Enhances wound healing: angiogenesis, fibroblast Proliferation, deposition of extracellular matrix Inhibits T- and B-cell proliferation Inhibits antibody secretion Primary inducer of isotype switch to IgA
GM-CSF	T cells Macrophages Endothelial cells Fibroblasts	Stimulates growth and differentiation of monocytes and granulocytes

Data from Murphy K, Weaver C. *Janeway's Immunobiology*. 9th ed. New York: Garland Science, Taylor & Francis; 2016.

Innate Immunity

As a first line of defense against anything nonself, the innate immune system includes physical and biochemical barriers both inside and outside the body, as well as immune cells designed for host defense responses and soluble factors.

Most infectious agents enter the body through the respiratory system, gut, or genitourinary tract. Innate defenses to combat infection from pathogens entering through the respiratory system include mucus secreted along the nasopharynx, the presence of lysozyme in most secretions, and cilia lining the trachea and main bronchi. Reflexes such as coughing, sneezing, and elevation in body temperature are also a part of innate immunity. Pathogens that enter the body via the digestive tract are met with severe changes in pH (acidic) within the stomach and commensal bacteria (i.e., microbiota) living in the intestines that play a protective role.

Cellular Components: Neutrophils, Macrophages, Dendritic Cells, Natural Killer Cells, NKT Cells, γδ T Cells, and B-1 B Cells—Several cell types are involved in innate immunity. Neutrophils (also known as polymorphonuclear leukocytes or PMNs) are phagocytic cells that develop from the myeloid lineage of HSCs. Neutrophils can pass between the endothelial cells of the blood vessels (i.e., extravasation) and thereby represent a primary line of defense against pathogens. They are excellent phagocytic cells and can eliminate most microorganisms through the release of various reactive oxygen species (ROS), such as superoxide, singlet oxygen, ozone, hydrogen peroxide, and hydroxyl radicals. Their ability to internalize foreign materials is greatly enhanced by the presence of complement and antibody deposited on the surface of the foreign target. Neutrophils are also important in the inflammatory response.

Macrophages develop from the myeloid lineage of HSCs (Fig. 12–2) and migrate to various tissues where they then differentiate into distinct macrophage populations. Tissue-specific macrophages include Kupffer cells (liver), alveolar macrophages (lung), microglial cells (CNS), and peritoneal and splenic macrophages. These tissue-specific macrophages have distinct properties and vary in the extent of surface receptors, oxidative metabolism, and expression of major histocompatibility complex class II (MHCII). Macrophages can be classified as classically activated macrophages (M1), which are pro-inflammatory, or alternatively activated macrophages (M2), which are efficient at apoptotic cell removal and tissue remodeling and repair.

Dendritic cells (DC) survey their environment for pathogens, then mature to become highly efficient antigen presenting cells (APCs). They provide a bridge between initial detection of an infectious agent and elicitation of adaptive immune responses.

NK cells are derived from a CLP (Fig. 12–2) and belong to a larger family of innate lymphoid cells (ILCs), which play roles in innate immunity and inflammation. NK cells have two major functions, cytokine production and destroying virus-infected or neoplastic target cells. NK cells are a predominant source of IFN-γ, which helps mature DCs and activates macrophages. NK cells mediate both antibody-independent and antibody-dependent cellular cytotoxicities.

The γδ T cells migrate predominantly to “exposed” tissues, including skin, lung, gut, and reproductive organs, and are also expressed highly in the liver. The γδ T cells acquire effector functions of cytokine production and cytotoxicity. There is also a subpopulation of B cells (i.e., $CD5^+$ or B-1 cells) that predominate in embryonic life and are later found mostly in the peritoneal and pleural cavities. B-1 cells are self-renewing and spontaneously produce polyspecific IgM antibodies (i.e., antibodies directed primarily against capsular polysaccharide

antigens) independent of T-cell help.

Pattern recognition receptors are recognized pathogen-derived molecules (pathogen-associated molecular patterns [PAMPs]) or cell-derived molecules produced in response to cellular stress (danger-associated molecular patterns [DAMPs]). Receptors that recognize PAMPs or DAMPs include Toll-like receptors (TLRs), NOD-like receptors (NLRs), and RIG-like receptors (RLRs). Several TLRs are expressed extracellularly, and several are expressed intracellularly in endosomes, to protect against extracellular and intracellular pathogens, respectively. Functional consequences of TLRs include expression of adhesion molecules, chemokines, or cytokines to stimulate T- or B-cell differentiation, enhance phagocytosis, or induce maturation of DCs.

Soluble Components: Acute-Phase Proteins, Granzyme, Perforin, and Complement —Acute-phase proteins, such as serum amyloid A, serum amyloid P, and C-reactive protein, participate in an acute-phase response to infection by binding bacteria and facilitating complement activation. Granzyme and perforin work in conjunction, with perforin disrupting the target cell membrane, allowing granzyme to enter and mediate cell lysis.

The complement system of about 30 serum proteins functions to destroy the membranes of infectious agents, facilitate phagocytosis of foreign materials, and promote an inflammatory response. Complement activation occurs with each component sequentially acting on other components. Proximal components of the cascade are often modified serine proteases, which activate the system but have limited substrate specificity. Several components are capable of binding to microbial membranes and serve as ligands for complement receptors on phagocytic cells to facilitate phagocytosis, although a primary pathway of complement activation occurs after antibody binds a pathogen. The final components, which are related structurally, are also membrane-binding proteins that form the membrane attack complex (MAC), which disrupts membrane integrity.

Three pathways have been identified that activate the complement cascade. The classical complement pathway is initiated when antibody binds antigen on the microorganism, and then proceeds by activating the cascade. The alternative pathway can be activated and can amplify the complement-mediated killing of the microorganism. Complement-mediated lysis is also activated through the lectin pathway, in which binding of mannose-binding lectin (MBL) to the surface of the microorganism activates the pathway. In addition to cytolysis and generation of pro-inflammatory mediators, complement activation induces opsonization, the process by which microorganisms are coated by opsonins (predominantly IgM and IgG antibodies and C3b), rendering them vulnerable to phagocytosis.

Antigen Recognition

A critical step in the initiation of acquired immunity is the ability of immune cells to recognize nonself. Nonself molecules need to be processed and presented by APCs to initiate the cell-mediated and humoral response by T and B cells, respectively.

Antigen—A nonself substance that can be recognized by the immune system is called an antigen, immunogen, or allergen. Antigens are usually (but not absolutely) biological molecules that can be cleaved and rearranged for presentation to other immune cells. Generally, antigens are about 10 kDa or larger in size. Smaller antigens are termed “haptens” and must be conjugated

with carrier molecules (larger antigens) to elicit a specific response. However, once an initial response is made, the hapten can induce subsequent responses in the absence of the carrier.

Antibodies—Antibodies are produced by B cells and are defined functionally by the antigen with which they react, and by their subtype (IgM, IgD, IgG, IgE, and IgA). Thus, an IgM antibody directed against sheep red blood cells (sRBCs) is called anti-sRBC IgM. Because the immune system generates antibodies to thousands of antigens with which the host may or may not ever come into contact, antibodies of unknown specificity are referred to as immunoglobulin (e.g., serum immunoglobulin or serum IgM) until defined by their specific antigen (e.g., anti-sRBC IgM).

All Igs are made up of heavy and light chains and of constant and variable regions. For the light chain genes, two separate gene segments (V and J) are combined to form the variable region, which is then joined to one constant region. For the heavy chain genes, three separate gene segments (V, D, and J) are combined to form the variable region, which is then joined to the constant region. There are several light chain V and J gene segments, and several heavy chain V, D, and J gene segments, which can rearrange in various combinations to contribute to the immense genetic diversity of the Ig genes. Finally, the five types of Ig are dependent on which heavy chain constant region is transcribed and translated (heavy chain genes μ, γ, ε, δ, or α encode for the IgM, IgG, IgE, IgD, or IgA heavy chain proteins, respectively). Immunoglobulin gene rearrangement occurs in the bone marrow during B-cell development (Fig. 12–3).

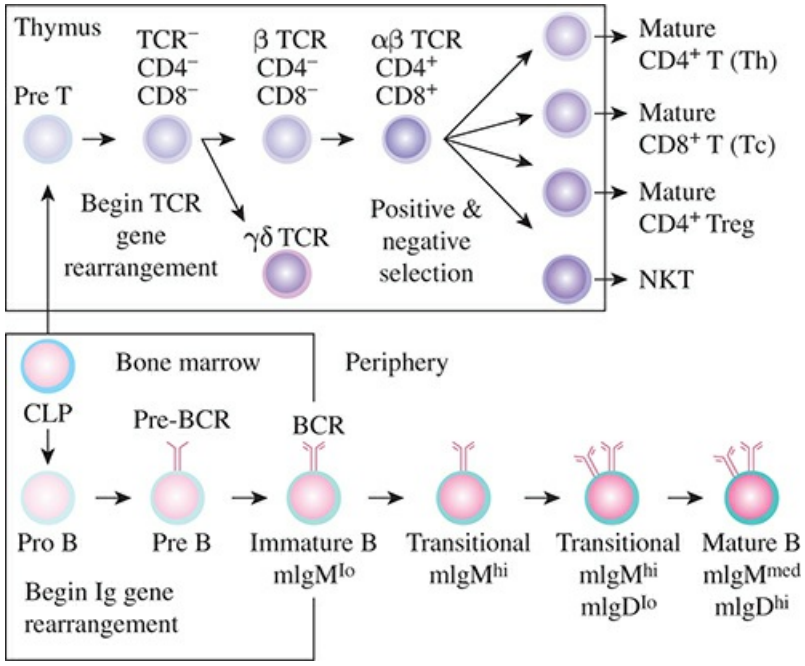

FIGURE 12–3 *Development and differentiation of T and B cells.* T cells develop in the thymus and B cells develop in the bone marrow. For T cells, an initial step is commitment to either αβ or γδ TCR. After positive and negative selection, T cells acquire either CD4 or CD8 to become Th or CTL, respectively. T cells can also become natural Tregs (nTregs) or NKT cells. The B-cell lineage depicted here is of the conventional B-2 lineage. B-1 cells are not depicted since these cells arise from a distinct precursor not found in the bone marrow, which might be a precursor that can generate B-1 or myeloid cells. mIg refers to membrane-bound

immunoglobulin.

The variable regions, which are contained within the Fab regions of the antibody molecule, determine antibody specificity and interact with antigen (Fig. 12–4). The Fc region mediates various effector functions, such as complement activation (IgM and some IgG subclasses) and phagocyte binding (via Fc receptors). Antibodies possess several functions: (1) opsonization of a pathogen with antibody to enhance Fc receptor–mediated phagocytosis; (2) initiation of the classic pathway of complement-mediated lysis; (3) neutralization of viral infection by binding to viral particles and preventing further infection; and (4) enhancement of the specificity of effectors of cell-mediated immunity (CMI) by binding to specific antigens on target cells, which are then recognized and eliminated by effector cells such as natural killer (NK) cells or cytotoxic T lymphocytes (CTLs).

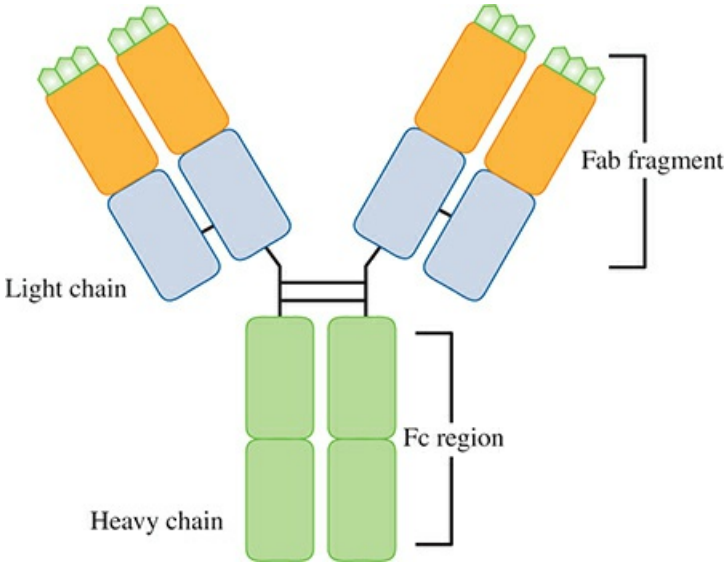

FIGURE 12–4 *Ig structure.* Igs comprise two heavy chains and two light chains, which are connected by disulfide bonds. Gold areas are variable regions and green areas are antigen recognition regions.

Antigen Processing—To elicit an acquired immune response, the particular antigen must be taken up and processed by accessory cells termed APCs and include macrophages, DCs, and B cells for presentation to lymphocytes. The most proficient APC is the DC. The several subtypes of DCs include plasmacytoid (pDCs), conventional, and specialized DCs in the skin called Langerhans cells. In addition, follicular DCs predominantly mediate T-cell-independent stimulation of B cells in germinal centers.

In most tissues, immature state DCs efficiently capture antigens either by phagocytosis, pinocytosis, or by receptor-mediated endocytosis (via antigen, Fc, or complement receptors), after which the DCs mature to express high levels of both classes of MHC (MHCI and MHCII) to stimulate innate and adaptive immune responses. Following internalization, antigen is processed (intracellular denaturation and catabolism) through several cytoplasmic compartments, and a piece of the antigen (peptide fragments about 20 amino acids in length) becomes physically associated with MHCII (Fig. 12–5). This MHCII–peptide complex is then transported to the surface of the cell and can interact in a specific manner with T lymphocytes.

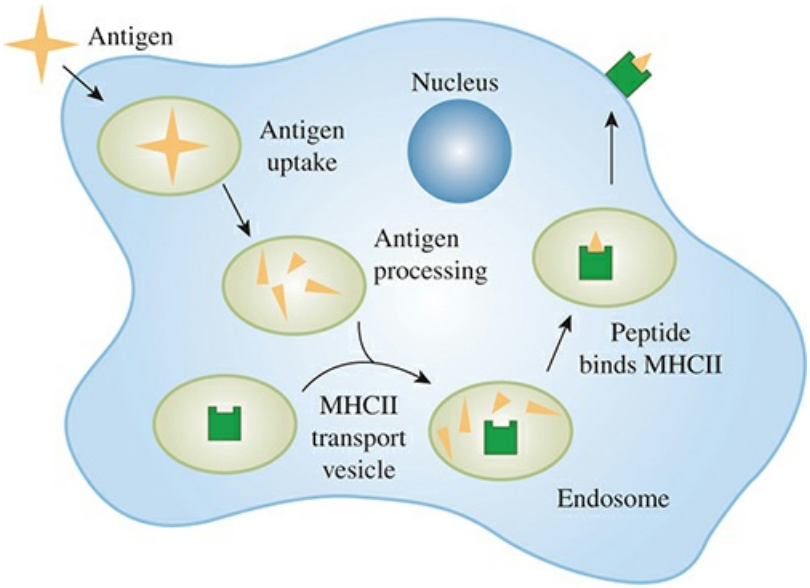

FIGURE 12–5 *Antigen processing by the MHCII pathway.* Antigen is engulfed by an APC (DC, macrophage, or B cell), degraded and loaded onto MHCII. The MHCII–peptide complex is then expressed on the surface of the APC for presentation to $CD4^+$ Th cells.

Some antigens may be processed and presented via MHCI. Although the pathways share some similarities in that short peptide fragments of antigens are generated, loaded onto MHC, and presented on the surface of cells to T lymphocytes, major differences between the MHCI and MHCII pathways are (1) the antigens processed and presented via MHCI are not limited to professional APCs; (2) all nucleated cells express MHCI; (3) the mechanisms by which the antigen is processed and loaded onto MHCI are slightly different than MHCII; (4) the MHCI antigenic peptides are usually smaller, often 8 to 10 amino acids in length; (5) the MHCI antigens to be processed are usually aberrantly expressed proteins, such as viral-associated proteins or mutated proteins; and (6) MHCI facilitates antigen presentation to $CD8^+$ T cells, whereas MHCII facilitates antigen presentation to $CD4^+$ T cells. MHCI antigen processing and presentation is the major pathway by which virally infected cells are detected and killed by the acquired immune system.

T cells can recognize antigen in the context of MHC with their T-cell receptor (TCR). Like Ig, the ability of T cells to specifically recognize thousands of antigens is due to somatic recombination. All TCRs are comprised of two different subunits, each encoded from a distinct gene (most abundant T-cell population expresses the αβ subunits, but γδ also exists). All TCR subunits are made up of constant and variable regions. For α subunits, two separate gene segments (V and J) are combined to form the variable region, which is then joined to a constant region. For β subunits, three separate gene segments (V, D, and J) are combined to form the variable region, which is then joined to a constant region. There are several α subunit V and J gene segments, and several β subunit V, D, and J gene segments.

With regard to T and B cells, key events that occur following antigen encounter are (1) specific antigen recognition either in the context of MHCI or MHCII for T cells or through the Ig receptor for B cells, (2) cellular activation and initiation of intracellular signaling cascades that contribute to production and release of cytokines and other cellular mediators, (3) clonal expansion (proliferation) of antigen-specific cells, and (4) differentiation of antigen-stimulated

lymphocytes into effector and memory cells. These processes are discussed in detail in the following section.

Acquired (Adaptive) Immunity

The acquired arm of the immune system produces a specific immune response to each infectious agent, remembers the pathogen, and protects the host from future infection by the same agent. Two key features that define acquired immunity are specificity and memory. Acquired immunity may be further subdivided into humoral and cell-mediated immunity (CMI). Humoral immunity depends on the production of antigen-specific antibody by B cells and involves the coordinated interaction of APCs, T cells, and B cells. CMI is that part of the acquired immune system in which effector cells, such as phagocytic cells, T helper cells, regulatory T cells, APCs, cytotoxic T cells, or memory T cells, play the critical role(s) without antibody involvement.

Mature, naive, or virgin lymphocytes (those T and B cells that have never undergone antigenic stimulation) are first brought into contact with exogenously derived antigens within the highly organized microenvironment of the spleen and lymph nodes, which are secondary lymphoid organs. The spleen serves as a filter for the blood, removing both foreign antigens and any circulating dead cells and cellular debris. The lymph nodes are part of a network of lymphatic veins that filter antigens from the fluid surrounding the tissues of the body. Other secondary lymphoid tissues associated with the skin, gut, bronchioles, or nasal cavity are referred to as associated lymphoid tissues (abbreviated SALT, GALT, BALT, and NALT, respectively). The associated lymphoid tissues tend to have more exposure to antigen and greater plasticity (i.e., increase or decrease in size and/or number) than lymph nodes.

Cellular Components: Antigen-Presenting Cells, T Cells, and B Cells— APCs include professional APCs such as B cells, macrophages, and DCs. Although all cells may act as APCs with internal antigen processing through the MHCI pathway, a professional APC can internalize external antigens and process them through the MHCII pathway for presentation to T helper cells.

B cells are the effector cells of humoral immunity, producing many Ig isotypes with varying specificities and affinities. The B cell develops in the bone marrow from the HSC and becomes committed to the B-cell lineage when the cell begins to rearrange its Ig genes (Fig. 12–3). Following successful rearrangement of the heavy chain, these cells express Ig heavy chains in their cytoplasm and are termed pre-B cells. Rearrangement of the light chain results in expression of membrane-bound IgM in association with Igα and Igβ to form the B-cell receptor (BCR). Expression of membrane IgM and IgD BCRs indicates a mature B cell. Upon antigen binding to the BCR, the mature B cell becomes activated, and then proliferates before it differentiates into either an antibody-forming cell (AFC) or a memory B cell. AFCs, also known as plaque-forming cells, antibody-secreting cells (ASCs), or plasma cells, actively secrete antibody specific for the antigen that initially bound and activated the BCR.

T-cell precursors migrate from the bone marrow to the thymus where they begin to rearrange their TCRs (Fig. 12–3). Most T cells express the two chain αβ TCR, which is critical for the recognition of antigens in the context of MHC. A much smaller population of T cells express the γδ TCR and directly bind intact host and pathogen proteins. T cells committed to the αβ lineage will express surface markers CD4 and CD8, which are co-receptors involved in the interaction of the T cell with MHC. T cells expressing αβ TCR and both CD4 and CD8 are termed immature

double-positive cells ($CD4^+/CD8^+$). These immature cells then undergo positive and negative selection to (1) select TCRs with affinity for self-MHC (positive selection) and eliminate cells that do not produce a functional TCR or produce TCRs with no affinity for self-MHC or (2) eliminate cells that strongly bind MHC plus self-peptide (negative selection). Double-positive cells that survive positive and negative selections will give rise to T cells that lose expression of either CD4 or CD8 and enter the periphery as mature single-positive cells ($CD4^+$ or $CD8^+$) with a high level of αβ TCR expression. CD4 will facilitate binding to MHCII expressed on APCs; T cells expressing CD4 (T helper cells [Th]) help activate other cells of the adaptive immune response. CD8 will facilitate binding to MHCI and mediate cell killing (cytotoxic T lymphocytes [CTL]). In contrast to αβ T cells, the γδ T cells do not express CD4 or CD8, do not interact with MHC, and do not undergo positive or negative selection.

Mature T cells are found in the lymph nodes, spleen, and peripheral blood. Upon binding of the TCR to antigen in the context of MHC, the T cell activates, proliferates, and differentiates into either an effector cell or a memory T cell. Effector Th cells can subsequently differentiate into several phenotypes depending on the cytokine milieu (Fig. 12–4); two of which, Th1 or Th2, dictate whether CMI or humoral immunity will predominate, respectively. Th1 cells predominantly express IL-2, IFN-γ, and lymphotoxin, which promote CMI and humoral defense against intracellular invaders. Th2 cells predominantly express IL-3, IL-4, IL-5, IL-6, IL-10, and IL-13, which promote humoral defense against extracellular invaders.

The ability of APCs, B cells, and T cells to communicate with each other depends on a variety of receptor–ligand interactions between cell types. These interactions help dictate the type of immune response (i.e., humoral vs. CMI) and the magnitude of the immune response. The duration and extent of an acquired immune response is also controlled by specialized regulatory cells found in both the T-cell and B-cell lineages (Fig. 12–6).

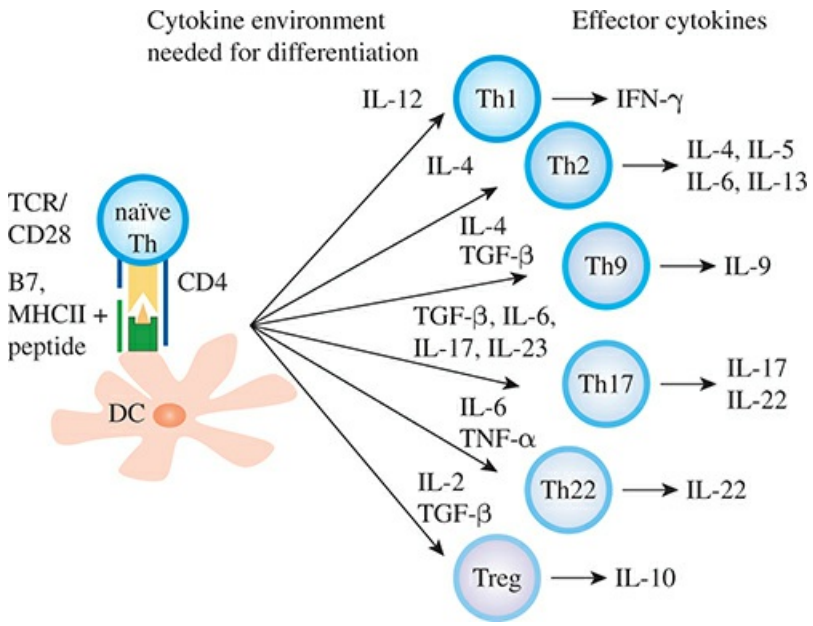

FIGURE 12–6 *The cytokine environment drives different Th populations.* Various Th cells (and T regulatory cells, Tregs) are differentially induced in response to the cytokine milieu. Th1 cells drive CTL responses; Th2 cells drive humoral immune responses; Th9, Th17, and Th22 produce pro-inflammatory cytokines; Tregs control other T-cell responses.

For the T-cell lineage, a small population of $CD4^+$ cells develops into T-regulatory cells (Tregs), which help control various immune responses including those directed against self. Tregs are generally identified as $CD4^+CD25^+Foxp3^+$, which are defined as natural Tregs (nTregs) if they develop in the thymus or induced (iTregs) if development occurs in the periphery in response to IL-2 and TGF-β. Tregs can suppress $CD4^+$ and $CD8^+$ IL-2 production and cell proliferation. Immune suppression by Tregs is likely a multistep process, which might involve direct cell killing via granzyme or perforin, induction of intracellular cAMP, or affecting cell surface expression of critical proteins, such as CD80/CD86. Treg induction may be one mechanism by which drugs and xenobiotics cause immune suppression. Several subsets of regulatory B cells (Bregs) have generally a suppressive role in hypersensitivity and autoimmune diseases such as allergic rhinitis, collagen-induced arthritis, chronic graft versus host disease, and autoimmune disease. The mechanism by which Bregs act involves induction of apoptosis-triggering molecules such as Fas ligand or production of immunosuppressive cytokines, IL-10 and IL-35. The regulatory T- and B-cell subsets also appear to reciprocally activate or suppress each other and may cooperatively control immune responses.

Humoral and Cell-Mediated Immunity—Humoral immunity is that part of the acquired immune system in which antibody is involved, and CMI is that part of the acquired immune system in which various effector cells perform a wide variety of functions to eliminate invaders (Fig. 12–7). The production of antigen-specific IgM requires 3 to 5 days after the primary (initial) exposure to antigen. Upon secondary antigenic challenge, the B cells undergo isotype switching, producing primarily IgG antibody, which is of higher affinity for the activating antigen. There is a higher serum antibody titer associated with a secondary antibody response.

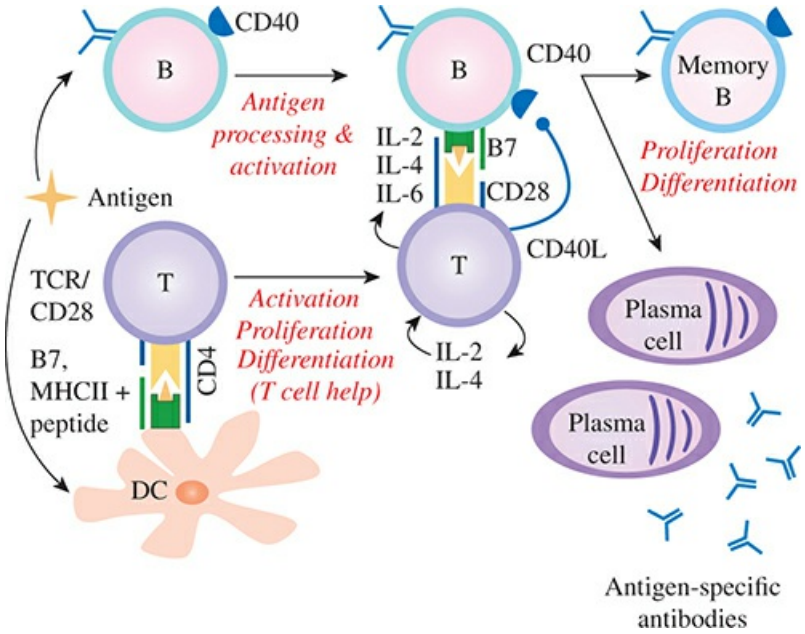

FIGURE 12–7 *Cellular interactions in the humoral immune response.* Antigen is engulfed by an APC (usually DC) and the antigenic peptide is presented to $CD4^+$ T cells in the context of MHC II. $CD4^+$ T cells then become activated, and then proliferate, and differentiate into Th cells, which release cytokines to help B cells that had also encountered the same antigen. B cells become activated, and then proliferate, and differentiate into memory B cells or antigen-

producing plasma cells.

CMI functions include delayed-type hypersensitivity (DTH) and cell-mediated cytotoxicity. Cell-mediated cytotoxicity responses may occur in numerous ways: (1) MHC-dependent recognition of specific antigens (such as viral particles or tumor proteins) by CTLs or iNKT cells (CTL depicted in Fig. 12–8), (2) indirect antigen-specific recognition by the binding of Fc receptors on NK cells to antibodies coating target cells, and (3) receptor-mediated recognition of complement-coated foreign targets by macrophages.

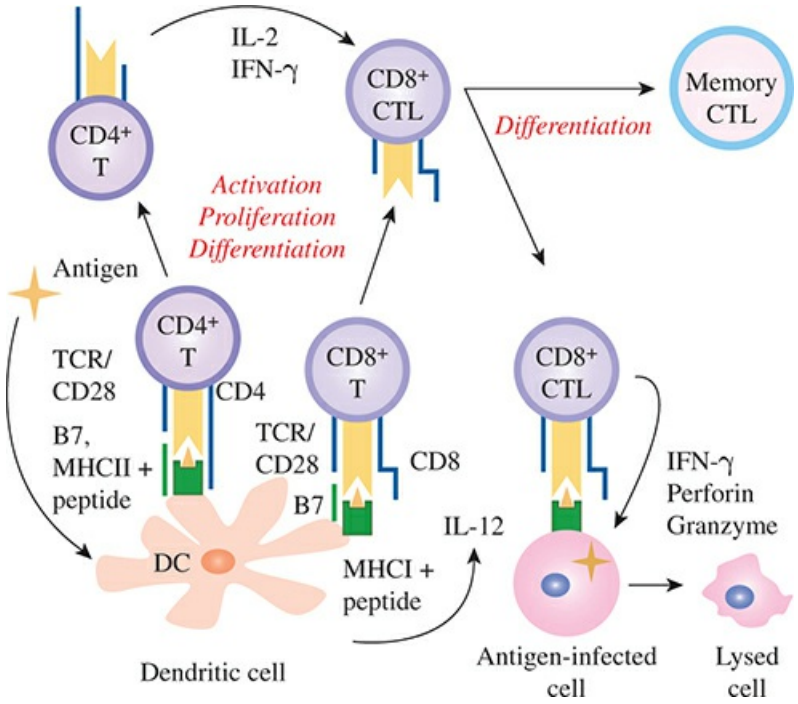

FIGURE 12–8 *Cellular interactions in the CTL response.* Antigen is engulfed by an APC (usually DC) and the antigenic peptide is presented to $CD4^+$ and $CD8^+$ T cells in the context of MHC II and I, respectively. $CD4^+$ T cells then become activated, and then proliferate, and differentiate into Th cells, which release cytokines to help $CD8^+$ T cells that had also encountered the same antigen. Especially in the presence of IL-12 produced by the DC, $CD8^+$ T cells become activated, and then proliferate, and differentiate into CTL that can kill other antigen-infected cells.

In cell-mediated cytotoxicity, the CTL, iNKT, or NK effector cell binds in a specific manner to the target cell. The majority of CTLs express CD8 and recognize either foreign MHCI on the surface of allogeneic cells or antigen in association with self-MHCI (e.g., viral particles). Once the CTL or NK cells interact with the target cell, the effector cell releases the contents of these granules onto the target cell. The target cell may be damaged by the perforins or enzymatic contents of the cytolytic granules. In addition, CTLs induce the target to undergo apoptosis through activation of the Fas and cytotoxic cytokine (i.e., TNF-α and lymphotoxin) pathways. Notably, many of the soluble factors critical to the innate immune system are also primary effectors in the acquired immune system, including ROS, granzyme, perforin, cytokines, and chemokines, again demonstrating the interplay among the various arms of the immune system.

Inflammation

Inflammation refers to a complex reaction to injury, irritation, or foreign invaders characterized by pain, swelling, redness, and heat. Inflammation involves various stages, including release of chemotactic factors following the insult, increased blood flow, increased capillary permeability, followed by either an acute resolution of tissue damage or persistence of the response that might contribute to fibrosis or subsequent organ failure. Whereas inflammation is a natural reaction to repair tissue damage or attack by foreign invaders, the process often results in destruction of adjacent cells and/or tissues. Inflammation plays a critical role in many diseases and exacerbates idiosyncratic reactions to drugs and other chemicals.

Cellular Components: Macrophages, Neutrophils, ILCs, and T Cells—Major cellular contributors to an inflammatory response are macrophages, neutrophils, ILCs, and T cells. Neutrophils are often the first, and most numerous, responders to sites of insult. In response to either host- or pathogen-derived signals, neutrophils secrete chemotactic factors to recruit other pro-inflammatory cells, such as macrophages, to the area.

Macrophages can be activated via TLR, pro-inflammatory cytokines, or recognition of opsonized particles by Fc receptors or complement receptors. Macrophages and neutrophils also induce apoptosis of cells in the insult area through the release of nitric oxide and ROS, resulting in disruption of extracellular structures that compromise tissue structure and function. Both neutrophils and macrophages are phagocytic cells that contribute to clearing of apoptotic cells.

Later in the inflammatory response, T cells are critical for generating an adaptive immune response. T cells, attracted to the insult area by adhesion molecules and integrins, are activated in response to antigen presented in the context of MHC, often by a DC. Depending on the signals that the T cell receives from the cytokine milieu, distinct subpopulations of T cells are induced (Fig. 12–6).

Other soluble factors that contribute to inflammation include a plethora of cytokines and chemokines. Pro-inflammatory cytokines (IL-1β and IL-18) are produced as a result of the activation of the inflammasome. The actions of various pro-inflammatory cytokines include inducing fever or activating T cells and macrophages (IL-1), stimulating B- or T-cell proliferation (IL-6), inducing neutrophilia (IL-17), and increasing vascular permeability or inducing apoptosis (TNF-α). Many pro-inflammatory cytokines induce C-reactive protein, which activates the classical complement cascade. Complement participates in inflammation through inappropriate and sustained activation of the cascade. Finally, prostaglandins and other eicosanoids possess various pro-inflammatory actions.

Immune-Mediated Disease

An individual's immune system may respond by producing self-induced disease states of: (1) hypersensitivity, or allergy, and (2) autoimmunity.

Hypersensitivity

Classification of Hypersensitivity Reactions—All four types of hypersensitivity reactions require prior exposure leading to sensitization in order to elicit a reaction upon subsequent challenge. In the case of Types I, II, and III, prior exposure to antigen leads to the production of allergen-specific antibodies (IgE, IgM, or IgG) and, in the case of Type IV, the generation of

allergen-specific memory T cells (Fig. 12–9).

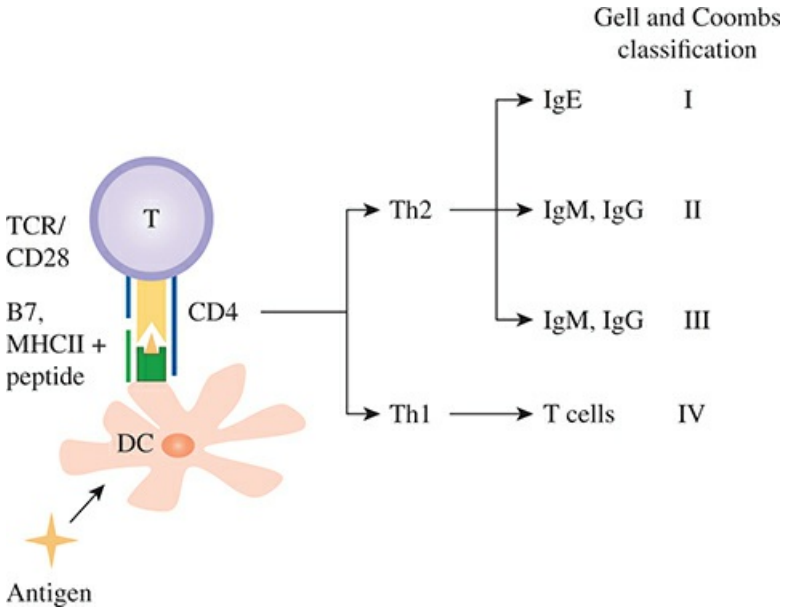

FIGURE 12–9 *Overview of classification of hypersensitivity reactions.* Hypersensitivity reactions are mediated via T cells and antibody production.

Type I (Immediate or IgE-Mediated Hypersensitivity)— Using penicillin as an example, Fig. 12–10 depicts the major events involved in a Type I hypersensitivity reaction. Sensitization occurs as the result of exposure to antigens by dermal contact or through the respiratory or gastrointestinal tract. Most people would mount an IgM, IgG, or IgA immune response to these antigens and clear them without causing any allergic symptoms. It is unclear why these antigens become allergens in certain individuals who respond by mounting an IgE immune response instead, but the mechanism appears to involve genetic and/or environmental determinants and likely some type of triggering event (e.g., acute pathogen exposure and emotional stress).

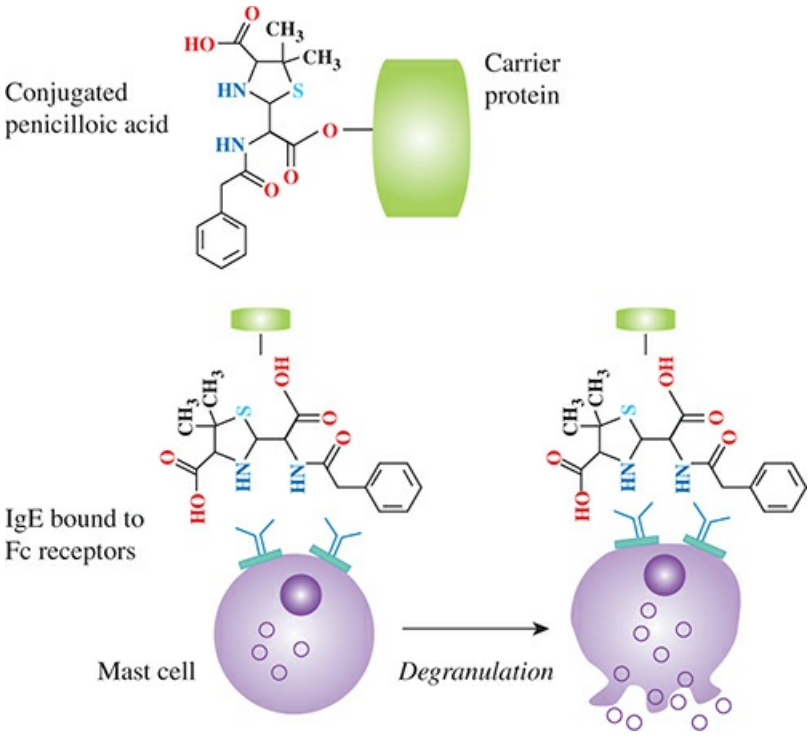

FIGURE 12–10 *Type I hypersensitivity reaction.* Metabolized penicillin is a hapten that conjugates with a protein. The conjugated hapten crosslinks IgE antibodies on mast cells. IgE crosslinking causes mast cell degranulation, releasing histamine and other pro-inflammatory mediators.

Soluble IgE binds to local tissue mast cells and enters the circulation, where it binds to circulating mast cells, basophils, and tissue mast cells at distant sites. Once an individual is sensitized, reexposure to the antigen results in binding to IgE on local mast cells and degranulation with the release of preformed mediators and cytokines that recruit and activate circulating eosinophils, basophils, macrophages, and neutrophils leading to the synthesis and release of more cytokines and of leukotrienes and thromboxanes. These mediators promote vasodilation, bronchial constriction, and inflammation. Clinical manifestations can vary from urticarial skin reactions (wheals and flares), signs of hay fever including rhinitis and conjunctivitis, and serious diseases like asthma to potentially life-threatening anaphylaxis. These responses may begin within minutes of reexposure to the offending antigen; therefore, Type I hypersensitivity is often referred to as immediate hypersensitivity.

Type II (Antibody-Dependent Cytotoxic Hypersensitivity)—Type II hypersensitivity is IgG or IgM-mediated. A foreign antigen attached to the surface of a cell or tissue may mediate the antibody response. Conversely, an antibody response could be mediated by an autoantibody and the resulting response would be part of an autoimmune disease (e.g., autoimmune hemolytic anemias and Goodpasture syndrome). Figure 12–11 shows the mechanisms of action for complement-independent and complement-dependent cytotoxic reactions. Tissue damage may result from the direct action of cytotoxic cells, such as macrophages, neutrophils, or eosinophils, linked through the Fc receptor to antibody-coated target cells (complement-independent) or by antibody-induced activation of the classic complement pathway.

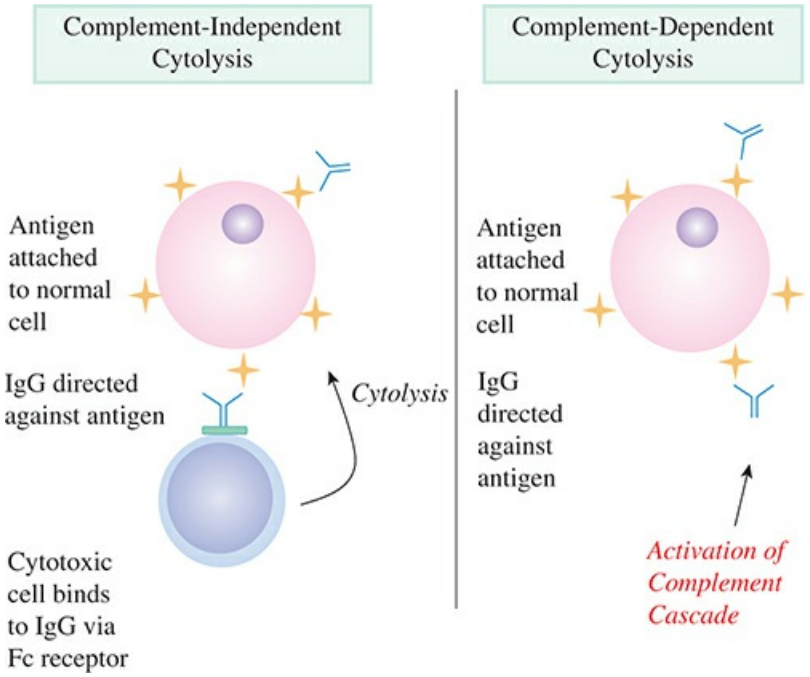

FIGURE 12–11 *Type II hypersensitivity reactions.* In complement-independent cytolysis, antigen becomes attached to a normal cell, which can be recognized by IgG. A cell capable of cytolysis (CTL, NK cell) binds to IgG via its Fc receptor and kills the antigen-coated cell. In complement-dependent cytolysis, antigen becomes attached to a normal cell, which can be recognized by IgG. Complement gets activated by the classical pathway (antigen–antibody complexes).

Type III (Immune Complex–Mediated Hypersensitivity)— Type III hypersensitivity reactions also involve IgG or IgM. The distinguishing feature of Type III is that Ig production is against soluble antigen in the serum (Fig. 12–12). This allows for the formation of circulating antigen-Ig complexes, which may result in widely distributed tissue damage in areas where the immune complexes are deposited. The most common location is the vascular endothelium in lungs, joints, and kidneys. The skin and circulatory systems may also be involved. Pathology results from the inflammatory response initiated by complement activation. Macrophages, neutrophils, and platelets attracted to the deposition site contribute to the tissue damage. Type III hypersensitivity can be induced in autoimmune diseases due to autoantibodies directed against soluble antigens such as double-stranded DNA or small nuclear proteins. In addition, antibody directed to protein therapeutics/biologics can form immune complexes with the drug, which results in Type III hypersensitivity responses.

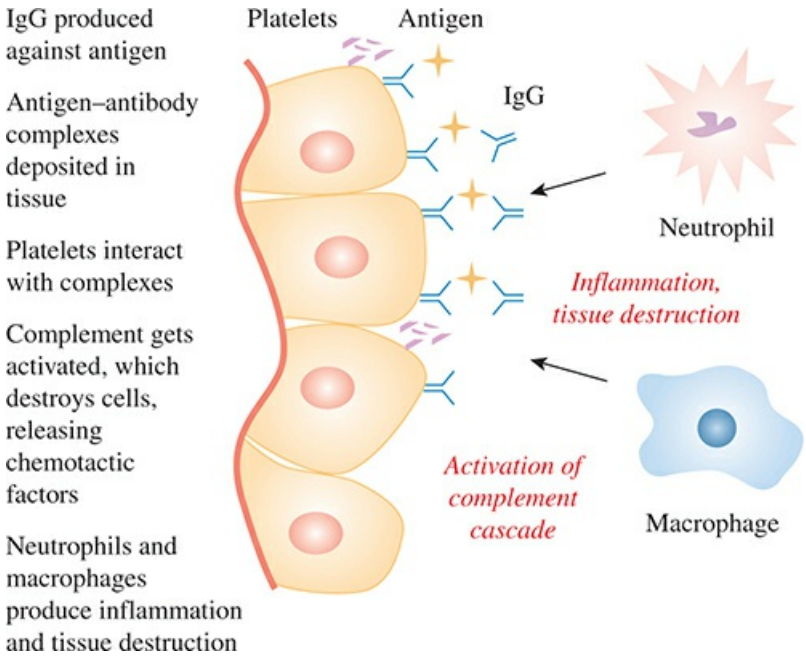

FIGURE 12–12 *Type III hypersensitivity reactions.* IgG is produced against an antigen and antigen–antibody complexes form, which can become deposited in tissue. Complement gets activated by the classical pathway (antigen–antibody complexes) and platelets also interact with complexes. Following complement-mediated cytolysis, released chemotactic factors attract neutrophils and macrophages, causing additional inflammation and tissue damage.

Type IV (Cell-Mediated Hypersensitivity)—Type IV responses can be divided into three classes: contact hypersensitivity, tuberculin-type hypersensitivity, and hypersensitivity pneumonitis. Contact hypersensitivity is initiated by topical exposure, and the associated pathology is primarily an eczematous reaction at the site of allergen contact and, like Type I through III responses, consists of two phases, sensitization and elicitation. However, in this case sensitization results from the development of activated and memory T cells as opposed to antibody production (Figs. 12–13 and 12–14). Sensitization occurs when a hapten penetrates the epidermis and forms a complex with a protein carrier. The hapten–carrier complex is processed by Langerhans DCs that migrate out of the epidermis to local lymph nodes. There, the APC presents the processed antigen to $CD4^+$ Th cells, leading to clonal expansion and the generation of memory T cells including memory $CD8^+$ CTL, which appear to play a major role in the elicitation phase.

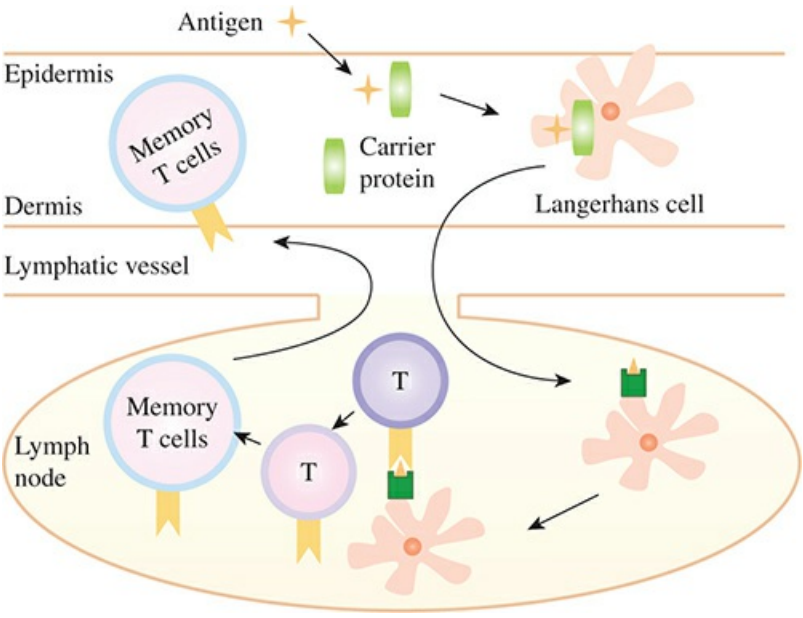

FIGURE 12–13 *Type IV hypersensitivity sensitization*. A hapten permeates the epidermis and complexes with a carrier protein. The conjugated hapten gets engulfed by Langerhans DCs, which migrate to the lymph node and present antigen to $CD4^+$ T cells in the context of MHCII. $CD4^+$ T cells activate, proliferate, and differentiate into Th cells and memory T cells.

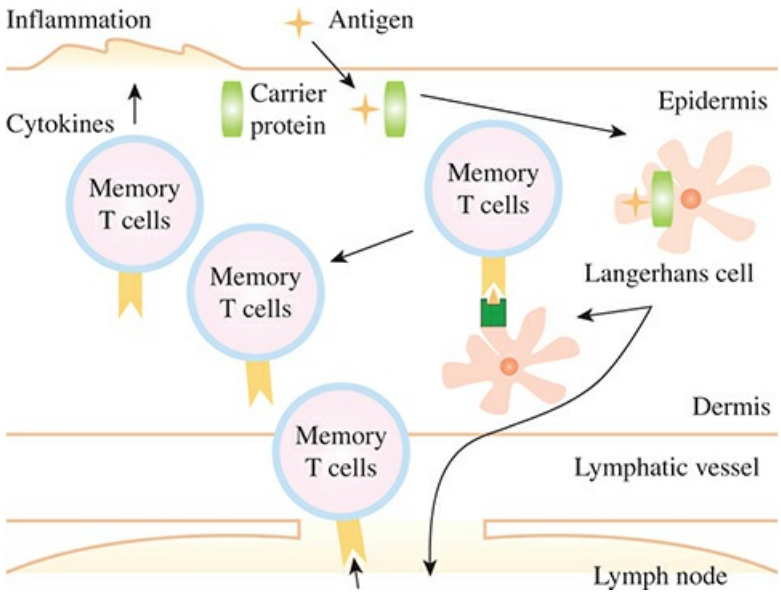

FIGURE 12–14 *Type IV hypersensitivity elicitation*. Upon secondary exposure to the hapten, Langerhans DCs either migrate to the lymph node or directly present antigen to memory T cells. Memory T cells become activated and produce cytokines to stimulate pro-inflammatory cytokine release from other cell types, such as keratinocytes.

Upon secondary contact, Langerhans DCs present the processed hapten–carrier complex to memory $CD8^+$ CTL in either the skin or the lymph nodes. These activated T cells then secrete cytokines that bring about further proliferation of T cells, generation of CTLs, as well as

increased expression of adhesion molecules on the surface of keratinocytes and endothelial cells in the dermis. Secretion of pro-inflammatory cytokines by T cells and keratinocytes facilitate the recruitment of inflammatory cells into the skin, resulting in erythema and the formation of papules and vesicles. Intracellular proteins can be modified by lipid-soluble chemicals that might readily cross the cell membrane. These cells then present modified peptides on their cell surface, which are recognized by $CD8^+$ cytotoxic T cells to cause tissue damage by either direct cytotoxic action (CTL) or the secretion of cytokines that further promote the inflammatory response.

Autoimmunity—Autoimmune disease occurs when the immune system acts against the body's own tissues because of a genetic susceptibility. These diseases may be tissue-specific or tissue-nonspecific. Both humoral immunity and CMI can be involved as effector mechanisms in causing the damage in autoimmune conditions. The terms "hypersensitivity" and "autoimmunity" are often confused and are certainly interrelated. Based on their definitions, a hypersensitivity response can be a mechanism by which an autoimmune disease is produced.

The precipitating mechanisms for true autoimmune disease are distinct from hypersensitivity. In cases of autoimmunity, self-antigens are the target of the immune response, and in the case of chemical-induced autoimmunity, the disease state is induced by a modification of host tissues or immune cells by the chemical and not the chemical acting as an antigen/hapten as in hypersensitivity reactions.

***Mechanisms of Autoimmunity*—**The rearrangement and recombination of the genes that comprise Ig and TCR result in tremendous diversity in the potential antigen recognition of B cells and T cells, respectively. Ideally, during development those lymphocytes recognizing self-antigens will largely be deleted by negative selection as central tolerance is established. Autoreactive clones that escape central tolerance and migrate to the periphery are normally controlled by peripheral tolerance mediated by various mechanisms that ultimately induce anergy or clonal deletion. For autoimmune disease to occur, (1) an autoreactive clone must escape central tolerance, pass into the periphery, and bind with specificity to its self-antigen; (2) mechanisms of peripheral tolerance must fail; and (3) the autoreactive clone must induce a detrimental immunological response.

Induction of autoimmunity is multifaceted and associated with several mechanisms primarily related to insufficient peripheral tolerance. Mechanisms associated with the breakdown of peripheral tolerance and prime events for the onset of autoimmune disease include inflammation; molecular mimicry by pathogen antigens; inherent defects in T or B cells including regulatory subsets, APCs, cytokines, or complement; and epitope spreading.

Effector mechanisms involved in autoimmune disease are similar to those described for Types II and III hypersensitivity, or they may involve $CD8^+$ CTL. Tissue damage associated with CTL may result from direct cell membrane damage and lysis, or from effects of cytokines produced and released by the T cell. TNF-β (lymphotoxin) can kill susceptible cells, and IFN-γ may increase the expression of MHCI on cell surfaces, making them more susceptible to $CD8^+$ cells. Cytokines may also be chemotactic for macrophages, which can cause tissue damage directly or indirectly through the release of pro-inflammatory cytokines. As with hypersensitivity reactions, autoimmune disease often results from multiple mechanisms working simultaneously.

Developmental Immunology

A sequential series of carefully timed and coordinated developmental events, beginning early in embryonic/fetal life and continuing through the early postnatal period, is required to establish a functional immune system in all mammals. Cells of the immune system develop initially from a population of pluripotent HSCs. The bone marrow and thymus are the primary sites of lymphopoiesis providing the microenvironmental factors necessary for the development of functionally competent immune cells (Fig. 12–2). Immune system development continues after birth as immunocompetent cells are produced from proliferating progenitor cells in the bone marrow and thymus. Mature immunocompetent cells leave these primary immune organs and migrate via the blood to the secondary immune organs: spleen, lymph nodes, and mucosal lymphoid tissues. Exposure to specific antigens during the perinatal period results in a rapidly expanding accumulation of lymphocyte specificities in the pool of memory cells in secondary lymphoid tissues. As thymic function wanes, this pool of memory B and T cells maintains immune competence for the life of the individual. Senescence of immunity is associated with reductions in both innate and acquired immune responses to antigens during the last quartile of life, which is due, in part, to a continual reduction in the production of newly formed cells, and to the decreased survival of long-lived memory cells in lymphoid tissues.

Defects in the development of the immune system due to heritable changes in the lymphoid elements have provided clinical and experimental examples of the devastating consequences of impaired immune development. Therefore, the effects of chemicals on the genesis of critical immune organs in the developing fetus may be more important than effects on these tissues after having been populated by hematopoietic and lymphoid cells. In contrast, the immune system of older people is perceived as declining in fidelity and efficiency with age resulting in an increased susceptibility to infectious diseases, pathological conditions relating to inflammation, or autoreactivity.

Neuroendocrine Immunology

Cytokines, neuropeptides, neurotransmitters, and hormones, as well as their receptors, mediate interactions between the CNS, endocrine, and immune systems. Because receptors for neuropeptides, neurotransmitters, and hormones are present on lymphoid cells, some chemicals exert their immunomodulatory effects indirectly on the immune system by acting to modulate the activity of the nervous or endocrine systems. In addition, immune cells secrete peptide hormones and neurotransmitters, which can have autocrine (immune system) and paracrine (endocrine and nervous systems) effects.

ASSESSMENT OF IMMUNOLOGIC INTEGRITY

Xenobiotics can exert significant effects on the immune system. Among the unique features of the immune system is the ability of immune cells to be removed from the body and to function in vitro. This unique quality makes it possible to comprehensively evaluate the actions of xenobiotics on the immune system employing in vivo, ex vivo, and in vitro approaches to dissect the cellular, biochemical, molecular mechanisms of action, and toxicity of xenobiotics. Employing a battery of functional assays allows different cell types to be evaluated for their

effector functions, potential participation as accessory cells in an immune response, and provide information concerning which cell type(s) within the immune system are targeted by a xenobiotic.

General Assessment

Standard toxicological studies, which allows any immunologic finding to be interpreted in conjunction with effects observed on other target organs, include body and selected organ weights, general observations of overall animal health, selected serum chemistries, hematologic parameters, bone marrow ability to generate specific colony-forming units, and histopathology of lymphoid organs. Because of the unique nature of the immune system, several experimental approaches may be taken to assess immunotoxicity and to evaluate the mechanisms of action of xenobiotics.

Functional Assessment

Innate Immunity—Innate immunity encompasses all those immunologic responses that are not induced through antigen receptors (i.e., TCR or BCR) and for which there is limited immunological memory. These responses include phagocytosis of pathogens; release of ROS as a killing mechanism by macrophages, neutrophils, and monocytes; recognition of tumor cells by NK cells; and the lytic activity of the complement cascade.

To evaluate phagocytic activity in vitro or ex vivo, plate-based or flow cytometric methods can be used to measure the amount of fluorophore-labeled foreign matter engulfed by phagocytic cells. For example, phagocytic cells, isolated from tissues including blood, peritoneal cavity fluid, and bronchial alveolar lavage fluid that adhere to tissue culture plates or whole blood containing monocytes and granulocytes, are incubated with fluorophore-labeled particles. To quantify the overall degree of fluorescence or the number of particles engulfed, cell samples are then analyzed by a microplate fluorimeter, fluorescent microscopy, or flow cytometry. During phagocytosis, the respiratory burst of ROS generated in response to soluble antigens to kill the pathogen may be measured by in vitro or ex vivo methods, which quantify the fluorescence elicited by oxidation of a fluorogenic substrate.

Phagocytosis can also be assessed in vivo. An older method involved intravenous injection of radiolabeled sRBCs (^{51}Cr-sRBCs), which are removed from the circulation by tissue macrophages and sequestered for degradation in the liver, spleen, lymph nodes, lung, and thymus. Clearance of the ^{51}Cr-sRBCs is monitored by sampling the peripheral blood. Newer methods to assess in vivo phagocytosis involve injection of fluorophore-labeled particles, including nanoparticles or bacteria.

Concomitant with antigen encounter by the phagocyte, there is an increase in the expression of MHCI and/or MHCII as well as the costimulatory molecules CD80/CD86. This upregulation can be readily quantified by flow cytometry after the activation of phagocytic cells, primarily those of the monocytic lineage. There are several methods to assay effects on NK activity.

Acquired Immunity: Humoral—The antibody forming cell (AFC) assay tests the ability of the host to mount an antibody response to a specific antigen. When a T-cell-dependent antigen (an antigen that requires T cells to help B cells make antibody) like sRBC is used, this response,

referred to as the T-cell-dependent antibody response (TDAR), requires the coordinated interaction of several different immune cells: APC (macrophages and DCs), T cells, and B cells. Therefore, functional impairment of any one of these cell types (e.g., antigen processing and presentation, cytokine production, proliferation, or differentiation) can alter the ability of B cells to produce antigen-specific antibodies. T-cell-independent antigens, such as dinitrophenyl-Ficoll or trinitrophenyl-LPS, can also be used that bypass the requirement for accessory T-cell help in eliciting antibody production by B cells, and thus produce a T-cell-independent antibody response (TIAR).

A standard AFC assay involves immunizing mice with sRBCs. The antigen is taken up in the spleen. The antibody response is quantified by enumerating the number of plasma cells (also known as AFCs or ASCs) present in the spleen that are secreting anti-sRBC antibodies. Data are usually presented as IgM AFCs per million splenocytes. IgG AFCs can also be enumerated by slight modifications of this same assay.

A second method to enumerate AFCs is by incubating splenocytes isolated from sRBC-sensitized mice on ELISPOT microtiter plates coated with either antimouse IgM or sRBC membranes to capture the secreted IgM. The site at which each IgM secreting cell was present in the well of the ELISPOT microtiter plate is visualized by the formation of a "spot" through the enzymatic conversion of the chromogenic substrate. An ELISPOT reader rapidly quantifies the number of spots (number of plasma cells) as well as the size and intensity (quantity of IgM being secreted) of the spots. The ELISPOT is especially useful for enumerating cytokine and chemokine secreting cells in which the desired endpoint is effector cell enumeration.

A third way the anti-sRBC humoral immune response can be evaluated is ex vivo using serum from peripheral blood of immunized mice and an enzyme-linked immunosorbent assay (ELISA) or other immunoassay methods. This approach takes into account antigen-specific antibody secreted by B cells in the spleen as well as B cells residing in the bone marrow and lymph nodes. Peripheral blood is collected from mice (or other experimental animals) 6 days postimmunization with sRBCs and incubated in microtiter plates that have been coated with sRBC membranes or antimouse IgM. An enzyme-conjugated monoclonal antibody (the secondary antibody) against IgM (or IgG) is added that recognizes the IgM (or IgG) and binds specifically to that antibody. After incubation and a wash step, the chromogenic substrate is added. Conversion of the substrate by the enzyme conjugated to the secondary antibody results in a color change that is determined by quantifying absorbance with a plate reader. In addition to evaluating serum, the supernatant from in vitro AFC assays can also be evaluated by ELISA.

Acquired Immunity: Cell-Mediated (CMI)—Of numerous assays used to assess CMI, three primary tests used routinely are the CTL assay, DTH response, and T-cell proliferative responses to TCR stimulation (anti-CD3 plus IL-2), mitogens (phytohemagglutinin [PHA] and concanavalin-A [ConA]), and allogeneic cell antigens (mixed lymphocyte responses [MLR]).

The CTL assay measures the in vitro ability of splenic T cells to recognize allogeneic target cells (MHC mismatched) by evaluating the ability of the CTLs to proliferate and then lyse the target cells. Splenocytes are incubated with P815 mastocytoma cells, which serve as target cells; the CTLs recognize the targets and undergo proliferation. Five days after sensitization, the CTLs are harvested and incubated in microtiter plates with radiolabeled (^{51}Cr) P815 mastocytoma cells. During this elicitation phase, the CTLs that have acquired memory recognize the foreign MHCI on the P815 cells and lyse the targets. At the end of the incubation, the percent cytotoxicity is calculated for each effector-to-target ratio and compared to that from control

animals. Several flow cytometry–based methods that avoid the use of radionucleotides have also been developed to assay CTL activity.

The DTH response evaluates the ability of memory T cells to recognize foreign antigen, proliferate, and migrate to the site of the infection, where they secrete cytokines and chemokines. The assay quantifies the influx of mononuclear cells into the sensitization site. During xenobiotic exposure, mice are sensitized with two doses of keyhole limpet hemocyanin (KLH). On the last day of exposure, mononuclear cells are labeled in vivo with an IV injection of ^{125}I-5-iododeoxyuridine. One day later, mice are challenged intradermally in one ear with KLH. Twenty-four hours after challenge, animals are euthanized, the ears are biopsied, and radiolabeled cells are counted. Data are expressed as a stimulation index, which represents the cpm of ^{125}I activity in the challenged ear divided by the cpm in the unchallenged ear. Alternative nonradioactive methods are available.

The ability of T cells to undergo clonal expansion through proliferation is critical to their central role in CMI. The MLR measures the ability of T cells to recognize foreign MHCI and MHCII on splenocytes from an MHC-incompatible mouse (allogeneic cells) and undergo proliferation.

Flow Cytometric Analysis—Flow cytometry is a method that employs light scatter, fluorescence, and absorbance measurements to analyze large numbers of cells (typically 5000 to 100,000 per sample) individually. Most commonly, fluorochrome-conjugated monoclonal antibodies raised against a specific protein of interest are employed for detection. The strength of the approach is that a wide variety of measurements can be made rapidly on large numbers of individual cells with a high level of precision. Additional methods allow for the analysis of specific proteins in cell-free preparations such as cell lysates, biological fluids, and culture supernatants. A broad selection of polyclonal and monoclonal antibodies to cell surface markers, intracellular proteins, and secreted proteins are available. This approach can provide insight into which specific T-cell subsets are targeted after exposure to a xenobiotic and to identify putative effects on T-cell maturation.

Another major advancement in flow cytometry–based analyses has been the development of fluorescent microspheres that are individually identified by the instrument. By coating the surface of microspheres with various concentrations of two fluorescent dyes, sets of microspheres can be generated with each set possessing a unique spectral signature. Various materials (i.e., proteins, antibodies, or nucleic acids) can be covalently conjugated to the surface of these microspheres in order to create unique detection systems. This technology is being widely applied for analyzing a broad variety of soluble cellular components including proteins in cell-free preparations by flow cytometry.

A recent flow cytometry advancement is the ability to quantify gene-specific mRNA levels in individual cells using a technique termed PrimeFlow®. The methodology is based on the principle of using antisense probes containing 20 to 40 oligonucleotides that hybridize to target mRNA. PrimeFlow® has a wide array of applications in immunotoxicology including identifying cell types that are the source of regulatory proteins (e.g., cytokine and chemokines), as these are typically made on demand and regulated at the transcriptional level.

Measurements of Cytokines and Cytokine Profiling— Development, maturation, differentiation, and effector responses of the immune system are highly dependent on a multitude of small, secreted proteins termed cytokines. In most cases, immunological processes are

controlled by the production of multiple cytokines. Xenobiotics that alter the production and release of these mediators can significantly affect immune competence. Quantification of multiple cytokines, often referred to as cytokine profiling, has become routine in immunotoxicology and can provide significant insights into the mechanisms by which a xenobiotic produces its immunotoxicity.

Cytokine Release Assays—Therapeutic monoclonal antibodies and pleiotropic cytokines have the potential to activate many immune cells (primarily T cells and monocytes/macrophages) by initiating a cascade that causes the release of cytokines, including TNF-α, IFN-γ, and IL-6. Cytokine release syndrome can be characterized by flu-like symptoms, fever, chills, headache, back pain, hypotension, and organ failure. Cytokine release is partly responsible for several drug-induced immune-mediated adverse effects, including first-dose reactions, infusion reactions, tumor-lysis syndrome, and systemic inflammatory response syndrome.

Host Resistance Assays—Host resistance assays assess how xenobiotic exposure affects the ability of the host to combat infection by a variety of pathogens. Although host resistance studies provide significant insight into the mechanisms by which an immunotoxicant is acting, these assays are not used as a first or only choice for evaluating immune competence. The results from host resistance assays are typically more variable than other immune function assays and therefore require markedly greater numbers of animals in order to obtain statistical power. In host resistance studies, it is important to consider the following: (1) strain, route of administration, and challenge levels of the pathogen; (2) strain, age, and sex of the host; (3) physiologic state of the host and the pathogen; and (4) time of challenge with the pathogen (prior to, during, or after xenobiotic exposure). All of these can have significant effects on the results from any individual study.

Host resistance models can study the effects of an immunotoxicant within the entire intact immune system encompassing all its diversity as it acts to protect the host against a bona fide pathogen. The immune system possesses significant redundancy (i.e., multiple mechanisms to provide defense against invading microorganisms). Even if one particular cell type or effector response has been compromised by exposure to an immunotoxicant, other components of the immune system might provide partial or complete protection to the host from pathogen challenge. Host resistance models permit the study of immunotoxicants and their effects within the environment and in the context of the tissue targeted by the pathogen.

Assessment of Developmental Immunotoxicology (DIT)—Any of a number of dynamic changes associated with the developing immune system might provide periods of unique susceptibility to chemical perturbation. This unique vulnerability may be manifested as a (1) qualitative difference, in the sense that a chemical could affect the developing immune system without affecting the adult immune system; (2) quantitative difference, in the sense that a chemical could affect the developing immune system at lower doses than the adult immune system; or (3) temporal difference, in the sense that a chemical could produce either a more persistent effect in younger animals than adults, or trigger a delayed effect (i.e., the consequences of early exposure are not manifested until early adulthood). It is also noteworthy that development of leukocyte progenitors from bone marrow stem cells ultimately giving rise to mature immunocompetent cells is a life-long cell renewal process not unique to early life stages.

A better understanding of the developing immune system, particularly of critical developmental hallmarks, has prompted speculation about five critical windows of vulnerability.

The first window encompasses a period of hematopoietic stem cell formation from undifferentiated mesenchymal cells. Exposure of the embryo to toxic chemicals during this period could result in failures of stem cell formation, abnormalities in production of all hematopoietic lineages, and altered immunocompetence. The second window is characterized by migration of hematopoietic cells to the fetal liver and thymus, differentiation of lineage-restricted stem cells, and expansion of progenitor cells for each leukocyte lineage. This developmental window is likely to be particularly sensitive to agents that interrupt cell migration, adhesion, and proliferation. The critical developmental events during the third window are the establishment of bone marrow as the primary hematopoietic site and the establishment of the bone marrow and the thymus as the primary lymphopoietic sites for B cells and T cells, respectively. The fourth window addresses the critical periods of immune system functional development, including the initial period of perinatal immunodeficiency, and the maturation of the immune system to adult levels of competence. The final window addresses the subsequent period during which mature immune responses are manifest, and functional pools of protective memory cells are established.

In constructing a DIT testing framework, several points should be considered. The rat has been identified as the preferred animal model for evaluations of DIT, because of its extensive use in developmental and reproductive toxicology testing. A second consideration concerns sex-specific effects, and the third is consideration of exposure. A fourth consideration concerns which specific endpoints to measure. Immune organs, such as the thymus, spleen, lymph nodes, and/or bone marrow, should be assessed in routine developmental and reproductive toxicology studies. As developmental immunotoxicity protocols are inserted into existing toxicology testing regimes, such as developmental and reproductive toxicology protocols, immunization protocols are often incorporated.

Assessment of Hypersensitivity Responses—Depending on the specific drug/chemical, the exposure conditions, the species being tested, and the immune parameter being measured, the outcomes can be manifested as decreases, increases, or no effect. The most frequent observation has been immune suppression, but immune stimulation can occur. The adverse consequences of exaggerated immune function reflect an inability to recognize self and are generally depicted as hypersensitivity and autoimmunity. This profile is consistent with the concept of immunotoxicology existing as a continuum (Fig. 12–1).

Drugs and chemicals that elicit an immune response are generally low-molecular-weight substances possessing some inherent reactivity. There is a genetic susceptibility to hypersensitivity responses in that not all individuals react to the same drugs and chemicals. For the most part, the xenobiotic becomes a hapten, which triggers the immune response in some tissue in the host. This property is called the sensitizing potential of the hapten and is associated with its inherent reactivity. Hapten-specific immune responses are triggered only in the presence of the hapten–carrier complex and can be mediated by either humoral immunity or CMI. The most frequently occurring hypersensitivity reactions to chemicals are Type I and Type IV, which are often manifested as respiratory hypersensitivity and contact sensitization, respectively.

Assessment of Respiratory Hypersensitivity in Experimental Animals—Current assays utilize two phases: induction/sensitization and challenge/elicitation. The induction phase usually includes multiple exposures to the test compound (sensitization) via the respiratory tract (i.e., inhalation and intranasal or intratracheal instillation) or by dermal contact. A chemical with the capability of being a respiratory sensitizer will trigger an IgE response regardless of its route of exposure because it “selects” or supports the development of a Th2-dependent response, with the

associated cytokine profile: IL-4, IL-5, IL-10, and IL-13. In contrast, a chemical that lacks the capability of being a respiratory sensitizer but can still trigger contact dermatitis will select or support a Th1-dependent response, with the associated cytokines including IL-2 and IFN-γ.

Guinea pig models are frequently used for detection of pulmonary reactions to chemicals because this species responds to appropriate stimuli by developing an asthmatic-like bronchial spasm. Like humans, the guinea pig demonstrates immediate- and late-onset allergic reactions as well as bronchial hyperreactivity, eosinophil influx and inflammation. The major difference in the mechanism of pulmonary responses between humans and guinea pigs is that the antibody involved in Type I reactions in humans is IgE but predominantly IgG1 in guinea pigs. In murine models, IgE is the major anaphylactogenic antibody.

Assessment of IgE-Mediated Hypersensitivity Responses in Humans—Type I hypersensitivity testing, in conjunction with a relevant history and physical exam, can be diagnostic of IgE-mediated pulmonary disease. Two skin tests, The prick–puncture test and the intradermal test, are available for immediate hypersensitivity testing. In both, the measured endpoint is a "wheal and flare" reaction. In vitro serologic tests, ELISAs, and radioallergosorbent tests may also be used to detect the presence of antigen-specific antibody in the patient's serum. Additionally, bronchial provocation tests can be performed by having the patient inhale an antigen into the bronchial tree and evaluating pulmonary response. Care must be taken in these test situations in that it is possible to trigger severe asthmatic reactions or anaphylaxis in sensitized individuals.

Assessment of Contact Hypersensitivity in Experimental Animals—The two most commonly utilized guinea pig models are the Büehler test and the guinea pig maximization test. These tests vary in their method of application of the test article, in the dosing schedule, and in the utilization of adjuvants. The test site is examined for signs of erythema and edema, two well-recognized indicators of cutaneous inflammation and contact dermatitis. These endpoints for evaluation in the guinea pig assays are visual and subjective, and it is difficult to assess irritating or colored compounds using these models.

More quantitative and immunologically based assay methods in other species focus mainly on the mouse, primarily because of the availability of reagents and techniques to conduct mechanistic studies. The mouse ear-swelling test uses a quantitative measurement of ear thickness as an endpoint. The mouse local lymph node assay (LLNA) identifies contact allergens as a function of the events occurring during the induction (sensitization) phase of a hypersensitivity response. Advantages of the LLNA include (1) the potential to reduce the number of animals required and reduce animal distress, (2) the provision of quantitative data that allows for statistical analysis, and (3) the provision of dose–response data. The LLNA permits comparisons of potency across individual chemicals and/or within a chemical class. The LLNA has application to risk assessments for contact dermatitis. Because the LLNA evaluates the induction phase of the immune response, it is more applicable to mechanistic studies.

Assessment of Contact Hypersensitivity in Humans—Skin patch testing in humans allows for the diagnostic production of acute lesions of contact hypersensitivity by the application of a suspected allergen to the skin. Patches containing specified concentrations of the allergen in the appropriate vehicle are applied under an occlusive patch for 48 hours in most test protocols. Once the patch is removed and enough time elapses for the signs of mechanical irritation to resolve (approximately 30 minutes), the area is read for signs of erythema, papules, vesicles, and edema. Generally, the test is read again at 72 hours and, in some cases, signs may not appear for

up to 1 week or more.

Assessment of Autoimmune Responses—The immune system can be a passive target for the enhancing effects of drugs and chemicals, such as occurs when a xenobiotic mimics or causes aberrant production of immunomodulatory cytokines, when a xenobiotic disrupts the regulatory mechanisms that serve to protect self, or when xenobiotics act as an adjuvant that nonspecifically enhances the immune response to an antigen. The classic adjuvant is complete Freund's adjuvant, which is a water-in-oil emulsion containing killed *Mycobacterium tuberculosis*.

In the context of testing strategies, the ability of drugs and chemicals to exacerbate or trigger autoimmune disease in either animal models or humans is poorly understood. Primarily because of the strong genetic component in the susceptibility to autoimmunity, deciphering the exact role of xenobiotics in the induction of these conditions has proven to be difficult.

Rodent models fall into three categories: genetically predisposed animal models, animal models in which the autoimmune disease is produced by immunization with specific antigens, and animal models in which the disease is chemically induced. Genetically predisposed animal models include the nonobese diabetic (NOD) mouse, the F1 cross between the New Zealand black (NZB) and New Zealand white (NZW) mouse, and the MRL/lpr mouse. The NOD model has been used to study type 1 diabetes, specifically the T-cell autoimmune response, the role of B-cell antigen presentation, and the role of cytokines in disease progression. The NZBxNZW F1 and the MRL/lpr mouse models have been used to study human SLE. The NZBxNZW F1 model has been used to map the specific susceptibility loci and to assess the importance of B-cell hyperactivity and T-cell involvement in autoantibody production in the development of SLE. The important role of apoptosis in negative selection has been studied in the MRL/lpr mouse model, in which a genetic defect results in a mutation in the *Fas* gene. The MRL/lpr mouse model exhibits rheumatoid factor autoantibodies and inflammatory joint disease characteristic of an arthritic response.

Arthritis can also be induced in susceptible rat strains by immunization with complete Freund's adjuvant. Immunization of susceptible mouse strains containing the H-2^q or H-2^r alleles of MHC with Type II collagen or cartilage glycoproteins, in the presence of adjuvant, can induce pathology similar to human rheumatoid arthritis. Experimental autoimmune encephalomyelitis can be induced in several species by immunization with myelin-derived peptides in complete Freund's adjuvant. This model has been used in rodents to characterize T helper cell–mediated autoimmune disease characterized by perivascular lymphocyte infiltration into the CNS and the destruction of the myelin nerve sheath, which is similar to human multiple sclerosis.

One of the most commonly used models of chemically induced autoimmunity is the Brown Norway rat model, in which animals are injected with mercuric chloride. Although dosing produces no overt signs of toxicity, Brown Norway rats develop an immunologically mediated disease characterized by T-cell-mediated polyclonal B-cell activation and autoantibodies to laminin, collagen IV, and other components of the glomerular basement membrane similar to human autoimmune glomerulonephritis.

Molecular Biology Approaches to Immunotoxicology

A primary application of molecular biology in immunotoxicology is the identification of genes whose expression has been altered by a xenobiotic, and/or to quantify the magnitude to which gene expression has been changed due to some treatment. Many immunological mediators

produced by leukocytes (e.g., cytokines, chemokines, and immunoglobulins) are regulated transcriptionally (i.e., synthesized and secreted on demand) rather than being maintained in cells as stored products. Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR), PrimeFlow®, and RNA-Seq methods have been used to assess changes in mRNA levels for specific genes in tissues and cells.

Reporter assays are being used to discern whether xenobiotic-induced changes in mRNA levels for specific genes are due to alterations at the level of transcription versus mRNA stability. Likewise, reporters can characterize the effects of xenobiotics on specific transcription factors acting through defined regulatory elements. Commonly used reporter genes such as firefly luciferase and bacterial β-galactosidase are typically used enzymes, because their expression can be easily assayed.

Two methods to achieve gene-specific silencing include RNA interference and CRISPR (clustered regularly-interspaced short palindromic repeats)-Cas9. Both methods are limited by transfection efficiency for effective delivery of the guide RNA and Cas9 protein.

Proteomics aims to identify, quantify, and classify the function of proteins produced by given genomes. A potentially important application of proteomics is in the characterization of protein–protein interactions, especially as they relate to the elucidation of signal transduction mechanisms.

Mechanistic Approaches to Immunotoxicology

A general strategy for characterizing mechanisms of immunotoxic action by xenobiotics involves the following steps: (1) identifying the cell type(s) targeted by the agent, (2) determining whether the effects are mediated by the parent compound or by a metabolite of the parent, (3) determining whether the effects are mediated directly or indirectly by the xenobiotic, and (4) elucidating the molecular events responsible for altered leukocyte function.

Identification of the Cell Type or Type(s) Targeted— Because the immune system is comprised of many cell types with broad, and often, overlapping effector functions, identification of the specific cell type(s) affected allows for the selection of appropriate approaches and techniques to further elucidate the mechanism of action.

Determination of Whether Immunotoxicity Is Mediated by the Parent or by a Metabolite of the Parent Compound—The role of metabolism is especially critical when studying immunotoxicants as it will dictate the experimental approaches that can be utilized. In general, leukocytes possess modest levels of drug metabolizing enzymes, especially those within the cytochrome P-450 family. Those xenobiotics that are metabolically bioactivated to an immunosuppressive form will not exhibit, in most cases, their immunotoxic profile of activity when added directly to cultured leukocytes.

Determination of Whether the Effects Are Mediated Directly or Indirectly by the Xenobiotic or a Metabolite of the Xenobiotic—Direct actions of immunotoxicants may include structural alterations in lymphoid organs, the cellular composition of lymphoid organs, the expression of regulatory molecules on the immune cell surface, and/or by altering intracellular biochemical or molecular events. Some xenobiotics mediate changes in immune competence indirectly, through the release of an immunomodulatory factor resulting from the actions of the

immunotoxicant on cells or tissues other than the immune system. One tissue most often implicated in indirectly modulating the immune system is the liver, as it is the source of a broad and extremely diverse group of proteins, including acute-phase proteins that are associated with downregulation of the immune system and are therefore believed to play a role in maintaining immune homeostasis. A second example of indirect actions on the immune system is stress-induced alteration in the regulation of the hypothalamic-pituitary-adrenal axis. Deregulation of hormonal homeostasis, especially increased circulating levels of glucocorticoids, can markedly decrease immune competence. The elucidation of indirect mechanisms of action by an immunotoxicant can be challenging because effects may be mediated by one or more circulating immunomodulatory serum factors.

Elucidation of the Molecular Mechanism—To evaluate cellular and molecular mechanisms of action, there is continuing availability of new technologies, new animal models including transgenic and knockouts, and an ever-expanding list of reagents and molecular probes.

When considering the molecular mechanism by which a xenobiotic alters the function of a mature lymphocyte, a practical strategy is to first identify at which stage of leukocyte function the agent is acting (i.e., antigen recognition/signaling through the antigen receptor, cellular activation, proliferation, or differentiation). Once the specific stage of lymphocyte function being altered by a specific xenobiotic has been established, experiments can be designed to identify the specific intracellular proteins affected by the xenobiotic and the molecular mechanism by which it is modulated.

Cell Line Models in Immunotoxicology—Cell line–based models are being widely used for identifying xenobiotics that are potential immunotoxicants as well as for elucidating molecular mechanisms for immunotoxicity. Although there are many advantages to cell line–based models, the most important characteristic is that all cells are derived from the same clone, thus providing a relatively homogenous cellular preparation. The homogeneity of the model is especially useful for studies directed at characterizing signal transduction pathways as well as gene expression profiling due to the greater likelihood of obtaining reproducible results.

Because a cell line is an abnormal population of cells that has undergone a change rendering it capable of dividing indefinitely in culture, most cell lines are not good models for studying immunotoxicants that act by altering cell proliferation and/or regulators of the cell cycle. The aberrant nature of cell lines may also extend to a loss of function through one or more of its cognate receptors. Lastly, it is critical that cell lines are carefully monitored and characterized for changes in function and morphology after repeated passage in culture. Nevertheless, many cell lines that have been extensively characterized and widely utilized are capable of induced effector functions, including cytokine production, antibody secretion, and release of various mediators.

Animal Models: Transgenic, Knockout, and Humanized/SCID—Manipulation of the embryonic genome has created transgenic and knockout mice allowing dissection of complex immune responses into their components. Mice engineered to express nonself genes (e.g., human MHCI) or overexpress self-genes (e.g., constitutively active transcription factors) are termed “transgenic,” and can be used to address the role of a certain protein in immunotoxicology. In contrast, mice lacking certain proteins (e.g., receptors, transcription factors, or cytokines) are termed “knockouts,” and can be used for similar mechanistic studies. Humanized mice refer to immune-deficient mice that have been reconstituted either with human hematopoietic stem cells to support the development of a fully human immune system or with mature cells to evaluate

immune regulation, hematopoiesis, hypersensitivity, and autoimmunity. Severe combined immunodeficient (SCID) mice, which are mouse T- and B-cell deficient due to a VDJ (variable region gene segments) recombination defect, are common models into which human immune cells/system are established.

Approaches to the Assessment of Human Immunotoxicity

Chemicals that produce immunotoxicity in animals have the potential to produce immune effects in humans. Distinct, although often subtle, differences in the composition and functional responses of the immune system occur between animal species. Whether these differences in immune system composition and response to stimuli result in significant differences across species in sensitivity to immunotoxicants is unclear.

An important goal of experimental immunotoxicity testing is to enable the best extrapolations between results generated in the laboratory animal models and potential risk of immunotoxicity in humans. The current strategy has been to utilize in vivo and in vitro data from animal studies and, when available, in vitro data using human leukocytes in order to predict the in vivo effects of an immunotoxicant in humans. If in vitro human data are not available, then safety decisions are made solely on animal data.

It is recognized that extrapolations of alterations in immune function observed in laboratory animals to human health is associated with various uncertainties. Experimental animals are often inbred, and laboratory studies in rodents are highly controlled for environment, diet, and health status. By contrast, humans are highly outbred species with a high degree of interindividual variability in immune response. In fact, the overall immunocompetence of the individual is affected by age, sex, genetic factors, use of certain medications, drug/alcohol use, smoking history, stress, and nutritional status, and that these factors can account for variability in the "normal" human population. The development, validation, and utilization of more predictive methods to assess immunotoxicology in humans will be increasingly important in the future.

Regulatory Approaches to the Assessment of Immunotoxicity

The National Toxicology Program (NTP) Tier Approach—Historically, immunotoxicity has been assessed using a battery of assays, which are typically structured in a multi-tiered approach. Importantly, studies conducted by the NTP have indicated that immunotoxicity can be assessed with a finite number of assays, which is summarized in Table 12–3.

TABLE 12–3 Tier Approach for Immunotoxicology Testing

Testing Level	Procedures
Tier I	Hematology Body weight Organ weights (spleen, thymus, kidney, liver) Spleen cellularity Bone marrow cellularity and CFU Immunopathology AFC assay Proliferative responses NK assay
Tier II	Surface marker analysis Secondary (IgG) AFC assay CTL assay DTH response Host resistance studies

Tier I tests assess general toxicity (e.g., immunopathology, hematology, and body and organ weights) as well as immune functional capability (e.g., proliferative responses, TDAR, CTL assay, and NK assay). Tier I was designed to detect potential immunotoxic compounds at concentrations that do not produce overt toxicity. Tier II tests were designed to further define an immunotoxic effect, and included functional tests for secondary antibody responses, enumeration of lymphocyte populations, and host resistance models.

Several testing configurations were ultimately defined that would minimize the number of immune tests needed yet could still provide a high degree of sensitivity for detecting potential immunotoxicants. Current approaches by the NTP reflect the state-of-the-science of immunotoxicology, in that two developmental immunotoxicity studies, four contact hypersensitivity studies, and one study assessing the potential of a xenobiotic-associated change in an autoimmune disease model are assessed each year.

The NTP emphasized the importance of recognizing that the “levels of evidence” statements that are described reflected only immunologic hazard. Five categories of evidence of immune system toxicity were used to summarize the strength of the evidence observed in each experiment being considered: two categories for positive results (clear evidence and some evidence), one category for uncertain findings (equivocal evidence), one category for no observable effects (no evidence), and one category for experiments that cannot be evaluated because of major design or performance flaws (inadequate study).

Historical Perspective on Regulatory Guidance in Immunotoxicology—The T-cell dependent antibody response (TDAR) has been the consensus choice for a functional endpoint to identify immunotoxicity hazard in most, if not all, regulatory guidelines. The TDAR requires the orchestrated coordination and cooperativity of multiple cell types (e.g., APCs, Th cells, and B cells) and the manifestation of most, if not all, of the biological functions important to immunocompetence.

Recent Advances in Regulatory Guidance on Immunotoxicology—Two areas over the last few years that have impacted the status of immunotoxicology testing are legislative demands and continued evolution of the science.

IMMUNE MODULATION BY XENOBIOTICS

The very nature of the immune system with its diversity of cell types, presence of various cell types in every tissue of the body, dependence upon proliferation and differentiation for effector functions, and necessity to maintain immune function homeostasis renders system susceptible to modulation by a wide variety of xenobiotics. Many of these chemicals are immunomodulatory; that is, they might produce both immune suppression and immune enhancement (in the absence of true hypersensitivity or autoimmunity) (Table 12–4). Regardless of the end effect (immune suppression, immune enhancement, hypersensitivity, or autoimmunity) of a xenobiotic on the immune system, several common themes exist regarding the mechanism by which these chemicals act. First, the mechanisms by which a xenobiotic affects immune function are likely to be multifaceted, involving several proteins, signaling cascades, or receptors. In fact, there is evidence to suggest that xenobiotic-specific effects on the immune system are both receptor-dependent and receptor-independent. Second, whether a xenobiotic produces a particular immune effect might depend on the concentration or dose of the xenobiotic, the mode and/or magnitude of cellular stimulation, and the kinetic relationship between exposure to the xenobiotic and exposure to the immune stimulant (i.e., antigen, mitogen, or pharmacological agent). Third, xenobiotic exposures rarely occur one chemical at a time; thus, the effects and/or mechanisms observed might be attributable to several chemicals or classes of chemicals. Finally, determination of immune system effects and/or mechanisms by xenobiotics in humans might be further confounded by the physiological or immunological state of the individual.

TABLE 12–4 Immunomodulatory Therapeutics and Biologics

Agent	Effect	Primary Mechanism of Action
Therapeutics		
NSAIDs	Anti-inflammatory	COX-1 and/or COX-2 inhibitor
Glucocorticoids	Anti-inflammatory Immune suppressant	Phospholipase A2 inhibitor Induction of IκB (NF-κB inhibitor)
Cyclophosphamide	Immune suppressant	Inhibition of cell replication
Azathioprine Lefluonomide		
Cyclosporin A	Immune suppressant	Calcineurin and NFAT inhibitor
FK506		
Rapamycin	Immune suppressant	mTOR signaling inhibitor
Tofacitinib	Immune suppressant	Janus kinase inhibitor
Fingolimod	Immune suppressant	Sphingosine-1-phosphate modulator
Biologics		
Etanercept	Anti-inflammatory Immune suppressant	TNF-α and TNF-β inhibitor
Infliximab	Anti-inflammatory	TNF-α inhibitor
Adalimumab	Immune suppressant	
Certolizumab Golimumab		
Muromonab	Immune suppressant	T-cell depletion following antibody opsonization
Natalizumab	Immune suppressant	α4 integrin inhibitor
Ipilimumab	Immune stimulant	CTLA-4 inhibitor
Nivolumab	Immune stimulant	PD1 or PDL-1 inhibitor
Pembrolizumab Atezolizumab		
Recombinant cytokines (IFN-α/IFN-γ/GM-CSF/IL-2/IL-12)	Immune stimulant	Similar mechanisms to cytokines as listed in Table 12–2

Few classes of xenobiotics have been as extensively studied for immunotoxicity as the halogenated aromatic hydrocarbons (HAHs). The HAHs include the polychlorinated biphenyls (PCBs), the polybrominated biphenyls (PBBs), the polychlorinated dibenzofurans (PCDFs), and the polychlorinated dibenzodioxins (PCDDs). Substantial evidence has demonstrated the immune system to be a sensitive target for toxicity by these chemicals. Derived from a variety of animal models, primarily rodents, this evidence includes thymic atrophy, pancytopenia, cachexia, immune suppression, and tumor promotion. The majority of the biochemical and toxic effects produced by the HAHs are mediated via HAH binding to the cytosolic aryl hydrocarbon receptor (AhR).

Pesticides include all xenobiotics whose specific purpose is to kill another form of life, including insects (insecticides), small rodents (rodenticides), or even vegetation (herbicides). Exposure to pesticides occurs most often in occupational settings, in which manufacturers, pesticide applicators, or harvesters of treated agricultural products are exposed (see Chapter 22).

Metals target multiple organ systems and exert their toxic effects via an interaction of the free metal with targets, such as enzyme systems, membranes, or cellular organelles (see additional details in Chapter 23). Most metals act as immunomodulators and are not consistently

immunosuppressive. Lead exposure is associated with inflammation and increased expression of inflammatory cytokines, the generation of ROS, increased pathogen susceptibility, and the potential for hypersensitivity and autoimmune reactions. Arsenic exposure causes oxidative stress, alters inflammatory and adaptive immune processes, but variations in the effects may be due to the speciation of arsenic and the target cell. Mercury compounds suppress immunological responses, but also induce autoimmunity. Depending on the concentration, mercury exposure could be cytotoxic or potentially inflammatory, and alter self-tolerance mechanisms. Cadmium also exhibits immunomodulatory effects.

Solvents and related chemicals can produce immune suppression. Chemicals included in this section are aromatic hydrocarbons, such as benzene, haloalkanes and haloalkenes, glycols and glycol ethers, and nitrosamines. Chapter 24 contains additional information. Benzene induced anemia, lymphopenia, and hypoplastic bone marrow may be a result of altered differentiative capacity in bone marrow–derived lymphoid cells. Benzene (oral and inhaled) exposure has been reported to alter both humoral and CMI parameters, including suppression of the anti-sRBC antibody response, decreased T- and B-cell lymphoproliferative responses (mitogens and alloantigens), and suppression of CTL activity. Nitrobenzene and nitrotoluenes have immunotoxic effects on erythrocytes and bone marrow. Carbon tetrachloride is immunosuppressive.

Mycotoxins are structurally diverse secondary metabolites of fungi and comprise such toxins as aflatoxin, ochratoxin, and the tricothecenes, notably T-2 toxin and deoxynivalenol (vomitoxin). As a class, these toxins can produce immune stimulation or immune suppression depending on dose, exposure frequency, or route of administration. These toxins can produce cellular depletion in lymphoid organs, alterations in T- and B-lymphocyte function, suppression of antibody responses, suppression of NK cell activity, decreased DTH responses, and an apparent increase in susceptibility to infectious disease.

Natural and synthetic hormones show a sexual dimorphism in the immune system. Females have higher levels of circulating Igs, a greater antibody response, and a higher incidence of autoimmune disease than do males. Males appear to be more susceptible to the development of sepsis and the mortality associated with soft tissue trauma and hemorrhagic shock. Specific natural sex hormones have been implicated to mediate this dichotomy. A recent cross-species meta-analysis of immune function supports a general immunosuppressive effect of testosterone versus an immune-enhancing effect of estrogen on humoral-mediated immune function and cytokine levels, but estrogens also induce an immunosuppressive effect on cell-mediated immune function. Physiologically, immune effects of sex hormones appear to be very tightly controlled, and profound changes in immune activity can result for slight changes in concentrations of hormones. More detailed discussion for estrogens, androgens, and glucocorticoids are given in Chapters 20–21.

Several classes of drugs of abuse exhibit immunosuppressive actions, including cannabinoids, opioids, cocaine, methamphetamine, and ethanol. The mechanisms by which drugs suppress immune function might depend on the development of tolerance or addiction to the drugs; the immune, withdrawal, and pain status of the individual; and levels of endogenous molecules (i.e., endorphins or endocannabinoids).

Both CB1 and CB2 cannabinoid receptors are expressed on immune system cells, but not all cannabinoid effects are mediated via the receptors. CB2 mediates suppression in germinal centers, memory B cells, and T-cell-independent responses. Cannabinoid suppression of macrophages and DCs is also mediated through the CB1 and/or CB2 receptors. Cannabinoid-

mediated suppression of host resistance includes dysregulation of cytokine profiles (shifts in Th1/Th2/Th17 balance), induction of apoptosis, and iTregs.

Opioids suppress immune responses by both G_i-coupled opioid receptors (μ, κ, and δ receptors), and opioid receptor–independent actions on the CNS, the autonomic nervous system, and the hypothalamic-pituitary-adrenal axis.

Cocaine and its derivatives have been shown to alter humoral and cell-mediated immune responses and host resistance. Cocaine-altered immune function involves a disruption of the Th1/Th2 balance and the stress response.

Methamphetamine immunotoxicity includes suppression of both CMI and humoral immunity in vivo.

Ethanol exposure is associated with an increased incidence of pulmonary infection. A consistent finding in abusers of ethanol is the significant change in PBMC. Ethanol suppresses signaling through TLRs, contributing to pleiotropic effects of ethanol on innate immunity. Moreover, signaling alterations via TLRs depend on whether the alcohol exposure is acute or chronic.

Ultraviolet Radiation

Ultraviolet radiation (UVR), especially mid-range UVB (290 to 340 nm), has been demonstrated to modulate immune responses in animals and humans. UVR is immunosuppressive. As a consequence of UV absorption by chromophores, epidermal and dermal cells, including keratinocytes, melanocytes, Langerhans cells, mast cells, dermal fibroblasts, endothelial cells, and skin-infiltrating cells (i.e., granulocytes and macrophages), produce and/or release many immunoregulatory mediators, including cytokines, chemokines, and neurohormones. The mediators include both pro- and anti-inflammatory cytokines, such as TNF-α, IL-1, IL-6, IL-10, IL-12, and IL-17, which can modify directly or indirectly the function of APCs. UVR induces a switch from a predominantly Th1 response (favoring DTH responses) to a Th2 response (favoring antibody responses). Also, UVR is associated with suppression of certain allergic and autoimmune reactions. Indeed, Ig isotypes that are linked to either Th1 or Th2 cells can be suppressed by UVR. UV exposure not only impairs Th1 responses but also impairs humoral immunity by inhibiting T_{FH} and inducing Bregs that secrete IL-10.

Therapeutic Drugs

Drugs from many classes affect the immune system. Nonselective NSAIDs, cyclophosphamide, azathioprine, leflunomide, cyclosporin, tacrolimus, rapamycin, tofacitinib, fingolimod, and antiviral therapies such as zidovudine, all exert various effects on the immune system.

XENOBIOTIC-INDUCED HYPERSENSITIVITY AND AUTOIMMUNITY

When an individual’s immune system responds in a manner producing tissue damage, it could result in hypersensitivity or autoimmunity, which could be exacerbated, or even induced, by a

xenobiotic. Figure 12–15 is a schematic delineating the possible cascade of effects that can occur when a chemical produces an immune-mediated disease.

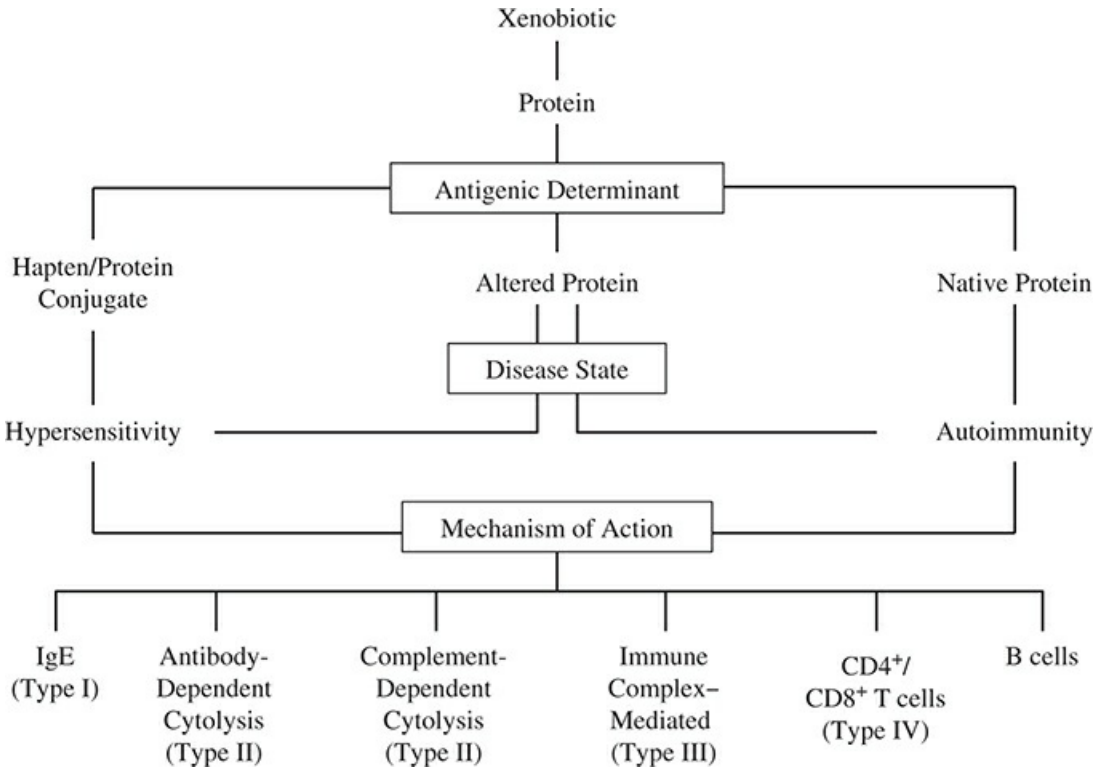

FIGURE 12–15 *Schematic diagram of xenobiotic induction of hypersensitivity or autoimmunity.* The mechanisms by which xenobiotics induce hypersensitivity or autoimmunity can overlap, although IgE production is most often associated with hypersensitivity.

Hypersensitivity

Polyisocyanates induce all four types of hypersensitivity responses and include toluene diisocyanate, methylene diphenyl diisocyanate, and hexamethylene diisocyanate. The acid anhydrides, such as trimellitic acid anhydride, cause all four hypersensitivity reactions. Metals, such as cobalt, chromium, nickel, and beryllium, and metallic substances produce contact and pulmonary hypersensitivity reactions. Natural rubber latex induces hypersensitivity via a Type I or Type IV reaction. The IgE responses may manifest as urticaria, asthma, or life-threatening anaphylaxis.

The most common food allergens are milk, egg, peanuts, tree nuts, fish, shellfish, soy, and wheat. Hypersensitivity to peanuts occurs primarily via a Type I reaction and the IgE responses may include shortness of breath, asthma, and anaphylaxis. At least 12 peanut proteins have been identified and antibodies to most can be detected in peanut-allergic patients.

Exposure to GMOs is becoming more widespread as biotechnological advances in food production are used to confer insect resistance, provide desired nutrients, or adapt to periods of drought. Allergenic determinants in GMOs result from the expression of novel proteins that might be recognized as nonself by the immune system.

Formaldehyde forms haptens with human proteins easily and is a contact sensitizer.

Pulmonary defenses against inhaled gases, such as ozone (O_3), sulfur dioxide (SO_2), nitrogen dioxide (NO_2), and phosgene, and particulates, asbestos, silica, and nanoparticles, are dependent upon both physical and immunologic mechanisms. Immune mechanisms primarily involve the complex interactions between neutrophils and alveolar macrophages and their abilities to phagocytose foreign material and produce cytokines, which not only act as local inflammatory mediators but also serve to attract other cells into the airways. Most reports demonstrate marked inflammation (and even autoimmune disease) in response to asbestos, silica, and various nanoparticles.

Therapeutic Drugs—Hypersensitivity responses to therapeutic drugs account for up to 10% of all adverse effects. Drugs that commonly induce hypersensitivity include sulfa drugs, barbiturates, anticonvulsants, insulin, iodine (used in many x-ray contrast dyes), and platinum-containing chemotherapeutics. Hypersensitivity to penicillin occurs through the formation of a neoantigen. The resultant penicilloylated protein now acts as a hapten to which the immune system mounts a response. Abacavir induces hypersensitivity with the expression of the HLA haplotype HLA-B*5701 and elevated $CD8^+$ CTL at the initiation of abacavir treatment.

Autoimmunity

Vinyl chloride, several organic solvents, crystalline silica (silicon dioxide) and hexachlorobenzene may perturb the immune system and produce schleroderma. Therapeutic agents, such as halothane, methyldopa, hydralazine, isoniazid, and procainamide, are capable of autoimmunity reactions.

IMMUNOTOXICITY OF BIOLOGICS

Biologics refer to those therapies that are derived in some manner from living organisms and include monoclonal antibodies, recombinant proteins, and adoptive cell therapies. Many biologics are intended to treat indications such as cancer, inflammatory/autoimmune diseases, or transplantation and thus are designed to interact with and modulate the immune system. They have the potential to induce immune-mediated toxicities due to immunosuppression that can lead to infections or malignancies or adverse immune stimulation that can cause infusion reactions, cytokine storms, or autoimmunity. In addition, due to their large size and/or foreignness to the host, biologics have the potential to be immunogenic and elicit an immune response to itself that can cause toxicities associated with hypersensitivity reactions or the neutralization of an endogenous protein due to cross-reactive antibodies.

Many monoclonal antibodies directed against certain molecules that are critical for inducing or sustaining an immune response can be divided into the following mechanistic categories: (1) bind and neutralize specific cytokines (TNF-α, IL-6); (2) bind cell-surface molecules to trigger lysis by the adaptive immune response (CD3 on T cells; CD25, α subunit of IL-2 receptor on activated T cells; CD20 on mature B cells; CD52 on B cells, T cells, NK cells, monocytes, macrophages); (3) bind cell-surface molecules and block costimulation signal from other cells (CD2 on T cells; CD80/CD86 on APCs, soluble BLyS/BAFF, CD40L on T cells); (4) bind cell-

surface molecules and stimulate costimulatory receptors or block inhibitory receptors from other cells (CTLA4, PD1, CD40 on T cells and PDL1, CD137 on APCs); and (5) bind cell-adhesion molecules and block lymphocyte trafficking (LFA-1 or α4 on leukocytes). A summary of the mechanisms by which these biologics alter immunity, including a comparison between small molecule therapeutics and biologics, is provided in Table 12–4.

NEW FRONTIERS AND CHALLENGES IN IMMUNOTOXICOLOGY

Characterization of the risk associated with xenobiotic-induced immunotoxicity represents one of the key challenges for this discipline. Risk must incorporate the hazard, including dose–response relationships, and exposure, including the amount of chemical involved and the time of its interaction with people and/or the environment. Significant advancement in immunological knowledge within immunology and cell biology is coupled with an explosion in methodological and technological capabilities. New tests reflecting potential impacts of immunotoxicity have emerged, and traditional tests have been improved. Quantifiable measurements can be made at the protein and mRNA levels in individual cells through the application of flow cytometry and cytometry time of flight (CyTOF) measurements. A second major recent advancement in flow cytometry using conventional fluorescent dye-based technology has been to measure changes in the full-emission spectra signature across all lasers for each fluorophore expanding the repertoire of simultaneous measurements.

Quantitative measurements of mRNA expression at the single cell level have also become possible on a routine basis utilizing PrimeFlow® and single-cell RNA sequencing (RNA-seq). Collectively, approaches for single-cell analysis will be critically important in the elucidation of molecular mechanisms by which xenobiotics modulate the immune system. As new insights into mechanisms of immune toxicity are gleaned, so will the identification of new biomarkers of immunotoxicity that can be incorporated into testing strategies.

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QUESTIONS

1. Which of the following cells or substances is NOT part of the innate immune system?
   - **a.** lysozyme.
   - **b.** monocytes.
   - **c.** complement.
   - **d.** antibodies.

e. neutrophils.

2. Myeloid precursor stem cells are responsible for the formation of all of the following EXCEPT:
   a. platelets.
   b. lymphocytes.
   c. basophils.
   d. erythrocytes.
   e. monocytes.

3. When an Rh− mother is exposed to the blood of an Rh+ baby during childbirth, the mother will make antibodies against the Rh factor, which can lead to the mother attacking the next Rh+ fetus. This is all possible because of which antibody's ability to cross the placenta?
   a. IgM.
   b. IgE.
   c. IgG.
   d. IgA.
   e. IgD.

4. Which of the following statements is FALSE regarding important cytokine function in regulating the immune system?
   a. IL-1 induces inflammation and fever.
   b. IL-3 is the primary T-cell growth factor.
   c. IL-4 induces B-cell differentiation and isotype switching.
   d. Transforming growth factor-β (TGF-β) enhances monocyte/macrophage chemotaxis.
   e. Interferon gamma (IFN-gamma) activates macrophages.

5. Which of the following is NOT a step performed during an enzyme-linked immunosorbent assay (ELISA)?
   a. A chromogen is added and color is detected.
   b. The antigen of interest is fixed to a microtiter plate.
   c. Radioactively labeled cells are added to the solution.
   d. Enzyme-tagged secondary antibodies are added.
   e. Test sera are added.

6. The delayed hypersensitivity response (DHR) test does NOT:
   a. evaluate memory T-cells' ability to recognize a foreign antigen.
   b. evaluate memory T-cells' ability to secrete cytokines.
   c. evaluate memory T-cells' ability to proliferate.
   d. evaluate memory T-cells' ability to lyse foreign target cells.
   e. evaluate memory T-cells' ability to migrate to the site of foreign antigen.

7. The number of alveolar macrophages in smokers is greatly increased relative to non-smokers. What is a characteristic of the alveolar macrophages found in smokers?

a. They are in an inactive state.
b. They are far larger than normal.
c. They have increased phagocytic activity.
d. They are incapable of producing cytokines.
e. They have decreased bactericidal activity.

8. Which of the following is NOT characteristic of a Type I hypersensitivity reaction?
a. It is mediated by IgE.
b. It involves immune complex deposition in peripheral tissues.
c. It involves mast-cell degranulation.
d. Anaphylaxis is an acute, systemic, and very severe Type I hypersensitivity reaction.
e. It is usually mediated by preformed histamine, prostaglandins, and leukotrienes.

9. Which of the following types of hypersensitivity is NOT mediated by antibodies?
a. Type I.
b. Type II.
c. Type III.
d. Type IV.
e. Type V.

10. Which of the following is NOT a common mechanism of autoimmune disorders?
a. subjection to positive selection in the thymus.
b. anergic T cells become activated.
c. interference with normal immunoregulation by $CD8^+$ suppressor T cells.
d. lack of subjection to negative selection in the thymus.
e. decreased self-tolerance.

CHAPTER 13

Toxic Responses of the Liver

Robert A. Roth, Hartmut Jaeschke, and James P. Luyendyk

KEY POINTS

- The liver's strategic location between intestinal tract and the rest of the body facilitates its maintenance of metabolic homeostasis in the body.
- The liver extracts ingested nutrients, vitamins, metals, drugs, environmental toxicants, and waste products of bacteria from the blood for catabolism, storage, and/or excretion into bile.
- Formation of bile is essential for uptake of lipid nutrients from the small intestine, protection of the small intestine from oxidative insults, and excretion of endogenous and xenobiotic compounds.
- Cholestasis is either a decrease in the volume of bile formed or an impaired secretion of specific solutes into bile, which results in elevated serum levels of bile salts and bilirubin.
- Hepatocytes have phase I enzymes that often convert xenobiotics to reactive electrophilic metabolites and phase II enzymes that add a polar group to a molecule and thereby enhance its removal from the body. The balance between phase I and phase II reactions determines whether a reactive metabolite will initiate liver cell injury or be safely detoxified.

INTRODUCTION

The liver performs many critical life functions, including processing of foods and other substances absorbed from the intestinal tract and subsequent delivery of processed nutrients to other organs in the body. The liver also contributes to immunity that protects mammals from harmful pathogens. It is the main organ where exogenous chemicals are metabolized for eventual excretion into bile and urine.

Knowledge of liver physiology and anatomy lends insight as to how the liver functions and provides underpinnings for understanding how toxicants cause liver dysfunction. Chemical-induced liver injury is typically initiated by one or more critical events, such as formation of a toxic metabolite, which trigger intracellular responses that can progress to dysfunction or death of hepatic parenchymal cells (i.e., HPCs, hepatocytes). These intrahepatocellular events can in turn prompt secondary events involving activation of nonparenchymal cells that magnify or attenuate the initial injury.

LIVER ANATOMY AND PHYSIOLOGY

Hepatic Functional Anatomy

The liver has a dual blood supply (Fig. 13–1). The hepatic artery supplies a minority of blood entering the liver. The hepatic portal vein, which comprises venous drainage from the stomach and intestine, is the major supplier of blood that contains food-borne xenobiotic agents absorbed into the blood from the gastrointestinal (GI) tract, and that is poorly oxygenated.

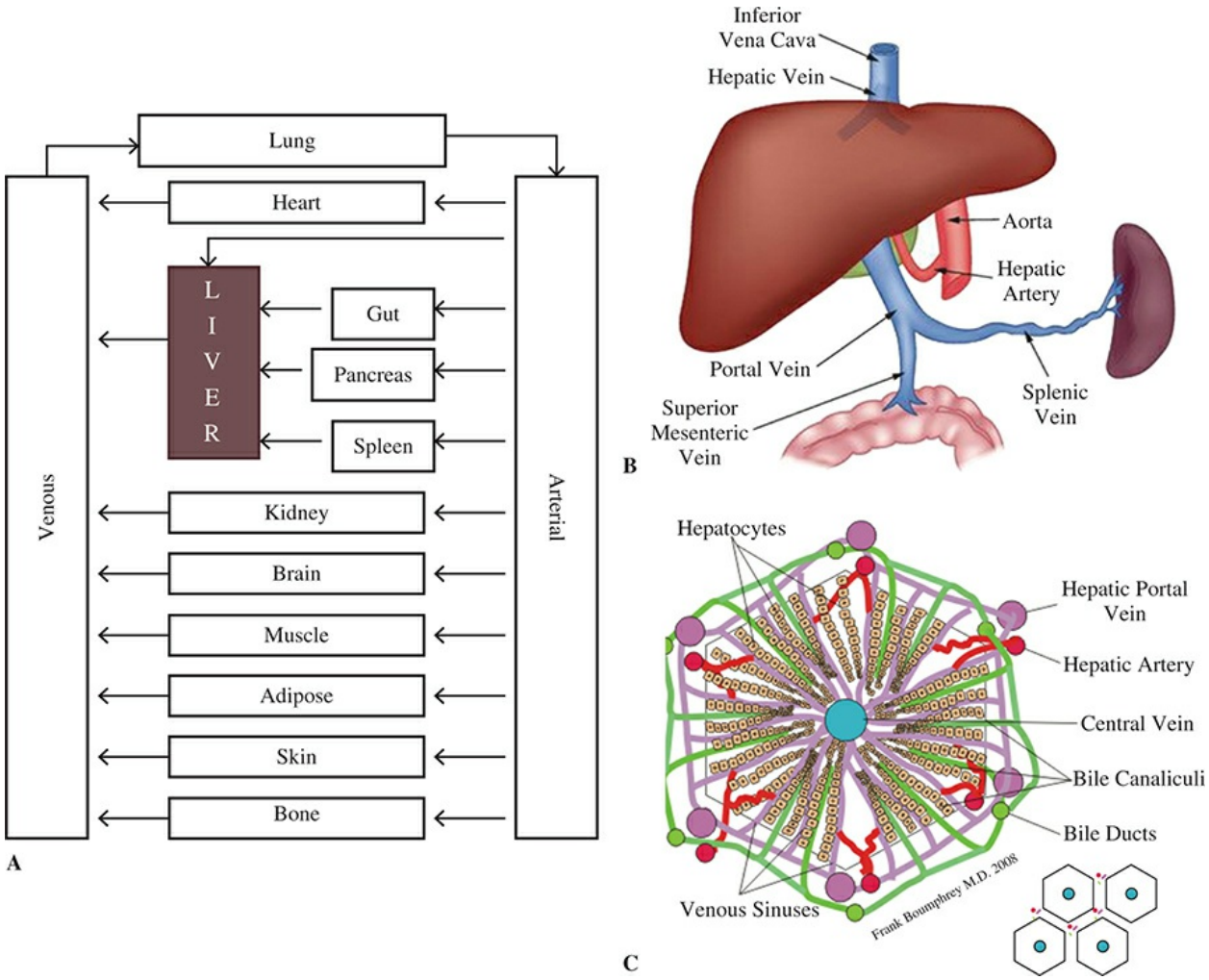

FIGURE 13–1 *Liver circulation and lobular organization.* (**A**) Physiologically based model of the body; the liver is unusual among organs in having a dual blood supply, one from arterial blood and the other from blood that drains the gastrointestinal tract. (Reprinted with permission from Kuo I, Akpa BS. Validity of the lipid sink as a mechanism for the reversal of local anesthetic systemic toxicity: a physiologically based pharmacokinetic model study. *Anesthesiology*. 2013;118(6):1350–1361.) (**B**) Blood enters the liver from the hepatic artery and from the portal vein, which arises from gastrointestinal and splenic drainage. (From http://basicmedicalkey.com/gastrointestinal-physiology-2/.) (**C**) The structure of the liver lobule. Blood from hepatic arterioles and portal venules mix as it enters the sinusoids; blood leaving the sinusoids enters central veins that drain into the vena cava. (From http://amedleyofpotpourri.blogspot.com/2015/03/liver.html.)

According to the classical lobular concept, the liver is organized into hexagonal lobules that are oriented around a central vein (also known as a terminal hepatic venule). At the corners of the lobule are portal triads, which contain a branch of the portal vein (portal venule), a hepatic arteriole, and one or more small bile ducts (Fig. 13–1C). The capillary equivalents in liver lobules are called “sinusoids.” Blood entering the portal tract via the portal vein and hepatic artery is mixed in the penetrating vessels, enters the sinusoids, and percolates along the chords of parenchymal cells. The blood then collects into terminal hepatic venules, which coalesce to form

the hepatic vein that connects to the vena cava (Fig. 13–1B). The lobule is viewed as having three regions known as periportal (nearest portal triad), centrilobular (surrounding the central vein), and midzonal (between periportal and centrilobular).

The acinus concept better reflects the way blood flows into the sinusoids; that is, some of the blood entering via the portal venule and hepatic arteriole mixes, then some flows laterally (between portal triads) before entering the sinusoid. The terminal branches of the portal vein and hepatic artery form the base of the acinus, which has three zones: zone 1 is closest to the entry of blood (i.e., cells near the portal triad), zone 3 abuts the central vein, and zone 2 is in between (Fig. 13–2). These zones correspond roughly to periportal, centrilobular, and midzonal areas of the classical lobule, respectively.

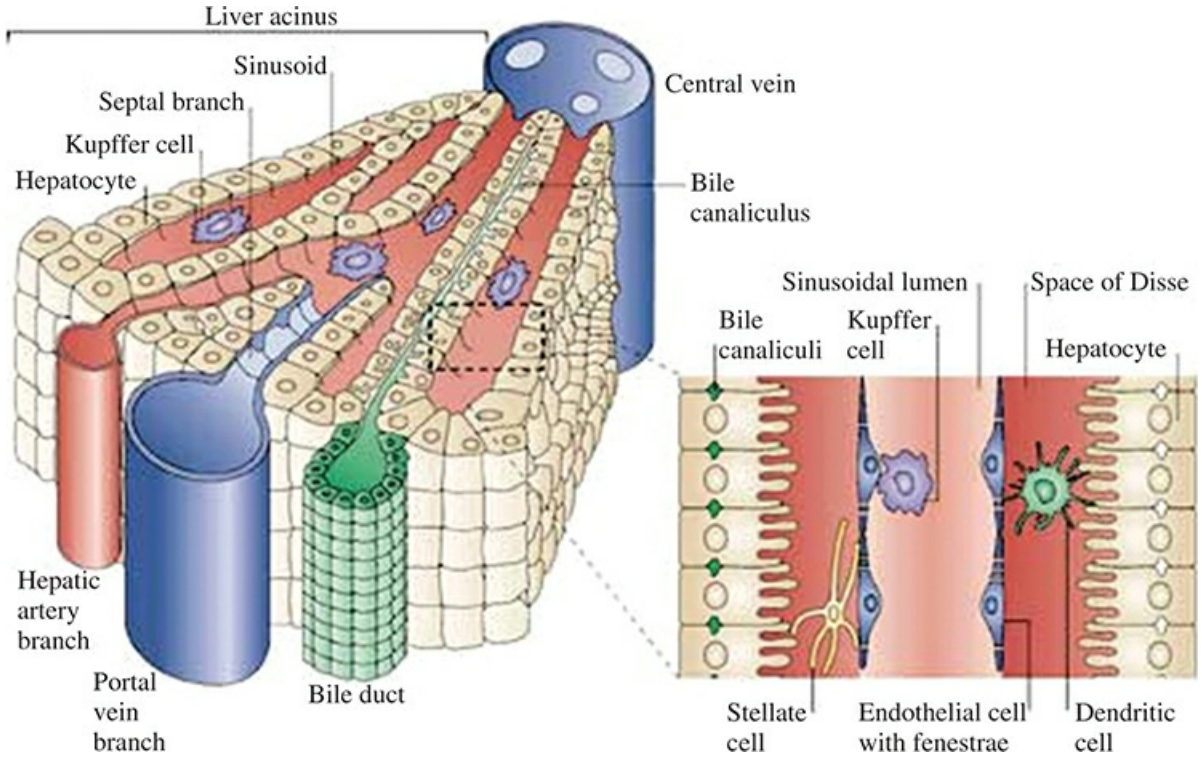

FIGURE 13–2 *Organization of cells in the hepatic acinus.* The insert on the right shows the position of major cells within the sinusoid. (Reprinted with permission from Adams DH, Eksteen B. Aberrant homing of mucosal T cells and extra-intestinal manifestations of inflammatory bowel disease. *Nat Rev Immunol*. 2006;6(3):244–251.)

Blood entering the acinus comprises mostly poorly oxygenated blood from the portal vein relative to the blood entering from the hepatic artery. Enroute to the central vein, oxygen rapidly leaves the blood to meet the high metabolic demands of the HPCs. Therefore, HPCs in zone 3 are exposed to substantially smaller concentrations of oxygen than those in zone 1. Liver lesions caused by chemical exposure usually appear preferentially in one of the zones.

The sinusoids are endothelium-lined channels between cords of HPCs through which blood flows on its way to the central vein. Sinusoids are larger and more irregular than typical capillaries. Three major types of cells in the sinusoids are sinusoidal endothelial cells (SECs), Kupffer cells, and stellate cells (Fig. 13–2). In addition, the liver contains dendritic cells and a substantial number of lymphocytes, especially natural killer (NK) and NKT cells.

Liver Cells and Their Functions

Since venous blood from the intestine flows into the portal vein and then through the liver before entering the systemic circulation, it is the first organ to encounter ingested nutrients, vitamins, drugs, environmental toxicants, as well as bacterial products that enter the portal blood after absorption in the gastrointestinal tract. Efficient uptake processes extract these absorbed materials from the blood for processing, storage, and/or excretion into bile.

Hepatic Parenchymal Cells—HPCs, also known as hepatocytes, are large epithelial cells that account for about 60% of the cells in the liver and about 80% of the liver volume. These cells occur in cords along the microvasculature of the liver (Fig. 13–2), with microvilli on their basal surface in apposition with the endothelium. The apical aspect of these cells is in contact with neighboring HPCs, which are joined by tight junctions. Here a channel, or canaliculus, is formed into which bile is secreted by HPCs (Fig. 13–2); this bile ultimately flows into bile ducts and from there into the small intestine. HPCs perform most of the many critical metabolic functions of the liver (Table 13–1).

TABLE 13–1 General Functions of Liver

- Processing of foods
  - Monosaccharides → glycogen or energy
  - Gluconeogenesis
  - Lipids → processing, energy
- Synthesis of circulating lipids
- Uptake of dietary lipids (e.g., cholesterol) and vitamins from blood
- Degradation of cholesterol and steroids
- Protein synthesis—for intrinsic and extrinsic proteins (e.g., albumin, coagulation, and complement factors, lipoproteins)
- Ammonia detoxification (urea formation)
- Heme synthesis
- Elimination of bilirubin
- Iron reutilization
- Xenobiotic metabolism (drugs, food-borne agents, etc.)
- Excretion via biliary tract (drugs, metals, etc.)
- Elimination of particulates and bacterial products from blood

Quantitative and qualitative differences in HPCs exist depending on their location along the sinusoid. The differences in exposure of HPCs to blood constituents from zone 1 to zone 3 may be one of several reasons why HPCs are functionally heterogeneous. For example, physiological concentrations of bile acids are efficiently extracted by zone 1 HPCs, leaving little bile acid left in the blood that flows past zone 3 HPCs. Moreover, there is difference in bile acid transporter expression among different zones. HPCs in the mitochondria-rich zone 1 predominate in fatty acid oxidation, gluconeogenesis, and ammonia detoxification to urea. Gradients of enzymes involved in the bioactivation and detoxification of xenobiotics have been observed along the acinus by immunohistochemistry. Of toxicological importance are greater concentrations of glutathione (GSH) in zone 1 and of cytochrome P450 proteins in zone 3. Metabolism of some

xenobiotic agents by HPCs can result in toxic metabolites that directly injure these cells. In fact, damage to HPCs and other liver cells from chemical exposure can lead not only to liver dysfunction but to dysfunction of other tissues (e.g., hepatic encephalopathy, coagulopathies).

Sinusoidal Endothelial Cells

Sinusoids are lined by thin, discontinuous endothelial cells with numerous fenestrae that allow molecules smaller than 250 kDa to cross the interstitial space of Disse between the endothelium and HPCs. In the space of Disse, sinusoidal endothelial cells (SECs) are separated from the HPCs by a basement membrane-like matrix. The numerous fenestrae and the attenuated basement membrane facilitate exchange of fluids and molecules, such as albumin, between the sinusoid and HPCs, but hinder movement of particles larger than chylomicron remnants. Endothelial cells are important in the scavenging of lipoproteins, denatured proteins, and advanced glycation endproducts. Hepatic endothelial cells also secrete biologically active molecules such as cytokines, prostanoids, nitric oxide, and endothelins and express intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) on the cell surface. These SEC-derived substances play important roles in liver injury and repair.

Kupffer Cells—Kupffer cells are the resident macrophages of the liver and constitute approximately 80% of the fixed macrophages in the body. They are situated within the lumen of the sinusoid, in apposition to SECs and with processes that extend through fenestrae to contact HPCs (Fig. 13–2). Kupffer cells ingest and degrade particulate matter (e.g., bacteria). Also, Kupffer cells are a major source of cytokines, eicosanoids, and reactive oxygen species (ROS) and can act as antigen-presenting cells (APCs).

Hepatic Stellate Cells—Hepatic stellate cells (HSCs) are located between endothelial cells and HPCs (Fig. 13–2). They are also known as Ito cells or fat-storing cells. Stellate cells are the major sites for vitamin A storage in the body. These cells express smooth muscle actin; they are contractile and appear to control local flow of blood in the sinusoids. When activated, especially during chronic liver injury, stellate cells can assume a myofibroblastic phenotype, synthesizing and secreting collagen and other extracellular matrix proteins and thereby initiate liver fibrosis.

Other Immune Cells of Liver Sinusoids—Also, the liver is home to a population of immune cells of diverse lineage and function. Specific immune cell content and organization within the liver sinusoid make the liver a true immune organ, capable of mounting both innate and adaptive immune responses both under normal conditions and during disease. Immune cells may become engaged in the progression of liver damage driven by inflammation and/or become activated in response to injury-associated changes, including inflammatory stimuli.

Natural Killer (NK) Cells and Natural Killer T (NKT) Cells—These subsets of lymphocytes accumulate in liver in response to injury and inflammatory challenges; they produce cytokines that modify the activity of several other cell types in the liver. Both NK and NKT cells can display quite dichotomous functions in various disease or injury settings.

A set of recently described resident liver immune cells likely to emerge as significant contributors to toxicity are new members of the innate lymphoid cell (ILC) family. Although functional overlap is observed between subtypes of ILCs and T lymphocytes, the stimuli required

to activate and engage ILCs in local inflammatory responses are different. For example, IL-33 has been identified as an inflammatory cue released by injured HPCs that drive ILC activation and subsequent liver fibrosis.

Bile Formation

Bile is a yellow aqueous fluid that contains bile acids, GSH, phospholipids, cholesterol, bilirubin and other organic anions, proteins, metals, ions, and xenobiotic agents. Adequate bile formation by HPCs is essential for uptake of lipid nutrients from the small intestine, for protection of the small intestine from oxidative insults, and for excretion of endogenous and xenobiotic compounds. Bile canaliculi are separated from the intercellular space between HPCs by tight junctions. Under physiological conditions, high concentrations of bile acids, GSH, bilirubin diglucuronide, and other organic anions accumulate in bile. The structure of the biliary tract is analogous to the roots and trunk of a tree, where the tips of the roots equate to the canalicular lumens. Canaliculi between HPCs connect to a series of increasingly larger channels (intrahepatic bile ducts, the large extrahepatic bile ducts) that merge to form the common bile duct. Bile originating in the lobules travels from the intrahepatic bile ducts to the common bile duct and then into the gallbladder to be stored and concentrated before its release into the duodenum. However, the gallbladder is absent in several species, including the horse, whale, and rat, so that bile flows directly into the duodenum from the common bile duct in these species.

Bile Formation and Transport— HPCs begin the process of bile formation by active transport into the canalicular lumen of bile acids, GSH, and other osmotically active compounds including xenobiotics and their metabolites. These molecules are the major driving force for the passive movement of water and electrolytes across the HPC epithelium into the bile. Concentration of solutes in bile depends on their active transport at the locus of the canalicular membrane.

On the basal (sinusoidal) side of the HPCs, there are sodium-dependent and sodium-independent uptake systems (Fig. 13–3). Most conjugated bile acids (taurine and glycine conjugates) and some unconjugated bile acids are transported into HPCs by sodium/taurocholate cotransporting polypeptide (NTCP). Sodium-independent uptake of conjugated and unconjugated bile acids is performed by members of the organic anion–transporting polypeptides (OATPs). OATP1B1 and OATP1B3 are predominantly expressed in liver and are capable of transporting conjugated and unconjugated bile acids, steroids, and many other organic anion drugs and hepatotoxicants. Several organic-anion transporters (OATs, in humans) transport small organic anions coupled to exchange of another organic anion. Organic cations are transported across the basolateral membrane by organic cation transporters (OCTs, especially OCT1 in humans). The driving force for transport by OCTs is the concentration gradient of the cation across the HPC membrane, so transport can be in either direction. In addition to the uptake or exchange systems, there are ATP-dependent efflux pumps located on the basolateral membrane of HPCs. These carriers are members of the multidrug resistance-associated proteins (MRPs; ABCC), which transport many different anions.

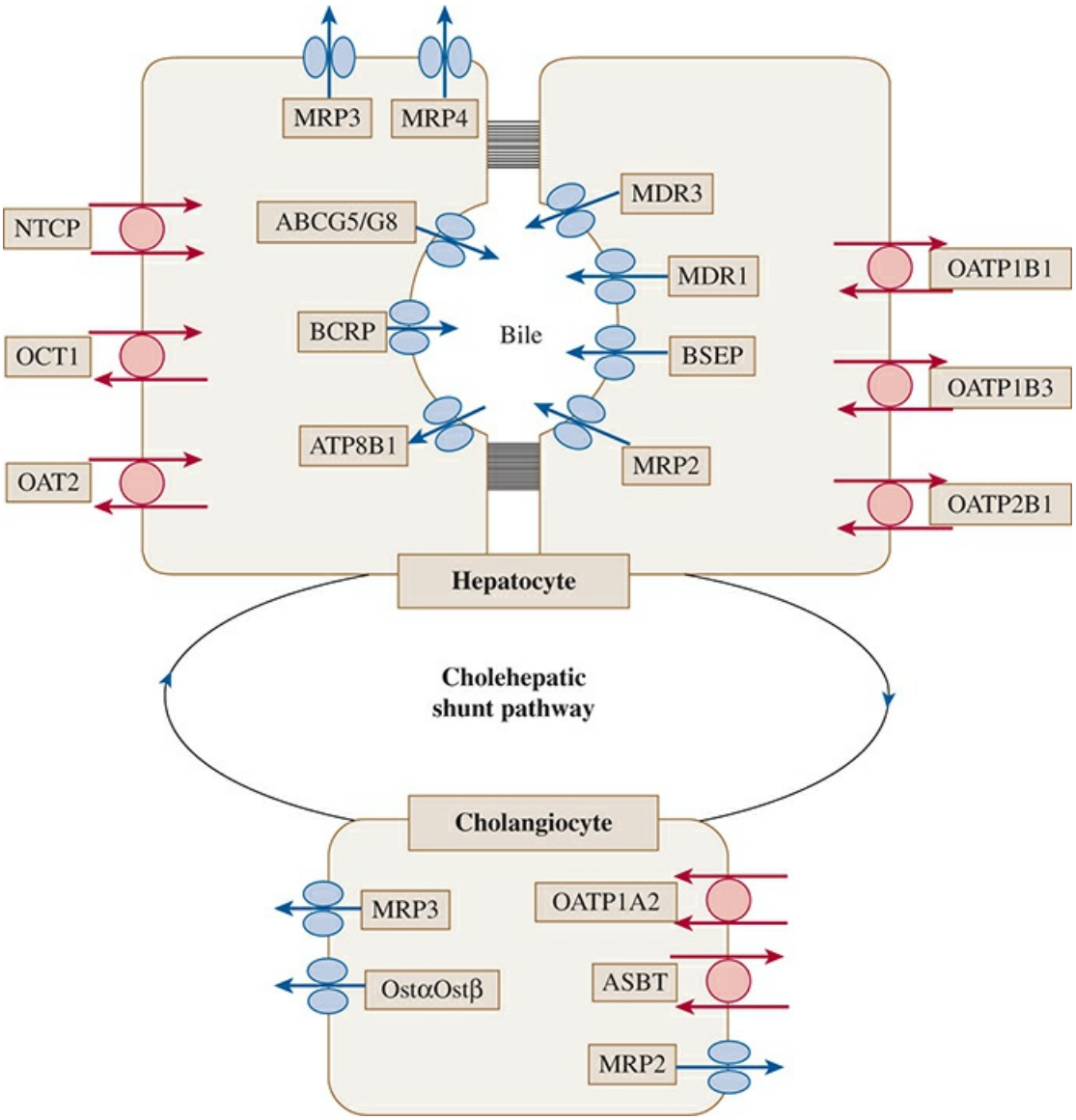

FIGURE 13–3 *Transport proteins in human hepatocytes and cholangiocytes.* Exporters relevant to canalicular secretion of toxic chemicals and their metabolites are the canalicular multiple organic anion transporter (MOAT) system and the multiple-drug resistant (MDR) P-glycoproteins. Note: MDR3 (ABCB4) flops phosphatidylcholine from the inner to the outer leaflet of the canalicular membrane. Phosphatidylcholine can then be extracted by bile salts. ATP8B1 together with the accessory protein CDC50A flips phosphatidylserine from the outer to the inner membrane to maintain the lipid asymmetry of the canalicular membrane and to protect against bile acids.

Efflux transporters (blue symbols): BSEP, bile salt export pump; MDR, multidrug resistance protein; MRP, multidrug resistance–associated protein; ABCG5/8; BCRP, breast cancer resistance protein; Ostα/Ostβ.

Uptake transporters (red symbols): ASBT, apical sodium-dependent bile salt transporter; NTCP, sodium taurocholate cotransporting polypeptide; OATP, organic anion–transporting polypeptide; OCT, organic cation transporter; OAT, organic anion transporter. Transporters

localized to the sinusoidal membrane extract solutes from the blood. Exporters localized to the canalicular membrane move solutes into the lumen of the canaliculus. (Reprinted with permission from Pauli-Magnus C, Meier PJ. Hepatobiliary transporters and drug-induced cholestasis. *Hepatology*. 2006;44:778–787.)

All unconjugated bile acids in HPCs are conjugated before being transported by the bile salt export pump (BSEP) across the canalicular (apical) membrane. Bile acid excretion is a major driving force of bile fluid formation (i.e., bile salt-dependent bile flow). Other solutes in bile are transported by members of the multidrug resistance (MDR) family such as MDR3 (ABCC2) that transports phospholipids, and the heterodimeric transporters ABCG5/ABCG8 that transport cholesterol and plant sterols into bile. In addition, MRP2 transports GSH, which is the main compound responsible for the bile salt-independent bile flow, as well as sulfated and glucuronidated bile acids, glutathione disulfide, glutathione conjugates, bilirubin diglucuronide, and many other conjugated drugs and chemicals. Other transport systems of the canalicular membrane include the breast cancer resistance protein (BCRP; ABCG2), which can contribute to the biliary excretion of bile acids and xenobiotics.

Canalicular lumen bile is propelled forward into larger channels by dynamic, ATP-dependent contractions of the pericanalicular cytoskeleton. The biliary epithelial cells that comprise bile ducts modify bile by absorption and secretion of solutes. Bile acids are taken up into biliary epithelial cells (cholangiocytes) by OATP1A2 (sodium-independent uptake) and by the sodium-dependent bile acid transporter ASBT (Fig. 13–3), and then secreted on the basolateral side mainly by MRP3 and heterodimeric organic solute transporter (OSTα/OSTβ). The bile acids secreted from biliary epithelial cells return to the portal circulation via the peribiliary plexus (cholehepatic shunt pathway). Biliary epithelial cells also express phase I and phase II xenobiotic metabolizing enzymes, which can contribute to the biotransformation of chemicals present in bile.

Secretion into bile ducts is usually, but not always, a prelude to toxicant clearance by excretion in feces. Exceptions occur when compounds undergo enterohepatic cycling. Secretion of toxic metabolites into the bile can be a prelude to the development of injury in extrahepatic tissues.

LIVER PATHOPHYSIOLOGY: TYPES OF LIVER RESPONSES TO CHEMICALS

Liver injury can arise from exposure to many types of chemicals, including drugs, environmental pollutants, occupational chemicals, plant toxins, and others. Major adverse responses of the liver are listed in Table 13–2.

TABLE 13–2 Types of Liver Responses to Xenobiotic Agents

Response	Representative Toxicants
Hepatocyte death	Acetaminophen, allyl alcohol, Cu, dimethylformamide, ethanol
Fatty liver (steatosis)	Amiodarone, CCl_4, ethanol, fialuridine, tamoxifen, valproic acid
Canalicular cholestasis	Chlorpromazine, cyclosporin A, 1,1-dichloroethylene, estrogens, Mn, phalloidin
Bile duct damage	Alpha-naphthylisothiocyanate, amoxicillin, methylenedianiline, sporidesmin
Sinusoidal endothelial damage	Cyclophosphamide, microcystin, pyrrolizidine alkaloids
Inflammation	Bacterial endotoxin, ethanol
Liver regeneration	Acetaminophen, ethanol, estrogens
Fibrosis and cirrhosis	CCl_4, ethanol, thioacetamide, vitamin A, vinyl chloride
Liver cancers	Aflatoxin, androgens, arsenic, thorium dioxide, vinyl chloride

Cell Death

Cell death from chemical exposure may occur by several different molecular pathways including oncotic necrosis, apoptosis, pyroptosis, necroptosis, etc. *Oncotic necrosis*, often referred to simply as "necrosis," is characterized by cell swelling, leakage of cellular contents, nuclear disintegration (karyolysis), and an influx of inflammatory cells. Cell contents released during oncotic necrosis include intracellular enzymes such as alanine aminotransferase (ALT) and aspartate amino transferase (AST). *Apoptosis* is characterized morphologically by cell shrinkage, chromatin condensation, nuclear fragmentation, and formation of membrane-bound cell fragments termed "apoptotic bodies." Because the latter are phagocytosed and digested by Kupffer cells or other neighboring cells, apoptosis is often not accompanied by an inflammatory response.

In the extrinsic pathway of apoptosis, ligands (e.g., Fas ligand, TNF-α) bind to their respective death receptor (Fas receptor, TNF receptor type I), which triggers assembly of the death-inducing signaling complex (DISC) that activates initiator caspases (caspase-8 or -10). In HPCs, the active initiator caspase cleaves Bid, and the truncated Bid translocates together with other Bcl-2 family members to the mitochondria. These proteins form pores in the outer membrane of the mitochondria and cause the release of intermembrane proteins such as cytochrome *c* and the second mitochondria-derived activator of caspases (Smac). Cytochrome *c*, together with apoptosis protease activating factor-1 (APAF-1), ATP, and procaspase-9, form the apoptosome, causing the activation of caspase-9, which then processes (and activates) downstream effector caspases, for example, caspase-3 and caspase-7. The effector caspases can

propagate the apoptotic signal by activating caspase-activated DNase (CAD) to initiate nuclear DNA fragmentation and by cleaving numerous cellular proteins critical to cell function and to the structural integrity of the cell and its nucleus. In addition to the direct propagation of the apoptosis signal by mitochondrial cytochrome *c* release, the simultaneous release of Smac ensures that the cytosolic inhibitors of apoptosis proteins (IAPs) are inactivated and do not interfere with the promotion of apoptosis. Thus, mitochondria are an important part of the extrinsic (receptor-mediated) apoptotic signal transduction pathway in liver cells after most stimuli.

The intrinsic or mitochondrial pathway of apoptosis is initiated independent of the TNF receptor family, caspase-8 activation, and formation of the DISC. Despite the upstream differences, the postmitochondrial effects are similar to the extrinsic pathway. The intrinsic pathway is generally triggered by a cytotoxic stress or DNA damage, which activates the tumor suppressor p53. This protein acts as a transcription factor to promote the formation of proapoptotic Bcl-2 family members, for example, Bax. The increased Bax translocation to the mitochondria induces the release of mitochondrial intermembrane proteins including cytochrome *c*, Smac, endonuclease G, and apoptosis-inducing factor (AIF).

Programmed necrosis termed “necroptosis” is generally initiated by death receptors, for example, TNF receptor 1, and the formation of complex 1 with various adapter molecules including receptor-interacting protein kinases 1 and 3 (RIP1 and RIP3). If caspase-8 is activated, it will cleave RIP1 and RIP3, and apoptosis will be initiated. However, if caspase-8 is inhibited, RIP1 and RIP3 activate a caspase-independent execution mechanism involving the phosphorylation of mixed-lineage kinase domain-like pseudokinase (MLKL), which then translocates to the cell membrane and forms pores.

Fatty Liver (Steatosis)

Fatty liver (steatosis) is an increased lipid (mainly triglyceride) content of HPCs. Free fatty acids (FFAs) are synthesized in HPCs mainly from carbohydrate-derived acetyl-coenzyme A and from the hydrolysis of absorbed fat. Additionally, adipocytes can release FFAs into the circulation. Once in the cytosol of HPCs, FFAs can be imported into mitochondria for degradation by β-oxidation or can be esterified into triglycerides for incorporation into very low-density lipoproteins (VLDL). FFA uptake into mitochondria depends on the activity of the mitochondrial carnitine palmitoyl transferase 1.

Although the most common cause of hepatic steatosis is insulin resistance associated with obesity and sedentary lifestyle, many hepatotoxicants can induce steatosis. Chemicals producing steatosis associated with lethality include valproic acid, fialuridine, carbon tetrachloride, ethionine, puromycin, and cycloheximide. Drugs that inhibit β-oxidation and mitochondrial respiration and are associated with steatosis include 4,4′-diethylaminoethoxyhexestrol, amiodarone, tamoxifen, perhexiline, amineptine, doxycycline, tetracycline, tianeptine, and pirprofen. In addition, drugs such as amineptine, amiodarone, tetracycline, pirprofen, and tianeptine can directly inhibit microsomal triglyceride transfer protein, which lipidates apolipoprotein B to form triglyceride-rich VLDL particles. Drugs with this dual effect on β-oxidation and VLDL secretion are generally highly steatogenic.

Canalicular Cholestasis

Cholestasis is characterized by elevated serum concentrations of bile salts and bilirubin. This form of liver dysfunction is defined as a decrease in bile formation or an impaired secretion of specific solutes into bile. Accumulation of the yellowish bilirubin pigment in skin and eyes produces jaundice. Excess bilirubin causes urine to become darker yellow or brown. Histological features of cholestasis can include dilation of bile canaliculi and the presence of bile plugs in bile ducts and canaliculi. Toxicant-induced cholestasis can be associated with cell swelling, cell death, and inflammation.

Molecular mechanisms of cholestasis are related to expression and function of transporter systems in the basolateral and canalicular membranes of HPCs. In principle, increased hepatic uptake, decreased biliary excretion, and increased biliary reabsorption (cholehepatic shunting) of a xenobiotic agent can contribute to its accumulation in the liver. For example, basolateral uptake by OATPs can contribute to the liver injury from phalloidin, microcystin, and amanitin. Inhibition of BSEP by rifampicin, bosentan, and troglitazone leads to accumulation of bile acids. Estrogen and progesterone metabolites inhibit BSEP from the canalicular side after excretion by MRP2. Inflammation caused by bile acid accumulation in the liver plays a major role in liver injury from some forms of cholestasis.

Increased bile acid concentrations in liver can trigger compensatory mechanisms that limit cholestatic injury. Bile acids activate the nuclear farnesoid X receptor (FXR), which stimulates the small heterodimer partner (SHP) that downregulates NTCP expression to limit bile acid uptake. In addition, FXR activation increases expression of BSEP and MDR3, which enhances bile acids and phospholipid transport into the canaliculus. Furthermore, the FXR-independent upregulation of the basolateral transporters MRP3 and MRP4 reduces intracellular bile acid and drug concentrations. Agonists of the constitutive androstane receptor (CAR) and pregnane X receptor (PXR) can induce MRP3 and MRP4 expression, resulting in enhanced export of bile acids during cholestasis. In biliary epithelial cells, OSTα/OSTβ is upregulated at the basolateral membrane during cholestasis, which mediates the enhanced return of bile acids from bile to the plasma. Thus, such adaptation of transporter expression may counteract some detrimental effects of cholestasis.

Bile Duct Damage

Damage to the intrahepatic bile ducts, or *cholangiodestructive cholestasis*, may be noted as a sharp elevation in serum alkaline phosphatase (ALP) activity. Serum levels of bile acids and bilirubin are elevated, as observed with canalicular cholestasis. Initial lesions following a single exposure to cholangiodestructive chemicals include swollen biliary epithelium, debris from damaged cells within ductal lumens, and inflammatory cell infiltration of portal tracts. Chronic administration of toxicants that cause bile duct destruction can lead to bile duct proliferation and fibrosis resembling primary biliary cholangitis (PBC).

Sinusoidal Endothelial Cell Damage

The sinusoid is a specialized capillary lined with endothelium with numerous fenestrae (holes) that allow for high permeability. Functional integrity of the sinusoid can be compromised by dilation/blockade of its lumen and by destruction of sinusoidal endothelial cells (SECs). Dilation of the sinusoid occurs when the downstream flow of blood is impeded. The rare condition of

primary dilation, known as *peliosis hepatis*, has been associated with exposure to anabolic steroids and danazol. Blockade can occur when the fenestrae enlarge to such an extent that red blood cells become caught in them or pass through with entrapment in the interstitial space of Disse. Gaps between endothelial cells can occur after exposure to acetaminophen, galactosamine/endotoxin, or an anti-Fas antibody. A consequence of SEC injury is the loss of barrier function with extensive blood accumulation in the liver parenchyma (i.e., hemorrhage).

Disruption of SECs is an early structural feature of the vascular disorder sinusoidal obstruction syndrome. Swelling and/or proliferation of SECs progresses to nonthrombotic occlusion of hepatic venules with cell debris and fibrotic material. The resulting congestion and medial hypertrophy of vessels contribute to progressive necrosis of parenchymal tissue and fibrosis. Pyrrolizidine alkaloid plant toxins cause pronounced SEC destruction and sinusoidal obstruction.

Inflammation

Injury-induced inflammatory response activates the innate immune system and involves circulating blood cells as well as the resident Kupffer cells, NK, NKT, and innate lymphoid cells (Fig. 13–4). Activated coagulation and complement cascades and alterations in microvascular function are also components of an acute inflammatory response. Accumulation and activation of platelets, neutrophils, lymphocytes, and monocytes within the damaged liver are well-recognized features of hepatotoxicity produced by many chemicals.

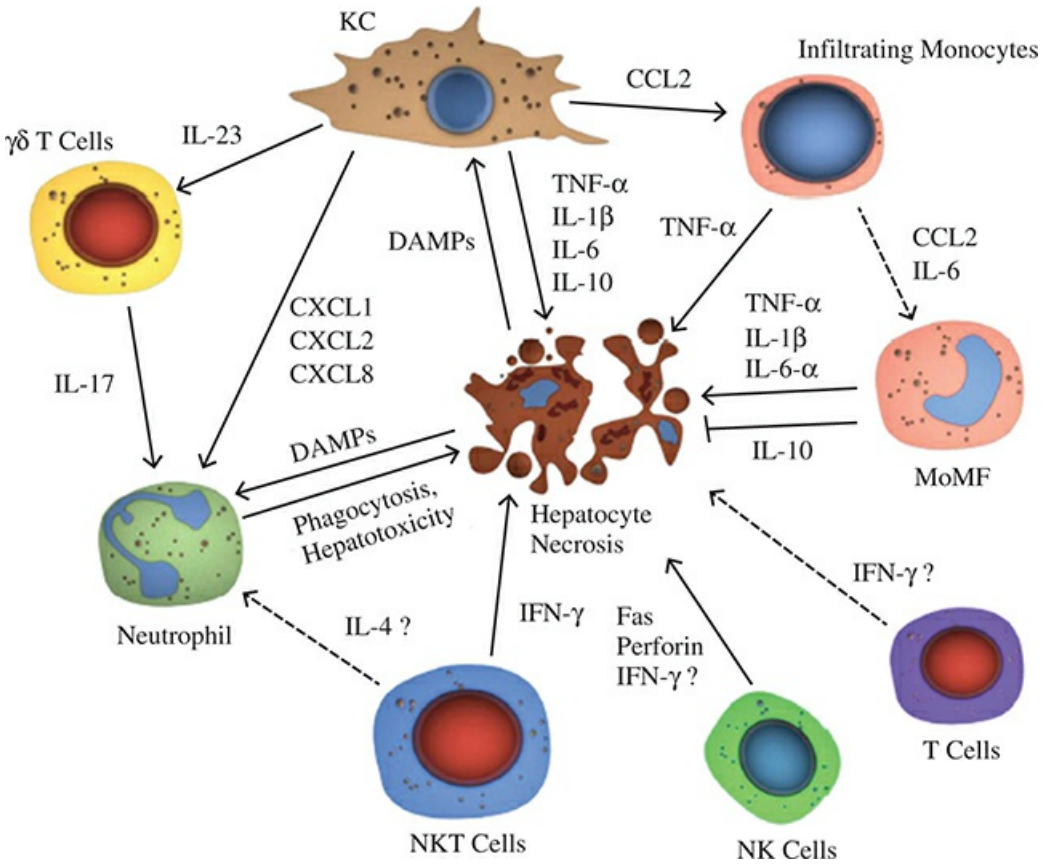

FIGURE 13–4 *Activation and crosstalk among resident and accumulating inflammatory cells in hepatotoxic responses.* Some immune cells such as Kupffer cells, natural killer (NK) cells, and stellate cells reside in healthy liver tissue. Others such as neutrophils, T cells, NK cells, and infiltrating monocytes (which differentiate in liver into monocyte-derived macrophages [MoMFs]) reach the liver via the blood and accumulate there upon exposure to inflammatory mediators such as chemokines (e.g., CXCLs, CCLs). Hepatocyte necrosis is associated with the release of damage-associated molecular patterns (DAMPs; e.g., HMGB1, Hsp70, free DNA), which are recognized by pattern recognition receptors (e.g., toll-like receptors) on Kupffer cells and neutrophils, resulting in activation of these cells and expression of cytokines and chemokines. Elaboration of the hepatic inflammatory response occurs through the recruitment and further activation of monocytes and other immune cells including various subsets of T cells, NK cells, and NKT cells. Coordinated amplification and tailoring of the hepatic inflammatory response is driven by cytokines and other inflammatory mediators produced by this collage of cell types. Depending on the context of hepatocellular injury and the timing, an inflammatory response can either exacerbate liver damage or contribute to repair processes. (Reprinted with permission from Krenkel O, Mossanen JC, Tacke F. Immune mechanisms in acetaminophen-induced acute liver failure. *Hepatobiliary Surg Nutr*. 2014;3(6):331-43.)

The mechanisms responsible for triggering inflammation within the injured liver are not fully understood. However, release of damage associated molecular pattern molecules (DAMPs; e.g., high mobility group box 1 [HMGB1]) from injured HPCs stimulates toll-like receptors on inflammatory cells. Lipopolysaccharide (LPS) from the intestine and other pathogen-associated molecular pattern molecules (PAMPs) act at toll-like and other cellular receptors to activate Kupffer cells and other sinusoidal cells. PAMPs also arise from bacterial or viral infections.

Kupffer cells and neutrophils are phagocytes that have a vital function in host defense and removal of cell debris. Production of reactive oxygen species by NADPH oxidase is a critical tool used by these cells for eliminating pathogens, but it can also contribute to damage of liver cells. Upon activation, Kupffer cells generate mainly hydrogen peroxide, which can diffuse into neighboring liver cells creating intracellular oxidant stress ultimately causing cellular injury. Kupffer cells generate inflammatory mediators such as cytokines and prostanoids that can aggravate injury by recruiting neutrophils into the liver; activation of neutrophils can cause cell death in susceptible HPCs. However, Kupffer cells can also generate anti-inflammatory mediators, such as prostaglandin E_2 and interleukin-10 that downregulate production of pro-inflammatory cytokines and thereby attenuate inflammatory liver injury. Thus, Kupffer cells can promote progression of injury or inhibit it by assisting in removal of cell debris and apoptotic bodies by phagocytosis.

Neutrophils in liver vasculature can become activated in response to bacterial infection or extensive cell injury. These cells remove bacteria and cell debris partly through interactions with resident macrophages. Neutrophils generate the potent oxidant and chlorinating species, hypochlorous acid, through the combined activities of NADPH oxidase and myeloperoxidase. The capability of neutrophils to migrate out of the vasculature, adhere to target cells, and generate potent cytotoxic agents renders this leukocyte an effective killer of invading microorganisms and a remover of dead or dying cells. However, if directed against viable liver cells, these cells can promote additional tissue injury via a multistep process: inflammatory mediators upregulate adhesion molecules such as Mac-1 (CD11b/CD18) on the cell surface

causing neutrophils to accumulate in liver sinusoids and prime them for reactive oxygen formation. If a chemotactic signal is received from the parenchyma, neutrophils will extravasate, adhere to the target, and become fully activated to release oxidants and proteolytic enzymes. Neutrophils are involved in liver injury from ischemia-reperfusion, alcohol, α-naphthylisothiocyanate, halothane, etc.

Regeneration and Repair

The liver has a high capacity to restore lost tissue and function by regeneration. Loss of HPCs due to hepatectomy triggers proliferation of all mature liver cells and restores the original liver mass. HPCs are normally quiescent in the G_0 phase of the cell cycle. Both cytokines and growth factors are involved in the activation of transcription factors that drive expression of cell cycle-regulating proteins. The coordinated expression of individual cyclins and inhibitors of CDKs guides HPCs through the various phases of the cell cycle. If HPC replication is blocked, liver regeneration relies on hepatic stem cells or oval cells, which proliferate and differentiate to replace lost parenchyma.

Fibrosis

Hepatic scaring occurs in response to chronic liver injury that overwhelms the capacity of the organ to repair. It is characterized by the accumulation of excessive fibrous tissue, specifically fibril-forming collagen types I and III, and a decrease in normal plasma membrane collagen type IV. Fibrosis can develop around central veins, portal tracts, or within the space of Disse. This progressive collagen deposition, marked by interconnecting fibrous scars, alters the hepatic cytoarchitecture. When the fibrous scars subdivide the remaining liver mass into nodules of regenerating HPCs, fibrosis has progressed to cirrhosis, and the liver has limited capacity to perform essential functions. The excessive extracellular matrix protein deposition and loss of sinusoidal endothelial cell fenestrae and of HPC microvilli limit exchange of nutrients and waste material between HPCs and sinusoidal blood.

The primary cause of hepatic fibrosis/cirrhosis in humans worldwide is viral hepatitis; however, biliary obstruction and alcoholic and nonalcoholic steatohepatitis are of growing concern. In addition, fibrosis can be induced by chronic exposure to carbon tetrachloride, thioacetamide, dimethylnitrosamine, aflatoxin, or other chemicals. Indeed, any chronic insult to the liver resulting in a rate of HPC death that exceeds the ability of the liver to repair itself can result in fibrosis.

Central to the development of fibrosis is the migration and activation of hepatic stellate cells and their differentiation into myofibroblast-like cells. Thus, stellate cells become the main cell type producing extracellular matrix proteins in the liver. Stellate cell activation is initiated by products formed during liver cell injury. For example, activating signals can be reactive oxygen species and lipid peroxidation products generated in injured HPCs. Kupffer cells can release reactive oxygen and pro-inflammatory cytokines during phagocytosis of cell debris or apoptotic bodies, thereby recruiting more inflammatory cells and enhancing the injury and oxidant stress. Stellate cell activation is also driven by the excessive production of extracellular matrix proteins induced mainly by TGF-β1. Damaged sinusoidal endothelial cells contribute to the activation of stellate cells by generating a splice variant of cellular fibronectin and by releasing urokinase-type

plasminogen activator, which processes latent TGF-β1. Furthermore, platelets at the site of injury can produce TGF-β1 and PDGF.

Upon activation, stellate cells undergo phenotypic changes that involve proliferation, fibrogenesis, matrix remodeling, and pro-inflammatory mediator expression, including production of chemotaxins for inflammatory cells. There is enhanced contractility due to the increased expression of α-smooth muscle actin. Increased expression of endothelin-1 (ET-1) receptors on stellate cells together with the general imbalance between vasodilator (nitric oxide, carbon monoxide) and vasoconstrictor (endothelin-1) formation contributes to the development of portal hypertension during fibrosis. During fibrogenesis, there is a qualitative change from the basement membrane-like matrix dominated by nonfibril-forming collagens (types IV, VI, and XIV) to one involving fibril-forming collagen types I and III. This effect involves the differential expression and release of matrix metalloproteinases (MMPs) and their inhibitors (tissue inhibitor of metalloproteinase [TIMPs]) from stellate and Kupffer cells. TIMP1 and TIMP2 are upregulated and MMP1 (collagenase I) is downregulated during fibrogenesis, leading to the reduced degradation of fibril-forming collagens (e.g., type I collagen). At the same time, MMP2 and MMP9 (collagenase IV) are activated, causing the accelerated degradation of nonfibril-forming collagens. The end result is enhanced deposition of fibril-forming collagens that characterize fibrosis.

Liver Cancers

Hepatocellular cancer has been linked to chronic abuse of androgens, alcohol, and consumption of aflatoxin-contaminated diets. In addition, viral hepatitis, metabolic diseases such as hemochromatosis, α_1-antitrypsin deficiency, and nonalcoholic steatohepatitis are major risk factors for hepatocellular carcinoma. A pronounced synergy between dietary aflatoxin exposure and hepatitis B infection is well recognized. The prevalence of hepatitis B and C viruses and environmental factors make hepatocellular carcinoma a common malignant tumor worldwide.

Nonparenchymal cells are the precursors for other types of cancers. Rare, highly malignant angiosarcomas are derived from sinusoidal lining cells and are associated with occupational exposure to vinyl chloride and arsenic. Exposure to radioactive thorium dioxide used as contrast medium for radiology (Thorotrast) has been linked to tumors derived from HPCs, sinusoidal cells, and bile duct cells (cholangiocarcinoma).

CLASSIFICATION OF LIVER INJURY

Liver injury can be characterized based on morphology (histopathology), serum or plasma biomarkers (clinical chemistry), or the nature of the toxic response (intrinsic vs. idiosyncratic). Each of these provides a different conceptual framework for classifying liver injury.

Histopathology

In experimental studies with animals, histopathological evaluation remains the “gold standard” for characterizing liver injury. Histopathology of liver biopsies is sometimes used to aid in diagnosis of liver injury in human patients, but only under certain circumstances because the

procedure involves some risk to the patient. Many toxicants, especially those that are metabolized to more toxic metabolites, cause a centrilobular necrosis because enzymes that bioactivate many chemicals to toxic metabolites occur in greater concentration in HPCs residing in centrilobular (zone 3) regions. Other determinants of zonal HPC heterogeneity such as supply of oxygen and concentration of cofactors for metabolism (e.g., NADPH, glutathione) can also contribute to distribution of lesions. Xenobiotic agents that cause centrilobular lesions include halothane, pyrrolizidine alkaloid plant toxins, carbon tetrachloride, acetaminophen, and many other chemicals. In contrast, liver lesions from chemicals such as aflatoxin B1 and allyl alcohol occur in periportal regions, whereas midzonal lesions predominate after systemic exposure to bacterial endotoxin.

Clinical Chemistry

When the liver is injured, various molecular constituents of damaged cells can be quantified in plasma or serum samples and serve as biomarkers that track the severity of ongoing liver injury. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are released into the plasma when HPCs are injured by chemicals or by liver diseases. Other enzymes that occur selectively in HPCs include mitochondrial glutamate dehydrogenase (GLDH), ornithine carbamoyltransferase (OCT), and various dehydrogenases (e.g., lactate dehydrogenase [LDH]). There are differences in these enzyme biomarkers of HPC injury with respect to their concentrations along the sinusoid, organellar associations, longevity in the plasma, occurrence in different species, and selective presence in liver relative to other organs. For example, ALT occurs relatively selectively in liver, whereas AST occurs also in muscle and other tissues; therefore, AST is less selective as a biomarker of HPC injury. There is interest in liver-selective microRNAs (e.g., miR-122), high mobility group box-1 (HMGB1) protein, and keratin-18 in plasma as HPC injury biomarkers.

Bilirubin undergoes uptake into HPCs, where it is conjugated with glucuronic acid and secreted into the bile by MRP2. Reflux of conjugated bilirubin into the plasma occurs during HPC injury or biliary obstruction. Reflux of bile acids (e.g., cholic acid, chenodeoxycholic acid) into the plasma can provide a biomarker of cholestasis. Increased serum alkaline phosphatase (ALP) activity is a biomarker of hepatobiliary injury. Gamma-glutamyl transferase (GGT) is localized to the plasma membrane of HPCs and biliary epithelial cells. Increases in plasma GGT activity are used in the diagnosis of biliary injury and to help confirm a liver source of ALP increase. 5′-Nucleotidase (5NT) is also localized to the canalicular and sinusoidal membranes of HPCs and released into plasma during liver injury. Notably, all of these enzymes occur in other organs and are therefore not specific for detection of liver injury.

Intrinsic versus Idiosyncratic Responses

“Intrinsic” toxic responses (aka, Type A responses or “toxic hepatitis”) are those that have a well-defined dose response relationship and injury is produced in all individuals. There is often some difference in sensitivity among animals or among humans, but all individuals respond with toxicity at some dose. Intrinsic liver toxicity is associated with distinctive liver lesions that depend on the specific chemical and that occur after a predictable latent period. These reactions are initiated by a direct effect of the chemical or its metabolite(s) and are reproducible in experimental animals.

"Idiosyncratic" drug reactions (Type B reactions) are adverse responses that occur in a minority of patients taking therapeutic doses of a drug, and the liver is frequently the target organ. Typically, these reactions occur only in a very small fraction of patients during drug therapy. With these reactions, the relationship to dose is usually unclear. It is likely that they are dose-related within individuals but that a relationship with dose is not apparent in populations because of the wide disparity in susceptibility among individuals. Unlike intrinsic hepatotoxicity, idiosyncratic, drug-induced liver injury (IDILI) is associated with variable liver pathology. Table 13–3 contrasts the characteristics of intrinsic and idiosyncratic reactions.

TABLE 13–3 Characteristics of Intrinsic and Idiosyncratic Hepatotoxicity

"Intrinsic" Toxicity (Type A)	"Idiosyncratic" Toxicity (Type B)
• With drugs, usually occurs in overdose • Distinctive lesion • Dose-related • Injury in all individuals • Predictable latent period • Caused by "direct" effect of agent or its metabolite • Reproducible in animal models	• Occurs at pharmacologic treatment regimens • Variable pathology • Dose relationship unclear • Susceptible (small) fraction of people • Relationship of exposure to onset of toxicity variable • Highly influenced by host contribution (e.g., immune response, metabolism polymorphisms, etc.) • Not reproducible in typical preclinical animal tests

INITIATION AND PROGRESSION OF HEPATOTOXICITY

The pathogenesis of chemical-induced liver injury comprises molecular initiating events that trigger other events within and outside of HPCs that result in progression to HPC death and to liver dysfunction (Fig. 13–5).

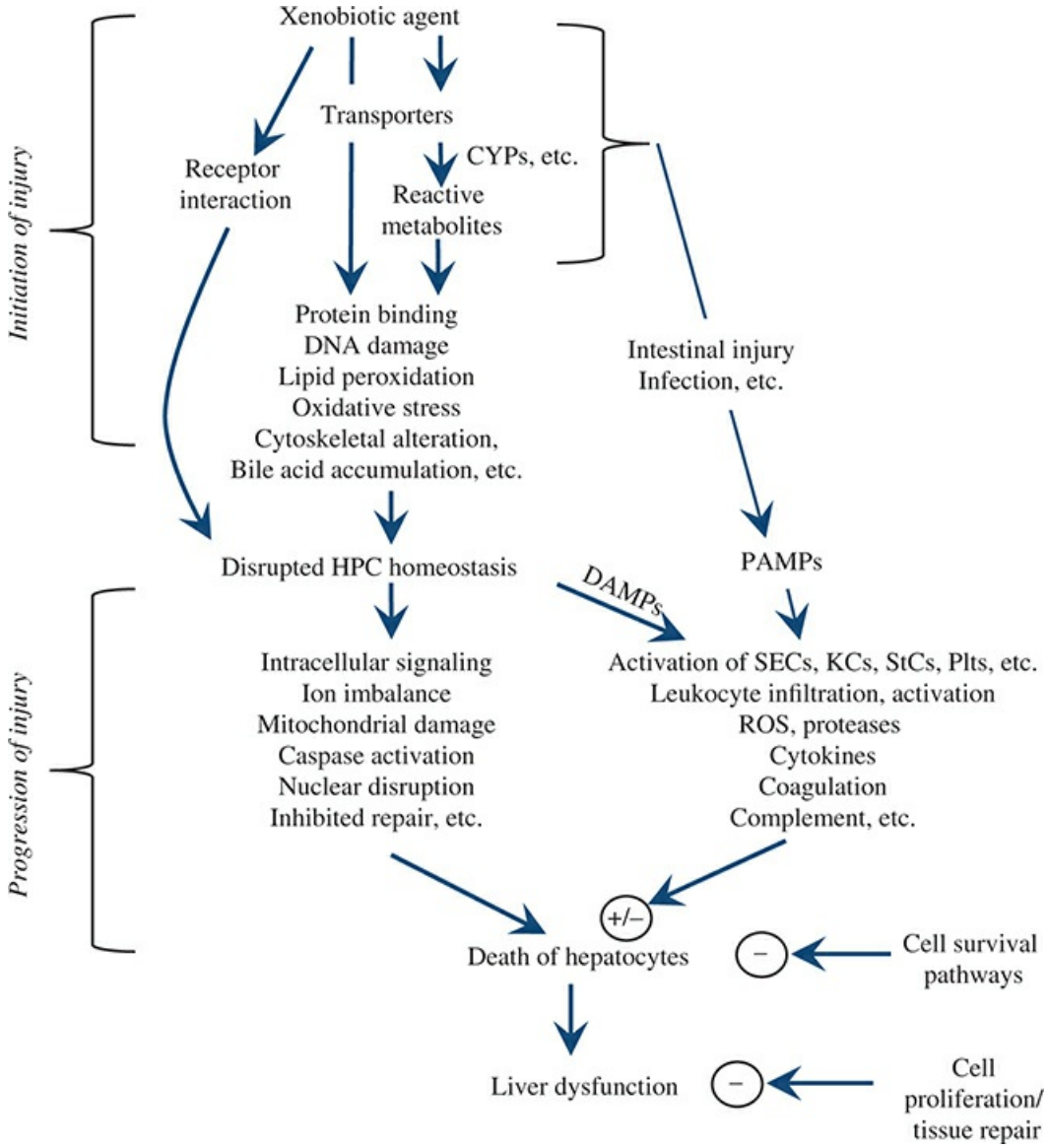

FIGURE 13–5 *Initiation and progression of hepatotoxicity.* A xenobiotic agent (drug, pollutant, etc.) or its metabolite(s) causes a molecular interaction with hepatocytes that is the initiating event in liver injury. This is followed by numerous events within hepatocytes (left) and/or within sinusoids (extrahepatocellular, right) that promote progression of injury. Accordingly, numerous mechanisms can contribute to hepatotoxicity from a single chemical. Cellular survival and repair mechanisms as well as extracellular events can modulate injury. Depending on the dose of the chemical, these complex and interdependent events can lead to recovery of tissue function or worsening of tissue injury. CYPs, cytochromes P450; HPC, hepatic parenchymal cell (hepatocyte); DAMP, damage-associated molecular pattern molecule; PAMP, pattern-associated molecular patternmolecule; SEC, sinusoidal endothelial cell; KC, Kupffer cell; StC, stellate cell; Plt, platelet; ROS, reactive oxygen species.

The initiation of injury typically begins with entry of a chemical into HPCs. Lipophilic compounds, particularly most drugs and environmental pollutants, readily diffuse into HPCs. Other toxicants are rapidly extracted from blood because they are substrates for uptake

transporters located on the sinusoidal membrane of HPCs. Once inside the cell, some toxicants exert their effects directly by binding reversibly to receptors or other proteins, whereas others are bioactivated to toxic metabolites. If the rate of production of a toxic metabolite exceeds the capacity of the HPC to detoxify it, HPC injury can ensue.

Initiating events often activates intracellular cell death signaling pathways. This culminates in cellular ion imbalance, mitochondrial dysfunction, altered gene expression, and/or DNA damage through activation of caspases or other mechanisms. Injured HPCs release factors that promote progression of liver injury such as calpain. Many so-called damage-associated molecular pattern molecules (DAMPs) released from injured HPCs activate pattern recognition receptors (e.g., toll-like receptors) present on nonparenchymal cells such as SECs, Kupffer cells, NK cells, and stellate cells. In addition, some hepatotoxic responses are associated with disturbance of the intestinal epithelium, which can result in the translocation into the portal circulation of pathogen-associated molecular pattern molecules (PAMPs; e.g., lipopolysaccharide, LPS) produced and released by intestinal flora. Like DAMPs, these PAMPs stimulate nonparenchymal cells, resulting in generation of ROS and NO and release of many inflammatory mediators including numerous cytokines, prostanoids, growth factors, proteases, etc. Expression of adhesion molecules and release of chemokines by activated nonparenchymal cells or by HPCs result in the infiltration into the liver of blood leukocytes and platelets. Activation of these cells prompts additional production of inflammatory mediators. In addition, the coagulation and complement cascades become activated.

EXAMPLES OF HEPATOTOXICANTS AND THEIR MECHANISMS OF ACTION

Acetaminophen

Acetaminophen (*N*-acetyl-*p*-aminophenol; APAP) overdose can cause severe liver injury and even liver failure in experimental animals and in humans. APAP-induced hepatotoxicity is a frequent cause of acute, drug-induced liver failure. APAP poisoning results in centrilobular hepatocellular necrosis in humans and in mice. At therapeutic doses, approximately 90% of APAP is conjugated with sulfate or glucuronide and excreted. The rest is metabolized by CYPs into a reactive, toxic metabolite, *N*-acetyl-*p*-benzoquinone imine (NAPQI). Most of the NAPQI is detoxified by conjugation with glutathione (GSH), thereby limiting its covalent binding to cellular proteins, which is the initiating event for HPC damage. The low levels of protein adducts formed after therapeutic doses are removed by autophagy.

After an overdose, overwhelmed sulfate and glucuronide conjugation pathways lead to break-through formation of large amounts of NAPQI, resulting in severe depletion of cellular GSH stores needed for NAPQI inactivation and thereby allowing extensive covalent binding of NAPQI to intracellular proteins (Fig. 13–6). Because protein binding can be prevented by conjugation of NAPQI with GSH, administration *N*-acetylcysteine (NAC) promotes the detoxification of NAPQI and limits cell injury. NAC treatment not only increases cytosolic GSH synthesis to detoxify NAPQI but also replenishes the depleted mitochondrial GSH, which scavenges reactive oxygen and peroxynitrite.

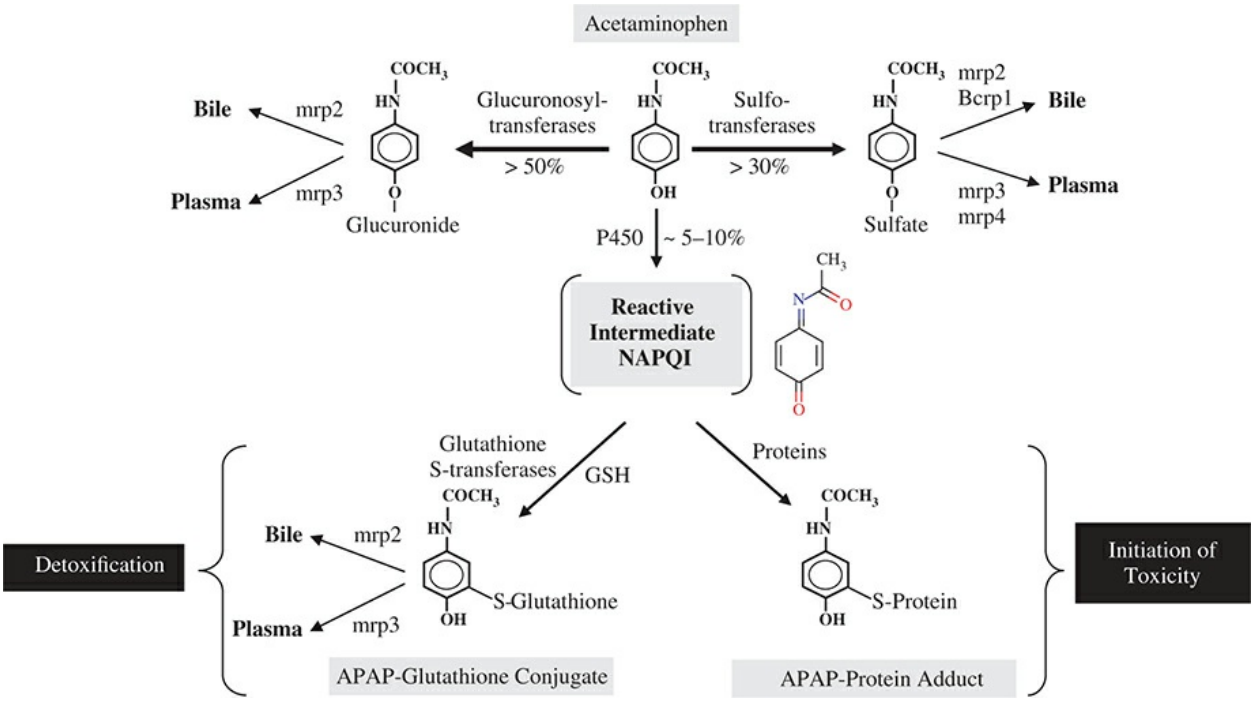

FIGURE 13–6 *Hepatic metabolism of acetaminophen.* At *therapeutic doses*, acetaminophen (*N*-acetyl-*p*-aminophenol) is metabolized in hepatocytes almost entirely by conjugation with sulfate and glucuronic acid (see percentages). These conjugates are transported into bile and into plasma for excretion by the kidneys. APAP is also deacetylated, but rapid reacetylation occurs in a "futile cycle," so that little *p*-aminophenol accumulates (not shown). A small fraction is metabolized by cytochromes P450 to *N*-acetyl-*p*-benzoquinone imine (NAPQI), which is detoxified by conjugation with glutathione (GSH). At *toxic overdoses*, the sulfate and glucuronide pathways are overwhelmed, leading to greater NAPQI formation. If enough of this reactive metabolite is formed, GSH is depleted, then NAPQI binds to intracellular proteins to initiate an hepatotoxic response.

Mitochondrial protein binding of NAPQI causes inhibition of mitochondrial respiration, mitochondrial oxidant stress and peroxynitrite formation, and declining ATP concentration in the liver (Fig. 13–7).

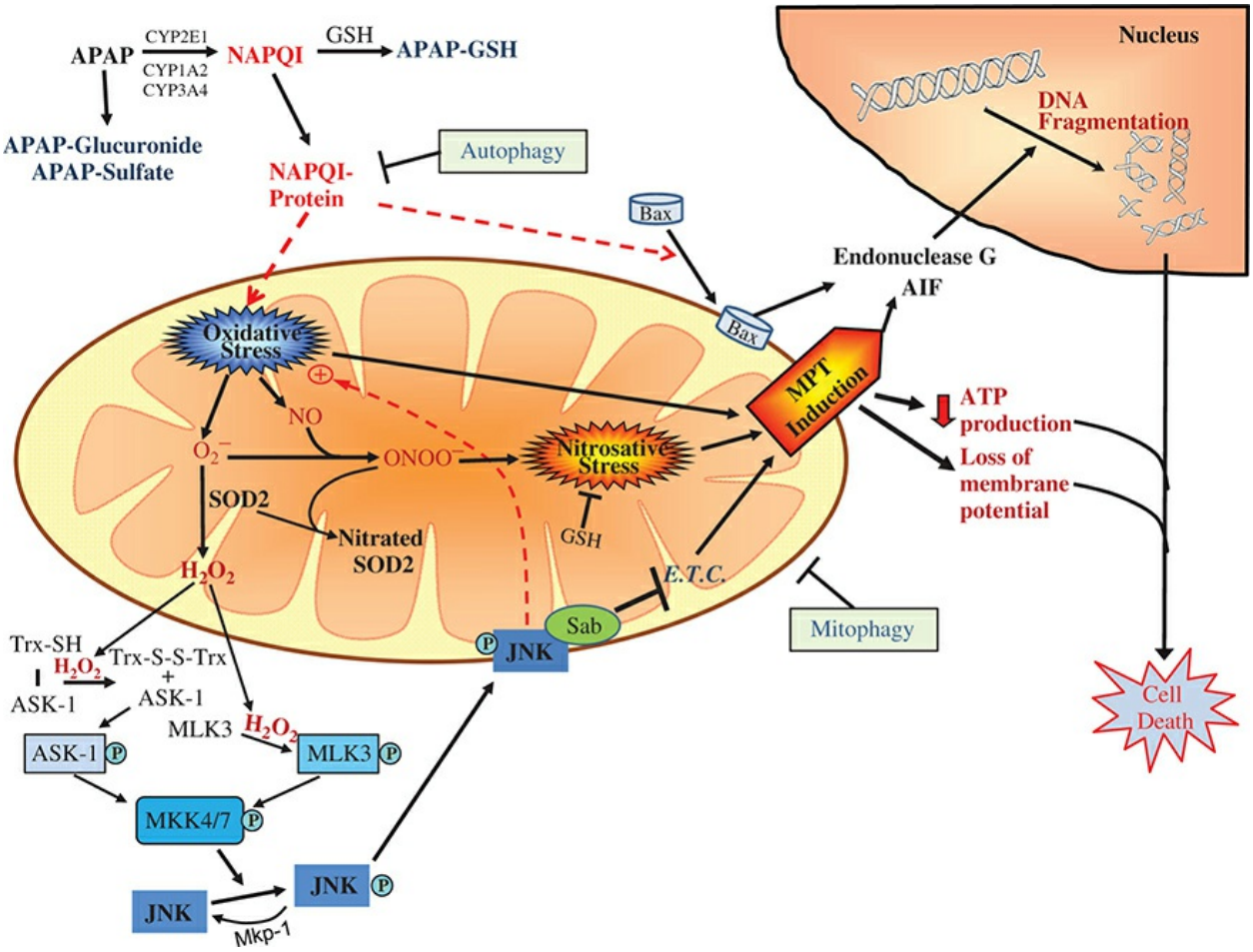

FIGURE 13–7 *Acetaminophen-induced cell death signaling in hepatocytes.* APAP is metabolized to reactive NAPQI, which binds to cellular proteins and initiates oxidative stress in mitochondria. This prompts activation of kinase signaling that results in phosphorylation of JNK, which translocates to the mitochondrial membrane where it interacts with Sab and inhibits the electron transport chain (E.T.C.) and enhances oxidative/nitrosative stress. The mitochondrial oxidative stress also results in the formation of peroxynitrite and consequent nitrosative stress. The combination of these events induces formation of the mitochondrial permeability transition (MPT) pore, which results in release of AIF and endonucleases that fragment nuclear DNA. These latter events are enhanced by the translocation of Bax to the outer mitochondrial membrane. In addition to DNA fragmentation, formation of the MPT pore results in reduced production of ATP and loss of mitochondrial membrane potential. Together, these events lead to compromised cell function and programmed necrosis. Depending on the magnitude of NAPQI-induced cell stress, autophagy and mitophagy can lead to cell survival, and GSH supplementation can also provide relief from the oxidative/nitrosative stresses. See text for abbreviations.

The early mitochondrial translocation of Bax and Bid, members of the Bcl-2 family of proteins, triggers mitochondrial pore formation and release of the mitochondrial intermembrane proteins endonuclease G and apoptosis-inducing factor (AIF). These endonucleases initiate nuclear DNA fragmentation. Continued exposure of GSH-depleted mitochondria to peroxynitrite

results in nitration of mitochondrial proteins and mitochondrial DNA modifications. The prolonged oxidant stress eventually triggers opening of the mitochondrial permeability transition (MPT) pore with breakdown of membrane potential, mitochondrial swelling, and rupture of the outer mitochondrial membrane. These events lead to the loss of mitochondrial ATP synthesis capacity, more extensive nuclear DNA fragmentation due to the amplified release of intermembrane proteins after the MPT, and eventually oncotic necrosis.

Inflammatory cells and hemostatic factors may not have singular roles in APAP-induced liver damage. The receipt of distinct extracellular cues can change the roles of these players from damaging to protective and vice versa. Additional understanding of how functions of inflammatory cells and blood elements can change under various circumstances might settle longstanding controversies about their roles in progression of injury and initiation of repair.

The multitude of events following the initial stress offers many opportunities for therapeutic interventions at later times during injury progression. Because these events do not occur in all cells to the same degree and at the same time, delayed interventions might not completely prevent cell damage but might limit the area of necrosis enough to allow tissue regeneration and prevent liver failure.

Ethanol

The early stage of ethanol abuse is associated with hepatic lipid accumulation (steatosis), which can progress to appreciable cell death with increasing hepatic inflammation (i.e., steatohepatitis). These pathologic processes drive replacement of functional liver mass with scar tissue, which impairs hepatic functions, including a progressive reduction in biotransformation capacity. Such hepatic dysfunction combined with defects in synthesis of key proteins, such as albumin and clotting factors, can ultimately drive multiple organ dysfunction and death.

Morbidity and mortality associated with the consumption of alcohol is mainly caused by the toxic effects of ethanol and its metabolites on the liver and on other tissues as well. More than 90% of a dose of ethanol is metabolized in the liver. The first and principal pathway of ethanol metabolism involves alcohol dehydrogenase (ADH), which oxidizes ethanol to acetaldehyde and the electrons are transferred to NAD^+, which leads to the production of NADH. Acetaldehyde is further oxidized to acetate in a NAD^+-dependent reaction by acetaldehyde dehydrogenase (ALDH). A second pathway of importance involves the alcohol-inducible enzyme, CYP2E1, which is also dependent on NAD^+ availability. The third pathway involves catalase contained in peroxisomes within HPCs in which ethanol functions as an electron donor for the reduction of hydrogen peroxide to water, as it is oxidized to acetaldehyde; less than 2% of an ethanol dose is metabolized through this pathway.

Hepatocellular steatosis is a common feature of chronic alcohol consumption as the excessive supply of acetate and NADH increases fatty acid synthesis. In addition, ethanol and acetaldehyde disrupt constitutive regulation of fatty acid metabolizing enzymes by impairing DNA binding of the transcription factor, peroxisome proliferator-activated receptor-α (PPAR-α). Ethanol exposure inhibits the transfer of triglycerides from liver to adipose tissue. Acetaldehyde inhibits the incorporation of triglycerides into VLDL and reduces VLDL release from HPCs by interfering with microtubular function.

Ethanol metabolism by CYP2E1 produces intracellular oxidant stress that can induce mitochondrial dysfunction and cell death of HPCs and activate stellate cells to promote fibrosis.

Gut-derived endotoxin and other bacteria-derived products leaking into the portal circulation from a leaky gut can activate Kupffer cells through toll-like receptor activation to produce reactive oxygen species and cytokines, such as TNF-α that can increase expression of inducible nitric oxide synthase (iNOS, NOS2) leading to the formation of peroxynitrite, a potent oxidant species capable of nitrating proteins. TNF-α can also directly promote cell death by acting on HPCs, which are rendered sensitive by ethanol-induced depletion of mitochondrial GSH. Inhibition of the proteasome pathway, a well-recognized feature of chronic alcohol exposure, can enhance chemokine formation in HPCs and promote inflammatory liver injury. Additional pro-inflammatory mediators and immune responses can be triggered by protein adducts of acetaldehyde and by lipid peroxidation products as well as by DAMPs released from HPCs and PAMPS (e.g., LPS) translocated from the intestine into the portal circulation (Fig. 13–8). Thus, alcoholic liver disease is a complex interplay between the activation of pro-cell death mechanisms (reactive metabolite formation, oxidant stress, protein adducts, and stimulation of pro-inflammatory and profibrotic innate immune responses) and activation of defense mechanisms (antioxidants, autophagy, and NK cell activation).

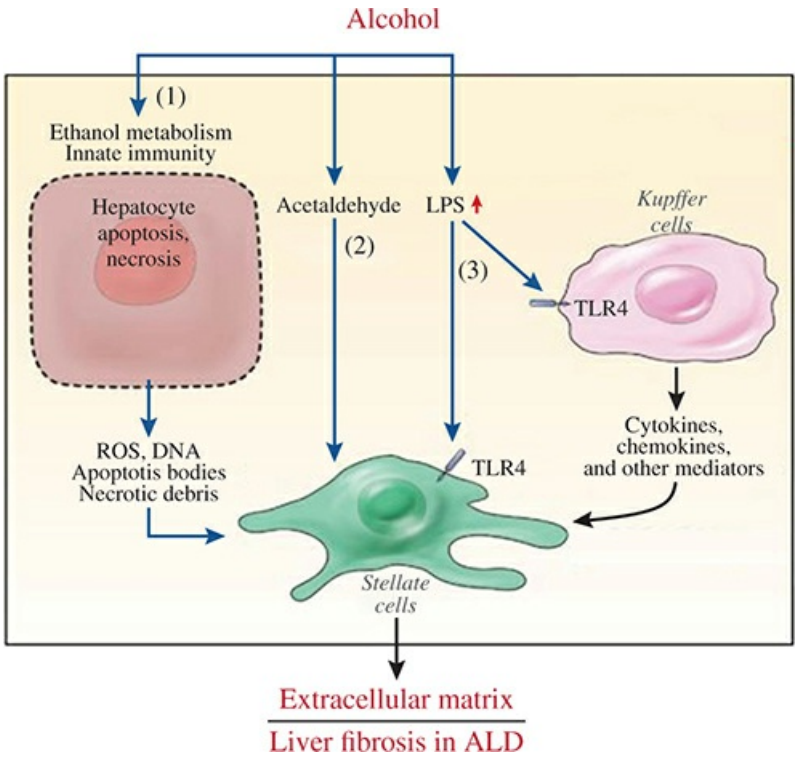

FIGURE 13–8 *Mechanisms of liver fibrosis in patients with alcoholic liver disease (ALD).* (1) Alcohol consumption causes hepatocyte damage, which leads to the release of a variety of mediators and the subsequent induction of stellate cell activation. (2) Acetaldehyde directly targets stellate cells and upregulates the expression of collagens by these cells. (3) Alcohol consumption results in elevation of gut-derived LPS levels in the liver. LPS can directly enhance stellate cell activation via upregulation of TGF-β signaling and can indirectly promote stellate cell activation via activation of Kupffer cells to release profibrotic cytokines and chemokines.

Allyl Alcohol

Allyl alcohol preferentially causes periportal (zone 1) hepatotoxicity that is produced by the ADH metabolite acrolein, a highly reactive aldehyde, which is oxidized further by ALDH to acrylic acid. The occurrence of allyl alcohol injury preferentially in zone 1 HPCs is caused by the predominant uptake of allyl alcohol in the periportal region and the oxygen dependence of the toxicity. Protein binding of the acrolein and subsequent adduct formation appears to be the main cause of liver cell death. Lipid peroxidation can become an important mechanism of cell injury under conditions of a compromised antioxidant status or in the presence of excess iron.

Carbon Tetrachloride

Acute exposure to CCl_4 causes centrilobular necrosis in animals and humans. Cytochrome P450-dependent conversion of CCl_4 to trichloromethyl free radical ($\bullet CCl_3$) and then to the trichloromethyl peroxyl radical ($CCl_3OO\bullet$) is a classic example of xenobiotic bioactivation to a free radical capable of initiating lipid peroxidation by abstracting hydrogen atoms from polyunsaturated fatty acids in phospholipid membranes. Metabolic activation of CCl_4 in vivo involves primarily CYP2E1. CCl_4-induced lipid peroxidation increases the permeability of the plasma membrane to Ca^{2+}, leading to significant mitochondrial damage, severe disturbances in calcium homeostasis, and consequent necrotic cell death. Kupffer cell activation can enhance liver injury via oxidant stress or TNF-α generation, which can lead to apoptosis.

Aflatoxins

Fungi of various species synthesize mycotoxins of several types that cause injury to numerous organs. Aflatoxins are mycotoxins produced by *Aspergillus* molds that grow on nuts and crops such as corn, wheat, and rice. Liver injury from acute toxic exposures (i.e., aflatoxicosis) has produced signs in humans including abdominal pain, emesis, steatosis, and necrosis. Biochemical alterations associated with necrosis of bile duct epithelial cells and parenchymal cells and bile duct proliferation have been documented in clinical and experimental studies in animals.

Although several aflatoxins occur in dietary sources, aflatoxin B1 (AFB1) occurs in the greatest concentration and in animal studies, it is one of the most potent mutagens and carcinogens known. All aflatoxins are metabolized in liver by oxidation, hydrolysis, reduction, and conjugation reactions, some of which lead to toxic products and others to inactive metabolites. Oxidation of the furan ring leads to a reactive epoxide that alkylates DNA and that can be further metabolized to a dialdehyde that alkylates proteins. The DNA adducts are thought to initiate liver cancer, whereas protein adducts appear to cause acute hepatotoxicity. Conjugation with GSH is an important pathway for detoxification of the reactive epoxide, which is also hydrolyzed both spontaneously and enzymically by epoxide hydrolases. Combinations of polymorphisms in several genes encoding enzymes that metabolize AFB1 are associated with increased risk of developing hepatocellular carcinoma.

Pyrrolizidine Alkaloids

Pyrrolizidine alkaloids are produced by many plant species throughout the world. Retrorsine, seneciphylline, and monocrotaline are examples of toxic pyrrolizidine alkaloids. Monocrotaline is taken into HPCs at least in part by OATP1 and is bioactivated by CYPs to monocrotaline pyrrole, a bifunctional alkylating agent capable of binding DNA and proteins to initiate hepatotoxicity.

Acute administration of monocrotaline to rodents results in centrilobular megalocytosis (cell enlargement) and death of HPCs, which succumb from both apoptosis and oncotic necrosis. The ability of monocrotaline pyrrole to cross-link DNA might be responsible for blockade of cell division at G2/M, failure to proliferate, and consequent increase in cell size. The pronounced destruction of SECs and endothelial cells of central venules leads to marked hemorrhage that is associated with centrilobular tissue hypoxia. Monocrotaline pyrrole is detoxified primarily by conjugation with GSH.

SEC injury leads to activation of the coagulation system, which contributes to tissue hypoxia and the progression of liver injury. The swelling and destruction of SECs and their dissection from HPCs lead to emboli comprising SEC aggregates, red blood cells, and monocytes, which progresses into the sinusoidal obstruction syndrome that characterizes chronic pyrrolizidine alkaloid toxicity. Human sinusoidal obstruction syndrome can be divided into acute, subacute, and chronic phases. The acute phase is characterized by a rapid onset of nausea, emesis, abdominal pain, hepatomegaly, and ascites. The subacute phase involves persistent hepatomegaly, prominent megalocytosis, and recurrent ascites. The chronic phase consists of cirrhosis and liver failure, which can occur many months after pyrrolizidine alkaloid exposure. Death typically occurs up to 2 years after exposure.

α-Naphthylisothiocyanate

Exposure to α-naphthylisothiocyanate (ANIT; $C_{10}H_7N{=}C{=}S$) recapitulates in rodents the features of both acute and chronic biliary diseases in humans. Other available experimental models of acute and chronic cholestatic liver injury rely on investigator-imposed genetic mutations in transporters (e.g., $Mdr2^{-/-}$ mice) or surgically mediated cholestasis (i.e., bile duct ligation). The biliary epithelium is a proximal target of ANIT. GSH conjugation plays a permissive role in the biliary transport and accumulation of ANIT. An ANIT-GSH conjugate produced within the HPC is transported into bile by the canalicular transporter, Mrp2. The GSH *S*-conjugate of ANIT is unstable at physiological pH and dissociates, resulting in large concentrations of free ANIT in the bile (Fig. 13–9).

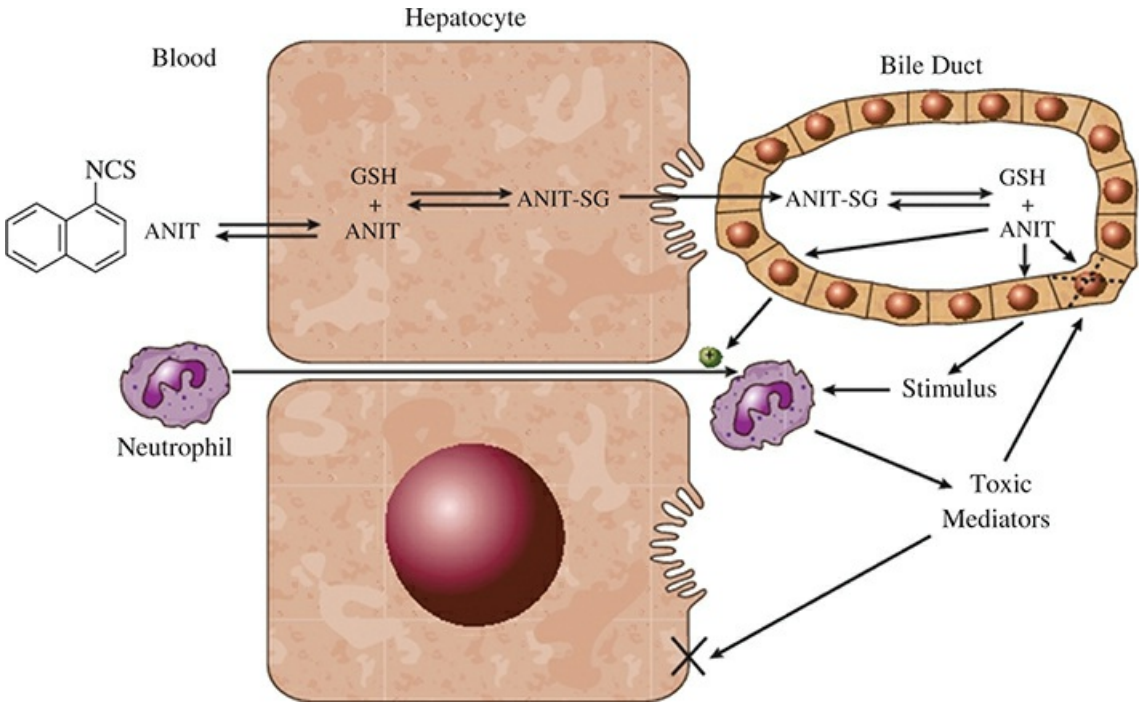

FIGURE 13–9 *Liver injury from α-naphthylisothiocyanate (ANIT).* Circulating ANIT enters hepatocytes where it forms a reversible conjugate with glutathione (GSH). The conjugate concentrates in bile, where it dissociates, leaving ANIT to injure bile duct epithelium and cause release of chemotactic stimuli that recruit neutrophils, which become activated and release cytotoxic mediators that amplify the injury.

A single, oral administration of ANIT produces acute biliary injury, disruption of bile flow, compensatory biliary hyperplasia, and portal neutrophilic inflammation. As the injury progresses, periportal hepatocellular necrosis becomes evident. Neutrophil accumulation and activation produce lesions that resemble so-called “bile infarcts.” ANIT causes bile duct epithelial cells to release a factor(s) that attracts neutrophils and stimulates them to injure HPCs (Fig. 13–9).

Persistent exposure of mice to ANIT for >4 weeks effectively recapitulates biliary fibrosis associated with chronic cholestatic liver diseases in humans, including primary sclerosing cholangitis. “Onion skin”-like layering of collagen is observed surrounding intrahepatic bile ducts after chronic ANIT challenge. Strong evidence indicates engagement of immune cells, biliary epithelium, and the hemostatic system in this model of biliary fibrosis.

Cyclic Peptide Hepatotoxins: Microcystins, Nodularins, Phallotoxins, Amatoxins

Several cyclic peptides synthesized by freshwater cyanobacteria or by toxic mushrooms are highly potent hepatotoxins, producing liver injury in humans and/or animals at doses below 1 mg/kg body weight.

Cyanobacterial Toxins—Microcystins are a group of more than 80 cyclic heptapeptide toxins

that are produced by cyanobacteria ("blue-green algae"; e.g., *Microcystis aeruginosa*) that bloom in freshwater lakes and ponds. Consumption of contaminated water from these blooms has resulted in injury to liver and other organs in fish, domestic animals, and humans. The microcystin toxins can cause liver failure or hepatocellular carcinoma.

Microcystin LR is transported into HPCs by the basolateral organic anion transporters OATP1B2 in mice and OATP1B1 and OATP1B3 in humans. Detoxification of microcystins occurs primarily via oxidation and conjugation with glutathione and cysteine. The conjugates are excreted in bile and urine. Acute injury is associated with blebbing and necrosis of HPCs, congestion, and hemorrhage. Chronic exposure is associated with hypertrophy, karyomegaly, and multinucleation of HPCs and can result ultimately in fibrosis and/or hepatocellular carcinoma. Microcystin LR affects hepatocellular microtubules. Acute exposure causes aggregation of cytokeratin-containing intermediate filaments and actin microfilaments, which detach from the plasma membrane, resulting in bleb formation. They also disrupt numerous signaling pathways critical for maintenance of normal homeostasis and cause liver injury at very small doses.

Mushroom Toxins—*Amanita phalloides* ("death cap") has been of greatest concern due to its ability to cause life-threatening toxicity. This mushroom produces several bicyclic heptapeptides known as phallotoxins and octapeptides known as amatoxins. Phalloidin and α-amanitin, respectively, have received the most attention as hepatotoxins.

Phalloidin is taken into HPCs by transporters of the OATP family and causes cholestasis and hemorrhagic necrosis in livers after intraperitoneal administration to rodents. Tight binding of phalloidin to actin filaments in HPCs prevents the disassembly phase of the normally dynamic rearrangement of the actin filament constituent of the cytoskeleton. The actin-rich web of cytoskeleton adjacent to the canalicular membrane of HPCs becomes accentuated and the canalicular lumen dilates.

α-Amanitin enters HPCs via OATPs, especially OATP1B3, on the basal membrane. Centrilobular hepatocellular necrosis and hemorrhage accompanied by a hepatorenal syndrome characterizes severe poisoning in humans. The lethal dose in humans has been estimated to be 0.1 mg/kg, an amount that can occur in one night-cap mushroom. α-Amanitin binds nuclear RNA polymerase II, thereby inhibiting RNA elongation with resultant impaired protein synthesis and cell death. In isolated HPCs, α-amanitin initiates apoptosis dependent on p53, and this might be connected to the inhibition of mRNA synthesis.

Metals

Exposure to metals occurs via several routes, including diet and inhalation of metal-containing particulates from occupational exposure or cigarette smoke. Most metals target numerous organs, among them the liver, with a wide spectrum of consequences. Metals are excreted into bile by a process that includes (1) uptake across sinusoidal membranes of HPCs by membrane transporters or receptor-mediated endocytosis; (2) storage in binding proteins or lysosomes; and (3) canalicular secretion via lysosomes, a GSH-coupled event, or by specific canalicular membrane transport via MRP2. Biliary excretion is important in the homeostasis of several metals, notably copper, manganese, cadmium, selenium, gold, silver, and arsenic.

Inability to export *copper* into bile is a central problem in Wilson's disease, an autosomal recessive inherited disorder characterized by a defect in or the absence of a copper transporting P-type ATPase (ATP7B). This carrier in the trans-Golgi network within HPCs transports copper

into the secretory pathway for binding to ceruloplasmin and then excretion into bile. Excessive copper accumulation in HPCs produces reactive oxygen species that initiate liver damage characterized by centrilobular necrosis and cholestasis. Kupffer cell activation and consequent inflammatory response contribute to liver injury.

Cadmium hepatotoxicity becomes manifest when cells exceed their capacity to sequester cadmium as a complex with the metal-binding protein, metallothionein (MT). Cadmium binding to sulfhydryl groups on proteins appears to be responsible for its toxic effect, but as with copper, activation of Kupffer cells and inflammation appear to play a role.

Acute *iron* toxicity is commonly observed in young children who accidentally ingest iron tablets. HPCs extract this essential metal from the sinusoidal blood by a receptor-mediated process and maintain a reserve of iron bound to the storage protein, ferritin. The cytotoxicity of free iron is attributed to its capacity as an electron donor for the Fenton reaction, in which hydrogen peroxide is reductively cleaved to the highly reactive hydroxyl radical, an initiator of lipid peroxidation. Accumulation of excess iron beyond the capacity for its safe storage in ferritin is initially evident in zone 1 HPCs, which are closest to the blood entering the sinusoid, and to the greater oxygen concentration in zone 1 that facilitates the injurious process of lipid peroxidation. Chronic accumulation of excess iron in cases of hemochromatosis is associated with a spectrum of hepatic disease including a greater risk for liver cancer.

DETERMINANTS OF SUSCEPTIBILITY

Pronounced variability in hepatotoxic response among individuals can occur upon exposure to xenobiotic agents. Underlying such differences in susceptibility and magnitude of response are many factors listed in Table 13–4.

TABLE 13–4 Determinants of Individual Susceptibility to Hepatotoxicants

Xenobiotic metabolism
Hepatobiliary transporters
Protective enzymes
Age
Sex
Tissue reserve and regeneration
Inflammatory stress
Nutritional status
Chemical–chemical interaction
Adaptive immune system activation

Xenobiotic Metabolism

Many chemicals are metabolized by the various CYP isoforms and conjugating enzymes to metabolites that are more or less toxicologically active than the parent molecule. Differences in the activity of such enzymes, polymorphisms in genes encoding the enzymes, genetic differences in receptors or transcription factors can markedly affect sensitivity to intoxication. The

expression and activities of many xenobiotic metabolizing enzymes are influenced by dietary constituents, exposure to drugs and environmental chemicals, nutritional status, liver diseases, inflammatory conditions, sex, and age.

Hepatobiliary Transporters

Neonates exhibit delayed development of bile acid synthesis and the expression of sinusoidal and canalicular transporters. Neonates are more prone to developing jaundice when treated with drugs that compete with bilirubin for biliary clearance. Individuals with genetic deficiency of certain hepatobiliary transporters are at risk for chronic liver injury and fibrosis and may be more susceptible to drugs and hepatotoxicants. Patients with bacterial sepsis frequently develop cholestasis, which is mainly caused by downregulation of multiple canalicular transport systems. Direct inhibition of the bile salt export pump (BSEP) (e.g., by the endothelin receptor antagonist, bosentan) can lead to retention of bile acids in the liver and consequent liver injury. The variation in the activities of many transporters can be an important determinant of individual susceptibility to intoxication.

Protective Enzymes

Polymorphisms and differences in expression of genes that encode enzymes that provide protection from reactive oxygen species can influence susceptibility to intoxication. DNA polymorphisms and/or variations in epigenetic or posttranslational control have been identified for GSH peroxidases, superoxide dismutases, and other enzymes as well as transcription factors such as Nrf2 that regulate protective cellular responses to oxidants.

Development and Aging

Age-related differences in hepatotoxicity have many underlying causes. For example, drug metabolizing enzymes generally are poorly expressed in newborn animals and people, and the capacity to metabolize increases at various rates during maturity. Elderly people typically have reduced liver blood flow, diminished xenobiotic metabolism, and differences in inflammatory responses. This results in differences in disposition of drugs and other xenobiotic agents, with consequent potential to affect toxicity.

Sex

Sex-related differences in hepatotoxic responses have been reported. For example, female sex is a risk factor for liver injury for several drugs and dietary supplements. Risk of halothane hepatitis is greater in female humans and mice, and the sex difference appears to be related to a difference in immune response. For most drugs, differences in xenobiotic metabolizing activity, drug dosing, body composition (e.g., lipid composition tends to be greater in females than males), or other factors may be involved.

Tissue Reserve and Regeneration

There is a large reserve capacity in the liver inasmuch as a large fraction of the liver volume can be lost without a serious decrement in function. This organ also has a remarkable capacity for regeneration. Interindividual variations in either of these can affect the magnitude of hepatotoxic response. Proliferative repair is an important response of liver to injury.

Inflammatory Stress

Inflammation is a common response to exposure to many hepatotoxicants, and genetic or environmental differences in one or more of the many factors involved in an inflammatory response occur among individuals. Modest inflammation is associated with alcohol consumption, pathogen infection, inflammatory diseases such as arthritis, surgery, etc., that by itself does not cause cell death or liver dysfunction. However, an independently initiated, modest inflammatory episode can qualitatively and/or quantitatively influence the magnitude of hepatotoxic response to drugs and other xenobiotic agents.

Nutritional Status

Fasting decreases GSH concentration in liver. Deficiencies in many nutrients have been shown to enhance the toxicity of numerous hepatotoxicants, and dietary supplementation can sometimes improve liver function in affected individuals. Examples of such dietary nutrients include essential amino acids and lipids, *S*-adenosylmethionine, zinc, and antioxidants. In contrast, excess consumption of some essential nutrients can exacerbate hepatotoxicity such as the hepatotoxic interaction of alcohol with iron overload.

IDIOSYNCRATIC, DRUG-INDUCED LIVER INJURY (IDILI): OCCURRENCE AND MODES OF ACTION

Although idiosyncratic, drug-induced liver injury (IDILI) occurs infrequently in human patients, it has importance because it is sometimes severe, requiring liver transplantation or causing death. Conventional preclinical safety testing in vitro and in animals often fails to detect IDILI liability of drug candidates. Many pharmaceuticals as well as herbal remedies and food supplements have been associated with IDILI in humans. Antibiotics are the drugs most commonly associated with human IDILI in Western countries. In Asia, IDILI is caused more commonly by dietary and herbal supplements.

IDILI is difficult to diagnose because the clinical presentation is often indistinguishable from liver injury from other causes. Hepatocellular injury may be the most common phenotype, but cholestatic and mixed injuries also occur. Many patients recover from liver injury when exposure to the offending drug is stopped, but others progress to acute liver failure culminating in death or a need for a liver transplant.

According to the *multiple determinant hypothesis*, IDILI occurs from the intersection of several genetic and/or environmental susceptibility factors present simultaneously within a

patient exposed to the offending drug. Such factors could be related to drug metabolism, transporters, nutrition, sex, age, concurrent exposure to other xenobiotic agents, physical activity, underlying disease, concurrent inflammation, etc. Each of the susceptibility factors is associated with a finite probability of occurrence (i.e., from 0 to 1), and the probability of an IDILI reaction would be related to the product of the individual probabilities. This would result in a small number and could explain why IDILI reactions are typically rare.

The *failure-to-adapt hypothesis* proposes that therapeutic doses of some drugs cause modest liver injury to which most patients adapt; those few who fail to do so progress to serious liver injury. For example, about 10% to 40% of patients taking the antibiotic, isoniazid, experience increases in serum ALT/AST that, in most patients, return to normal despite continued drug treatment. However, isoniazid is well known to cause life-threatening liver injury in about 1% of patients taking the drug, so it seems plausible that these patients failed to adapt to the modest initial insult.

Many conditions, such as inflammatory diseases, alcohol consumption, infection, etc., involve episodic exposure of the liver to PAMPs or DAMPs and consequent activation of the innate immune system (Fig. 13–10). According to the *inflammatory stress hypothesis*, drug exposure interacts with a modest, noninjurious inflammatory episode, resulting in liver damage. One can picture a patient on maintenance drug therapy progressing well until an inflammatory episode interacts with the drug, precipitating liver injury. Such a scenario can account for many of the bizarre characteristics of idiosyncratic reactions (see Table 13–3).

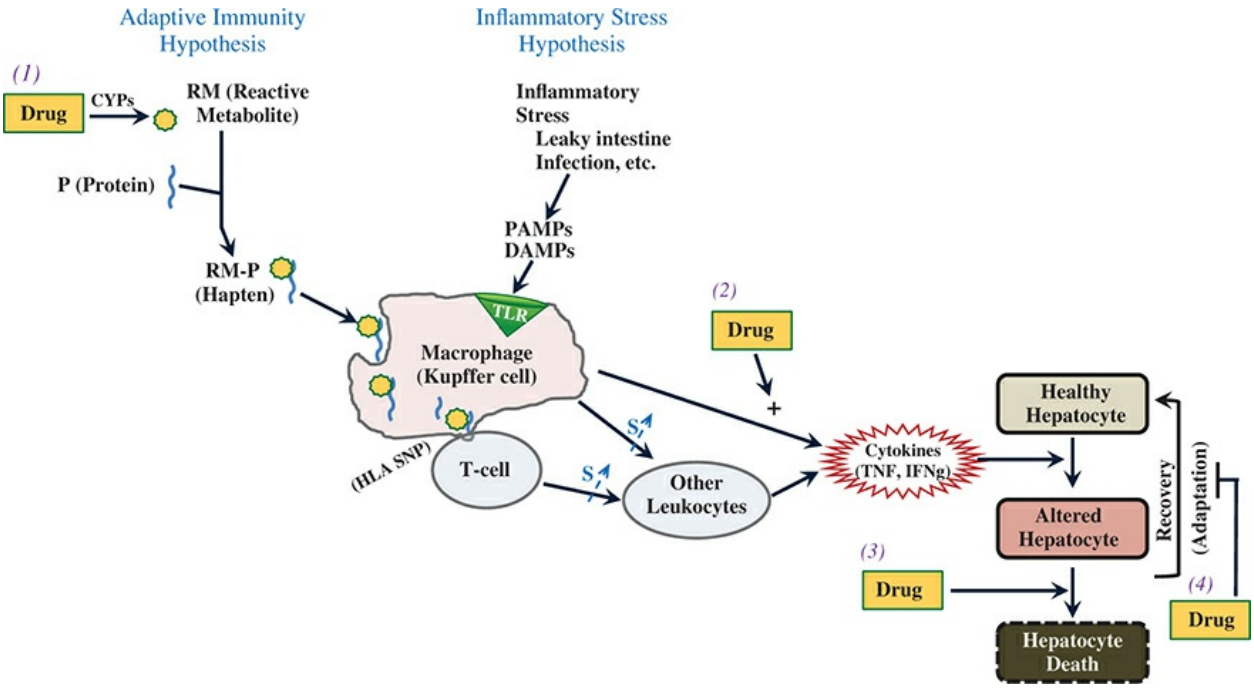

FIGURE 13–10 *Potential mechanisms by which drugs contribute to IDILI responses. (1)* Drugs can be metabolically bioactivated by cytochromes P450 (CYPs) to reactive metabolites that bind to cellular proteins to initiate an adaptive immune response. *(2)* Activation of adaptive or innate immune systems leads to the production of cytokines, which is enhanced by some IDILI-associated drugs. *(3)* One of the pleiotropic effects of cytokines is to activate cell death pathways, which is enhanced by numerous IDILI-associated drugs in vitro. *(4)* Some drugs can interfere with the ability of the liver to adapt to (recover from) modest injury,

thereby leading to injury progression.

According to the *adaptive immunity hypothesis* (or *hapten hypothesis*), IDILI reactions are initiated when drugs are metabolized to reactive metabolites that bind covalently to proteins (Fig. 13–10). These altered proteins are detected as antigens by antigen-presenting cells that process the antigens and present them to T-cells, which become activated, leading to an adaptive immune response that results in activation of inflammatory cells, release of cytokines, and other events (Fig. 13–10). Normally, this does not damage host tissue. However, in the presence of "danger signals" (e.g., DAMPs released from cells that are injured) and elimination of "immune tolerance," the liver becomes poised for injury.

The *pharmacological interaction (PI) hypothesis* proposes that certain drugs can activate lymphocytes directly to prompt an adaptive immune response without the need for haptenization. There is mounting evidence that drug-induced, sublethal stress renders HPCs sensitive to the killing capacity of cytokines and that this might be important in IDILI reactions that are initiated by immune responses (Fig.13–10). IDILI likely occurs by several different mechanisms depending on the drug and characteristics of the patient. It should be borne in mind that the hypotheses described in the preceding section are not mutually exclusive.

CONCLUSION

The liver comprises several cell types and has numerous functions, many of which relate to metabolism of endogenous and xenobiotic agents, elimination of toxic substances, immune surveillance, and supplying other organs with energy sources. Its blood supply arising predominately from intestinal drainage exposes the liver to large concentrations of components in the diet that are absorbed into the blood, and this in part renders the liver susceptible to toxicity. Responses to chemical insults include steatosis, death of various cell types, inflammation, fibrosis, cancer, and proliferative repair. The mechanisms that result in liver injury are complex and can be dose-related; that is, different mechanisms may apply at small and large exposures to the same toxicant. Toxic mechanisms typically begin with an insult to HPCs that initiates intracellular signaling, which can lead to cell death. Activated sinusoidal cells and blood elements can encourage progression of injury, and these extrahepatocellular factors add to the complexity of mechanisms. Injury initiation and progression are counterbalanced by adaptation and regeneration mechanisms, and together these determine the seriousness of the toxic outcome. Recently developed and emerging tools promise to contribute to improved detection of hepatotoxicity and greater understanding of mechanisms in the near future.

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QUESTIONS

1. The impairment of hepatic function can have numerous negative consequences. Which of the following is likely NOT caused by impaired hepatic function?
   a. jaundice.
   b. hypercholesterolemia.
   c. hyperammonemia.
   d. hyperglycemia.
   e. hypoalbuminemia.

2. All of the following statements regarding the liver are true EXCEPT:
   a. The major role of the liver is to maintain metabolic homeostasis of the body.
   b. The liver encounters ingested nutrients before the heart does.
   c. Hepatic triads contain a branch of the hepatic portal vein, a branch of the hepatic artery, and a bile ductile.
   d. The liver manufactures and stores bile.
   e. The large fenestrae of hepatic sinusoids facilitate exchange of materials between the sinusoid and the hepatocyte.

3. Activation of which of the following cell types can result in increased secretion of collagen scar tissue, leading to cirrhosis?
   a. hepatocyte.
   b. Ito cell.
   c. Kupffer cell.
   d. endothelial cell.
   e. β-cell.

4. Wilson's disease is a rare genetic disorder characterized by the failure to export which of the following metals into bile?
   a. iron.
   b. zinc.
   c. silver.
   d. lead.
   e. copper.

5. Which of the following is NOT characteristic of apoptosis?
   a. cell swelling.
   b. nuclear fragmentation.
   c. lack of inflammation.
   d. programmed death.
   e. chromatin condensation.

6. A patient suffering from canalicular cholestasis would NOT be expected to exhibit which of the following?

a. increased bile salt serum levels.
b. jaundice.
c. increased bile formation.
d. dark brown urine.
e. vitamin A deficiency.

7. Which of the following statements regarding liver injury is FALSE?
a. Large doses of acetaminophen have been shown to cause a blockade of hepatic sinusoids.
b. Hydrophilic drugs readily diffuse into hepatocytes because of the large sinusoidal fenestrations.
c. There are sinusoidal transporters that take toxicants up into hepatocytes.
d. Hepatocellular cancer has been associated with androgen abuse.
e. In cirrhosis, excess collagen is laid down in response to direct injury or inflammation.

8. The inheritance of a “slow” aldehyde dehydrogenase enzyme would result in which of the following after the ingestion of ethanol?
a. high ethanol tolerance.
b. little response to low doses of ethanol.
c. low serum levels of acetaldehyde.
d. nausea.
e. increased levels of blood ethanol compared to an individual with a normal aldehyde dehydrogenase.

9. Which of the following is NOT a common mechanism of hepatocellular injury?
a. deformation of the hepatocyte cytoskeleton.
b. mitochondrial injury.
c. cholestasis.
d. interference with vesicular transport.
e. increased transcytosis between hepatocytes.

10. Ethanol is not known to cause which of the following types of hepatobiliary injury?
a. fatty liver.
b. hepatocyte death.
c. fibrosis.
d. immune-mediated responses.
e. canalicular cholestasis.

CHAPTER 14

Toxic Responses of the Kidney

Rick G. Schnellmann

SPECIFIC NEPHROTOXICANTS

- **Heavy Metals**
  - Mercury
  - Cadmium
- **Chemically Induced α_{2u}-Globulin Nephropathy**
- **Halogenated Hydrocarbons**
  - Chloroform
  - Tetrafluoroethylene
- **Aristolochic Acid and Fungal Toxins**
- **Therapeutic Agents**
  - Acetaminophen
  - Nonsteroidal Anti-Inflammatory Drugs
  - Aminoglycosides
  - Amphotericin B
  - Cyclosporine
  - Cisplatin
  - Radiocontrast Agents

KEY POINTS

- The kidney contributes to total body homeostasis via its role in the excretion of metabolic wastes, the synthesis and release of renin and erythropoietin, and the regulation of extracellular fluid volume, electrolyte composition, and acid–base balance.
- Xenobiotics in the systemic circulation will be delivered to the kidney in relatively high amounts.
- The processes that concentrate urine also serve to concentrate potential toxicants in the tubular fluid.
- Renal transport, accumulation, and biotransformation of xenobiotics contribute to the susceptibility of the kidney to toxic injury.
- Numerous nephrotoxicants cause mitochondrial dysfunction via compromised respiration and ATP production, or some other cellular process, leading to either apoptosis or necrosis.
- Vitamin D_3 is metabolized to the active 1,25-dihydroxy vitamin D_3 form.

FUNCTIONAL ANATOMY

A sagittal section of the kidney reveals three clearly demarcated anatomic areas: the cortex, medulla, and papilla (Fig. 14–1). The cortex receives a disproportionately higher percentage (90%) of blood flow compared to the medulla (about 6% to 10%) or papilla (1% to 2%). When a blood-borne toxicant is delivered to the kidney, a high percentage of the material will be delivered to the cortex and will have a greater opportunity to influence cortical rather than medullary or papillary functions. However, medullary and papillary tissues are exposed to higher luminal concentrations of toxicants for prolonged periods of time, a consequence of the more concentrated tubular fluid and the more sluggish flow of blood and filtrate in these regions.

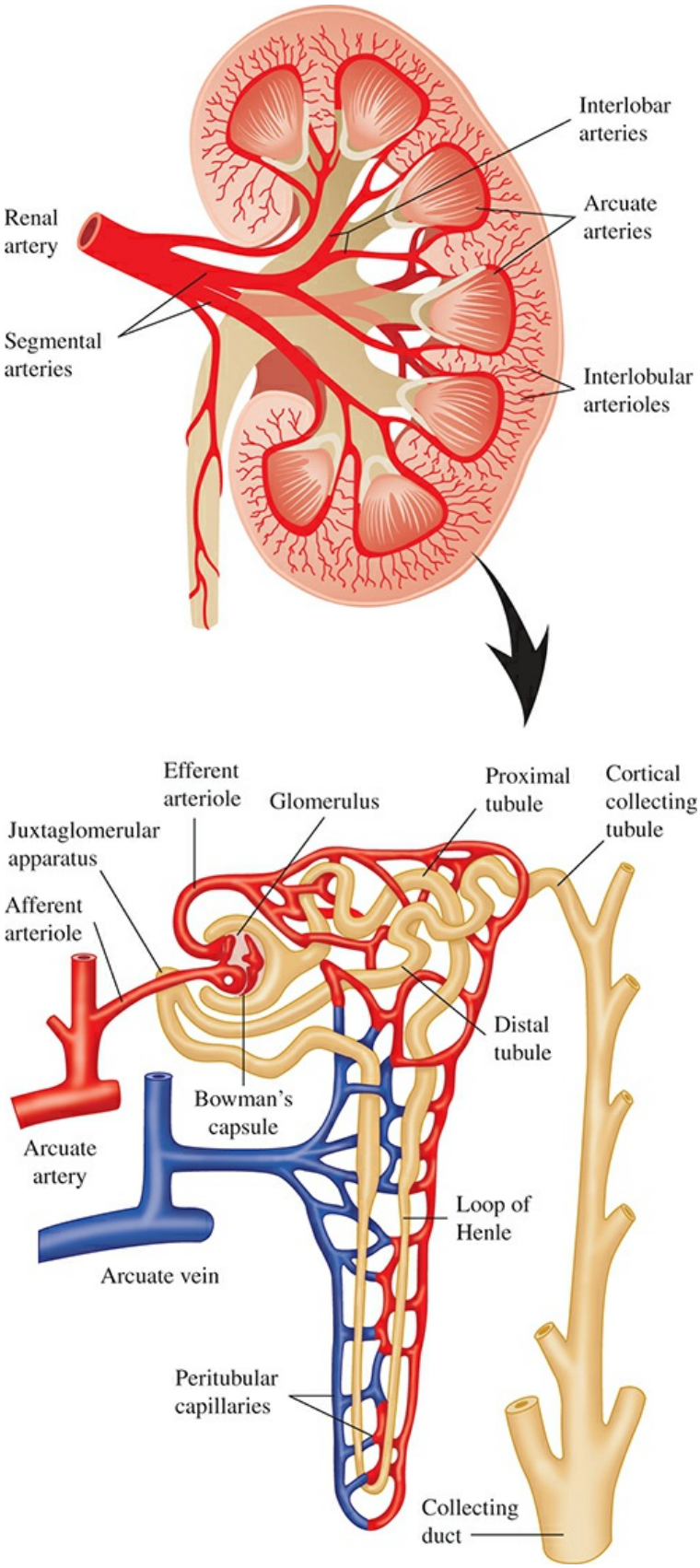

FIGURE 14–1 Schematic of the human kidney showing the major blood vessels and the microcirculation and tubular components of each nephron. (Reprinted from Guyton AC, Hall JE, eds. *Textbook of Medical Physiology*. Philadelphia, PA: WB Saunders; 1996: p. 318.)

Renal Vasculature and Glomerulus

The renal artery branches successively into the afferent arterioles (Fig. 14–1), which supply the glomerulus. Blood leaves the glomerular capillaries via the efferent arteriole. Both the afferent and efferent arterioles control glomerular capillary pressure and glomerular plasma flow rate. These arterioles are innervated by the sympathetic nervous system and contract in response to nerve stimulation, angiotensin II, vasopressin, endothelin, adenosine, and norepinephrine decreasing glomerular filtration rate and renal blood flow. The efferent arterioles draining the cortical glomeruli branch into a peritubular capillary network, whereas those draining the juxtamedullary glomeruli form a capillary loop, the vasa recta, supplying the medullary structures. These postglomerular capillary loops provide delivery of nutrients to the postglomerular tubular structures, delivery of wastes to the tubule for excretion, and return of reabsorbed electrolytes, nutrients, and water to the systemic circulation.

The glomerulus is a complex, specialized capillary bed that fractionates blood into a virtually protein-free and cell-free ultrafiltrate, which passes through Bowman's space and into the tubular portion of the nephron. The formation of such an ultrafiltrate is the net result of the Starling forces that determine fluid movement across capillary beds, that is, the balance between transcapillary hydrostatic pressure and colloid oncotic pressure. An additional determinant of ultrafiltration is the effective hydraulic permeability of the glomerular capillary wall, or the ultrafiltration coefficient (K_f).

Although the glomerular capillary wall permits a high rate of fluid filtration (approximately 20% of blood entering the glomerulus is filtered), it provides a significant barrier to the transglomerular passage of macromolecules; thus, small molecules, such as inulin (molecular weight [MW] ~5500), are freely filtered, whereas large molecules, such as albumin (MW 56,000 to 70,000), are restricted. Filtration of anionic molecules tends to be restricted compared to that of neutral or cationic molecules of the same size. Charge-selective properties of the glomerulus appear to be related to the anionic groups of the glomerular basement membrane (GBM) coupled with the anionic coating of the epithelial and endothelial cells (Fig. 14–2). These highly anionic components produce electrostatic repulsion to hinder circulation of polyanionic macromolecules, thereby retarding passage of these molecules across the filtration barrier. Toxicants that neutralize or reduce the number of fixed anionic charges on glomerular structural elements impair the charge and/or size-selective properties of the glomerulus, resulting in urinary excretion of polyanionic and/or high-molecular-weight proteins.

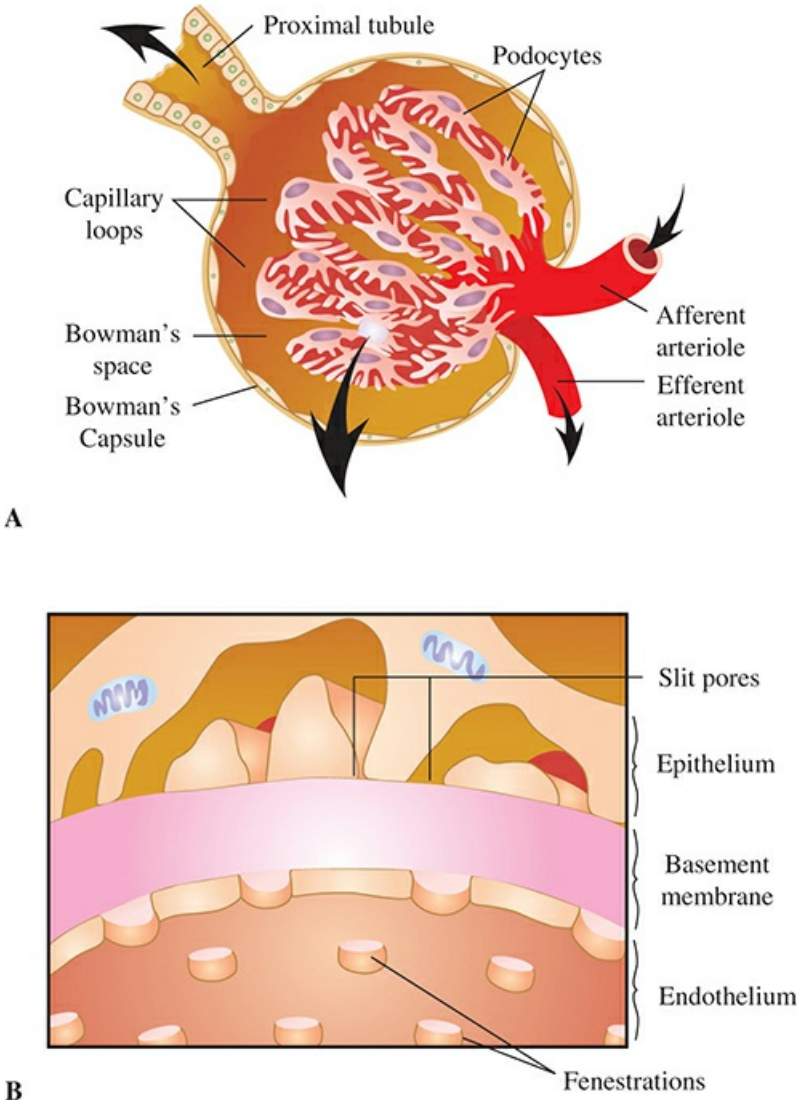

FIGURE 14–2 (***A***) Schematic of the ultrastructure of the glomerula-capillaries. (***B***) Cross-section of the glomerular capillary membrane with the capillary endothelium, basement membrane, and epithelium podocytes. (Reprinted from Guyton AC, Hall JE, eds. *Textbook of Medical Physiology*. Philadelphia, PA: WB Saunders; 1996: p. 32.)

Proximal Tubule

The proximal tubule consists of three discrete segments: the S_1 (pars convoluta), S_2 (transition between pars convoluta and pars recta), and S_3 (the pars recta) segments. The formation of urine is a highly complex and integrated process in which the volume and composition of the glomerular filtrate are progressively altered as fluid passes through each of the different tubular segments. The proximal tubule reabsorbs approximately 60% to 80% of solute and water filtered at the glomerulus by numerous transport systems capable of driving reabsorption of Na^+, K^+, HCO_3^-, Cl^-, PO_4^{3-}, Ca^{2+}, Mg^{2+}, amino acids, glucose, and citric acid cycle intermediates. The proximal tubule also reabsorbs virtually all the filtered low-molecular-weight proteins by specific

endocytotic protein reabsorption processes. An important excretory function of the proximal tubule is secretion of weak organic anions and cations by specialized transporters.

Loop of Henle

Approximately 25% of the filtered Na^+ and K^+ and 20% of the filtered water are reabsorbed by the segments of the loop of Henle. The tubular fluid entering the thin descending limb is iso-osmotic to the renal interstitium; water is freely permeable, and solutes, such as electrolytes and urea, may enter from the interstitium. In contrast, the thin ascending limb is relatively impermeable to water and urea, and Na^+ and Cl^- are reabsorbed by passive diffusion. The thick ascending limb is impermeable to water, and active transport of Na^+ and Cl^- is mediated by the $Na^+/K^+/2Cl^-$ cotransport mechanism, with the energy provided by Na^+, K^+-ATPase.

Distal Tubule and Collecting Duct

The macula densa comprises specialized cells located between the end of the thick ascending limb and the early distal tubule, near the afferent arteriole. Under normal physiologic conditions, increased solute delivery or concentration at the macula densa triggers a signal resulting in afferent arteriolar constriction leading to decreases in GFR (and hence decreased solute delivery). This regulatory mechanism is a powerful volume-conserving mechanism that decreases GFR to prevent massive losses of fluid/electrolytes due to impaired tubular reabsorption. The early distal tubule reabsorbs most of the remaining intraluminal Na^+, K^+, and Cl^- but is relatively impermeable to water.

The late distal tubule, cortical collecting tubule, and medullary collecting duct reabsorb the remaining Na^+ in conjunction with K^+ and H^+ secretion. The combination of medullary and papillary hypertonicity generated by countercurrent multiplication and the action of antidiuretic hormone (ADH, vasopressin) serve to enhance water permeability of the medullary collecting duct. Chemicals that interfere with ADH synthesis, secretion, or action may impair concentrating ability. Additionally, because urinary concentrating ability is dependent upon medullary and papillary hypertonicity, chemicals that increase medullary blood flow may impair concentrating ability by dissipating the medullary osmotic gradient.

PATHOPHYSIOLOGIC RESPONSES OF THE KIDNEY

Acute Kidney Injury

A common manifestation of nephrotoxic damage is acute kidney injury (AKI), a group of syndromes with multiple causative factors and with varied clinical manifestations ranging from a minimal elevation in serum creatinine to anuric renal failure. AKI classification is based on the extent of serum creatinine increases or changes in urine output.

Any decline in GFR may result from prerenal factors (renal vasoconstriction, intravascular volume depletion, and insufficient cardiac output), postrenal factors (ureteral or bladder

obstruction), and intrarenal factors (glomerulonephritis, tubular cell injury, death, and loss resulting in back leak; renal vasculature damage; interstitial nephritis) (Fig. 14–3). Pre- and postrenal factors can lead to decreased GFR. If a chemical causes tubular damage directly, then tubular casts can cause tubular obstruction, increased tubular pressure, and decreased GFR. The tubular damage may result in epithelial cell death/loss, leading to back leak of glomerular filtrate and a decrease in GFR. If a chemical causes intrarenal vascular damage with hemodynamic alterations that lead to vasoconstriction, the resulting medullary hypoxia may cause tubular damage and/or decreases in perfusion pressure, glomerular hydrostatic pressure, and GFR. If a chemical causes intrarenal inflammation, then tubular and vascular damage may follow with decreases in GFR. Finally, a chemical may disrupt glomerular function, resulting in decreased glomerular ultrafiltration and GFR. Table 14–1 provides a partial list of chemicals that produce AKI through these different mechanisms.

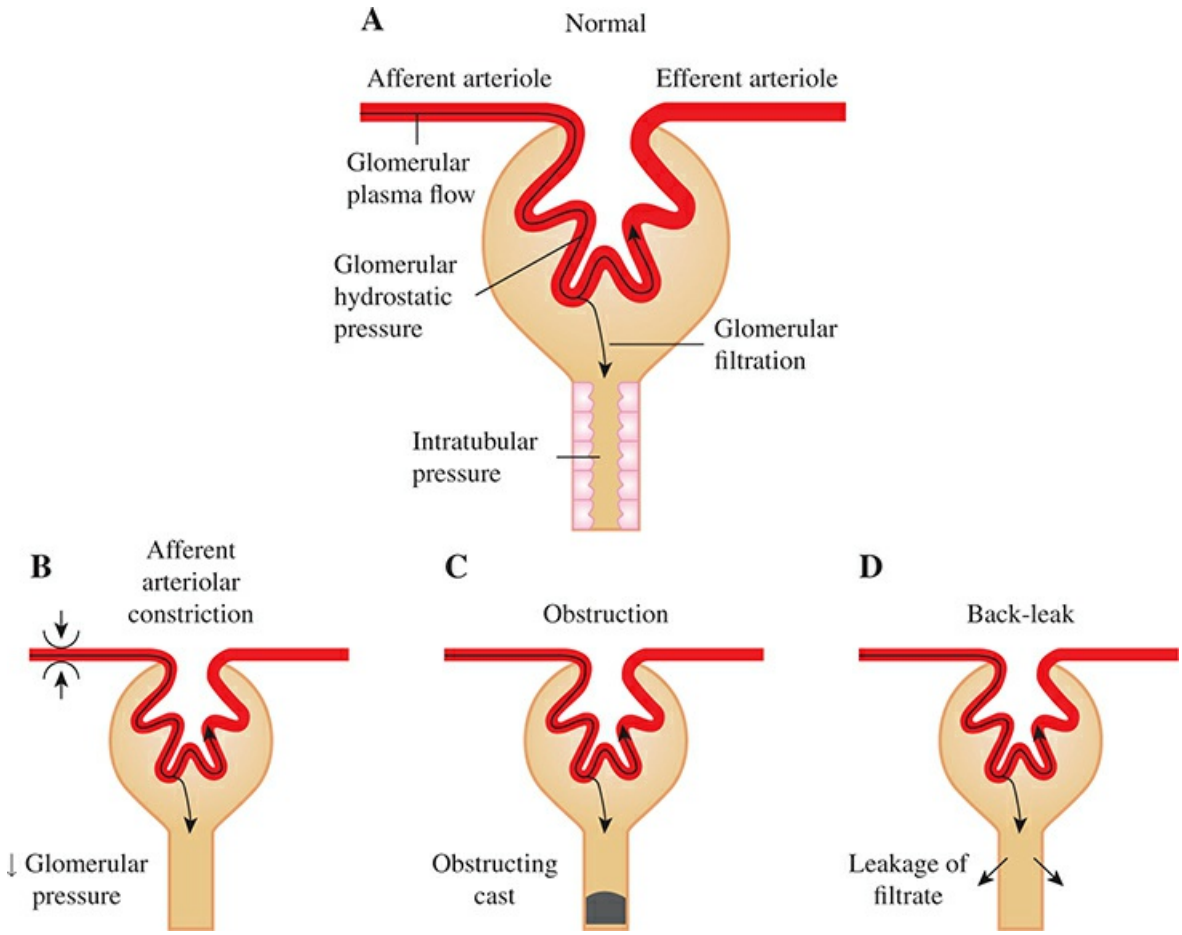

FIGURE 14–3 Mechanisms of reduction of the GFR. (**A**) GFR depends on four factors: (1) adequate blood flow to the glomerulus, (2) adequate glomerular capillary pressure, (3) glomerular permeability, and (4) low intratubular pressure. (**B**) Afferent arteriolar constriction decreases GFR by reducing blood flow, resulting in diminished capillary pressure. (**C**) Obstruction of the tubular lumen by cast formation increases tubular pressure; when tubular pressure exceeds glomerular capillary pressure, filtration decreases or ceases. (**D**) Back-leak occurs when the paracellular space between cells increases and the glomerular filtrate leaks into the extracellular space and bloodstream. (Reprinted with permission from Molitoris BA,

Bacallao R. Pathophysiology of ischemic acute renal failure: cytoskeletal aspects. In: Berl T, Bonventre JV, eds. *Atlas of Diseases of the Kidney*. Philadelphia, PA: Current Medicine; 1999.)

TABLE 14–1 Mechanisms of Chemically Induced Acute Renal Failure

Prerenal	Vasoconstriction	Crystalluria	Tubular Toxicity	Endothelial Injury	Glomerulopathy	Interstitial Nephritis
Diuretics Angiotensin receptor antagonists Angiotensin-converting enzyme inhibitors Antihypertensive agents	Nonsteroidal anti-inflammatory drugs Radiocontrast agents Cyclosporine Tacrolimus Amphotericin B	Sulfonamides Methotrexate Acyclovir Triamterene Ethylene glycol Protease inhibitors	Aminoglycosides Cisplatin Vancomycin Pentamidine Radiocontrast agents Heavy metals Haloalkane- and haloalkene-cysteine conjugates	Cyclosporine Mitomycin C Tacrolimus Cocaine Conjugated estrogens Quinine	Gold Penicillamine Nonsteroidal anti-inflammatory drugs	Antibiotics Nonsteroidal anti-inflammatory drugs Diuretics

The maintenance of tubular integrity depends on cell-to-cell and cell-to-matrix adhesion; these interactions are mediated in part by integrins and cell adhesion molecules (Fig. 14–4). After a chemical or hypoxic insult, adhesion of nonlethally damaged, apoptotic, and oncotic cells to the basement membrane is compromised, leading to gaps in the epithelial cell lining, potentially resulting in back-leak of filtrate and diminished GFR. These detached cells may aggregate in the tubular lumen (cell-to-cell adhesion) and/or adhere or reattach to adherent epithelial cells downstream, resulting in tubular obstruction.

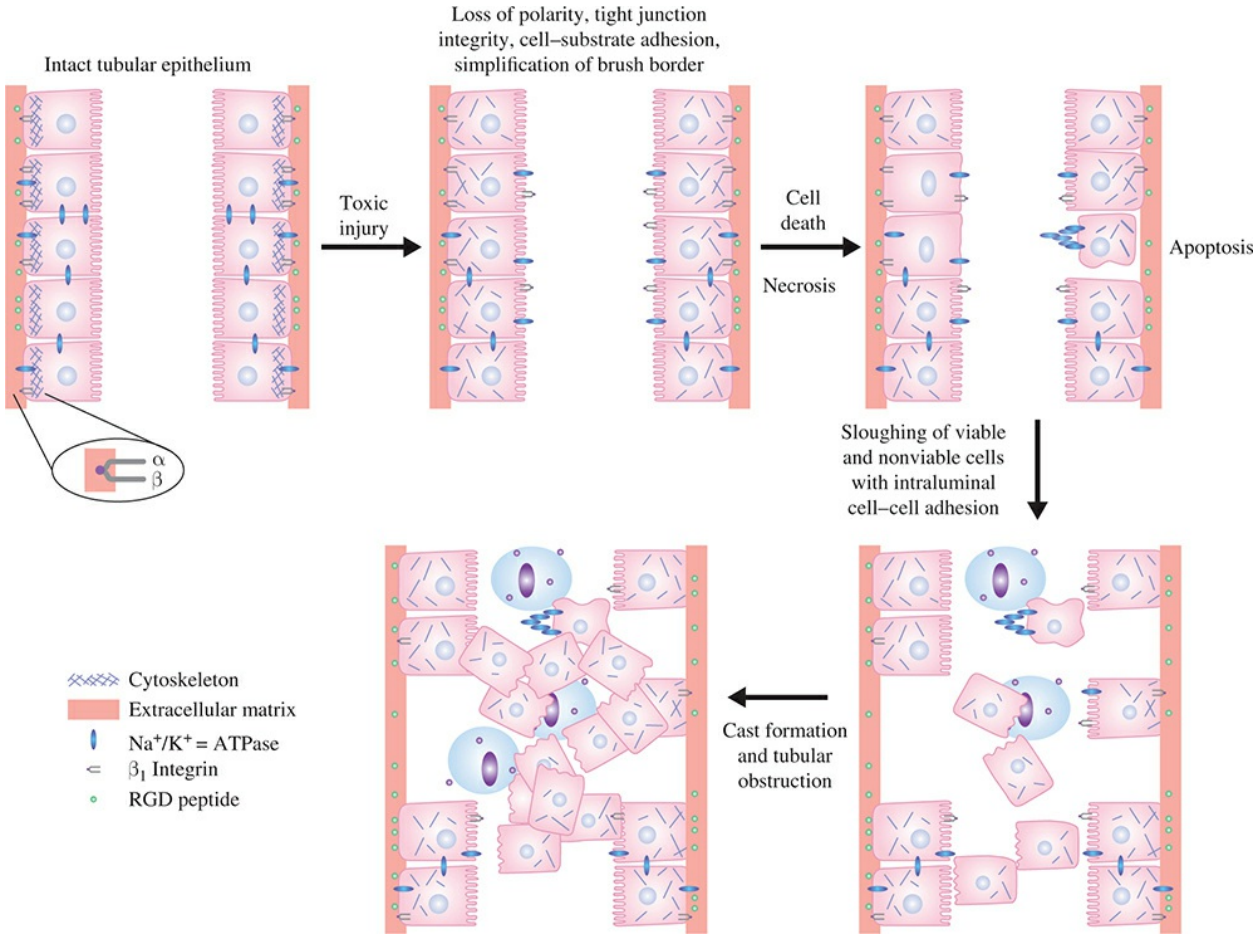

FIGURE 14–4 *Alterations can occur in the cytoskeleton and in the normal distribution of membrane proteins such as Na+, K+-ATPase, and β1 integrins in sublethally injured renal tubular cells*. These changes result in loss of cell polarity, tight junction integrity, and cell–substrate adhesion. Lethally injured cells undergo oncosis or apoptosis, and both dead and viable cells may be released into the tubular lumen. Adhesion of released cells to other released cells and to cells remaining adherent to the basement membrane may result in cast formation, tubular obstruction, and further compromise the GFR. (Reprinted with permission from Schnellmann RG, Kelly KJ. Pathophysiology of nephrotoxic acute renal failure. In: Berl T, Bonventre JV, eds. *Atlas of Diseases of the Kidney*. Philadelphia, PA: Current Medicine; 1999.)

Extensive evidence supports the idea that renal endothelial injury and inflammatory cells are involved in ischemia-induced AKI. Injury to the renal vasculature endothelium results in chemokine and pro-inflammatory cytokine production and neutrophil adhesion (Fig. 14–5). Adhesion of neutrophils to the vascular endothelium leads to capillary damage/leakage and vascular congestion.

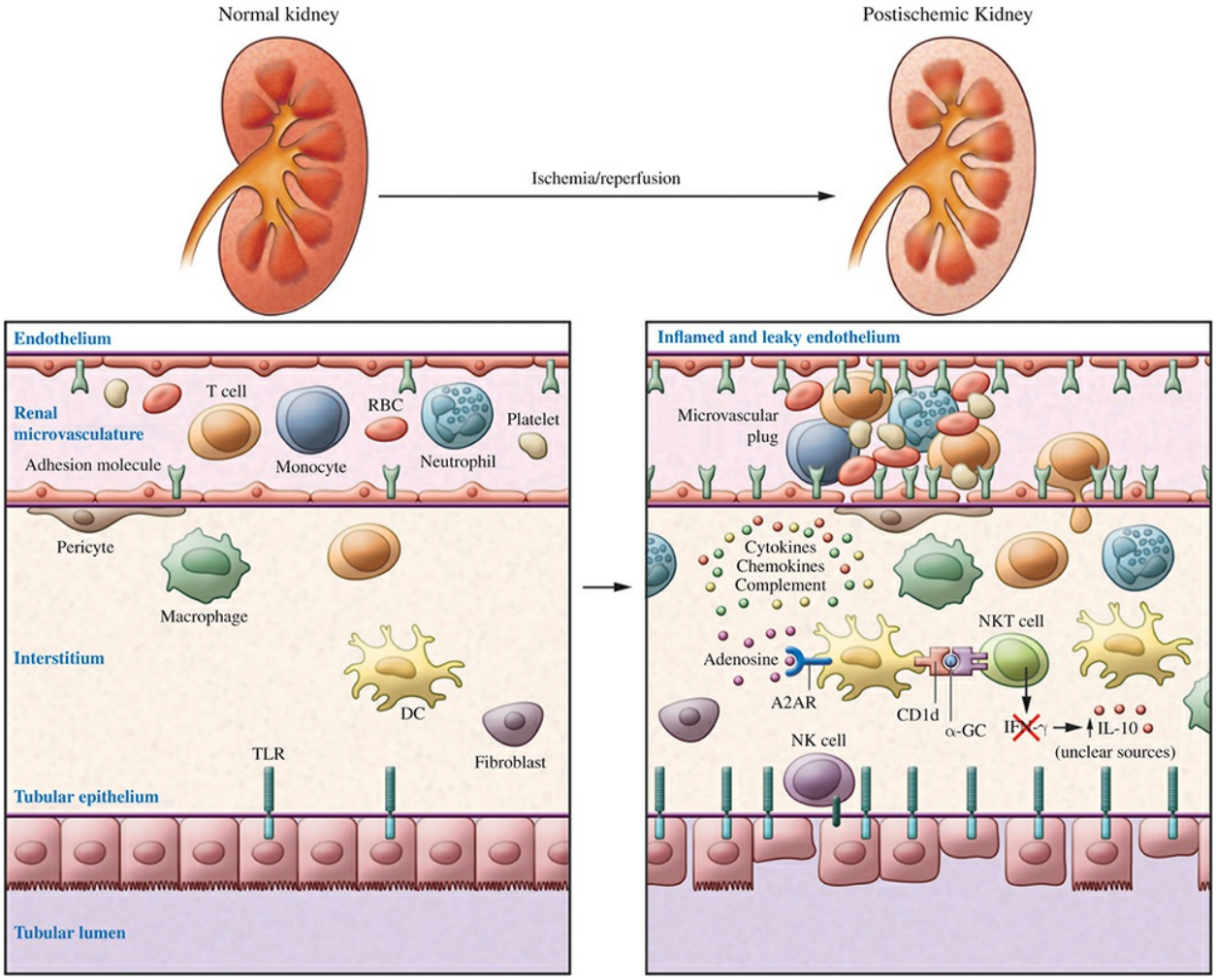

FIGURE 14–5 *Immune cells mediate AKI.* Immune cells likely mediate AKI while in circulation and when localized in the renal microvasculature, renal interstitium, and lymphoid tissue. While in the renal microcirculation during reperfusion, these cells increase their adhesiveness and adhere to activated endothelium and other cells, accentuating the "plug" that contributes to the no-reflow phenomenon. Some immune cells migrate into the interstitium, but there are also well-described resident renal immune cells. T and B cells, DC, NK and NKT cells, macrophage, and neutrophil crosstalk accentuate the postischemic inflammation. These cells produce and respond to cytokines, chemokines, oxygen-free radicals, complement, coagulant factors, and other mediators. Adenosine, acting via $A_{2A}R$, activates DCs, which in turn modulate NKT cell function by decreasing IFN-γ secretion. This triggers increased IL-10 levels, which subsequently downregulate postischemic inflammation. Panels depict the outer medulla. (Reprinted with permission from Rabb H. The promise of immune cell therapy for acute kidney injury. J Clin Invest. 2012;122:3852–3854.)

Renal dendritic cells found in the interstitial extracellular space are involved in the initiation of innate immunity after renal injury. Influx of macrophage and dendritic cell subpopulations, neutrophils, and lymphocytes (e.g., $CD4^+$ B and T cells) is mediated by cytokines and chemokines produced by the resident dendritic cells and macrophages. These various cells can contribute to kidney injury and promote inflammation. Once injury has occurred, regulatory T

cells and macrophage subsets can decrease the injury and/or promote repair processes.

Adaptation Following Toxic Insult

The kidney has a remarkable ability to compensate for a loss of renal functional mass. Following a unilateral nephrectomy, GFR of the remnant kidney increases by approximately 40% to 60%. Compensatory increases in single-nephron GFR are accompanied by proportionate increases in proximal tubular water and solute reabsorption; the glomerulotubular balance is therefore maintained and overall renal function appears normal by standard clinical tests. Consequently, chemically induced changes in renal function may not be detected until these compensatory mechanisms are overwhelmed by significant nephron loss and/or damage.

There are several cellular and molecular responses to a nephrotoxic insult. After a population of tubular epithelial cells is exposed to a toxicant, a fraction of the cells will be severely injured and will undergo cell death by apoptosis or oncosis. Cells that are nonlethally injured may undergo cell repair and/or adaptation and contribute to the recovery of the nephron (Fig. 14–6). In addition, the population of cells that are uninjured may undergo compensatory hypertrophy, adaptation, and proliferation. Tubular epithelial cells are primarily responsible for the structural and functional recovery of the nephron following injury and that surviving tubular epithelial cells replace dead and detached cells through dedifferentiation, proliferation, migration, and redifferentiation.

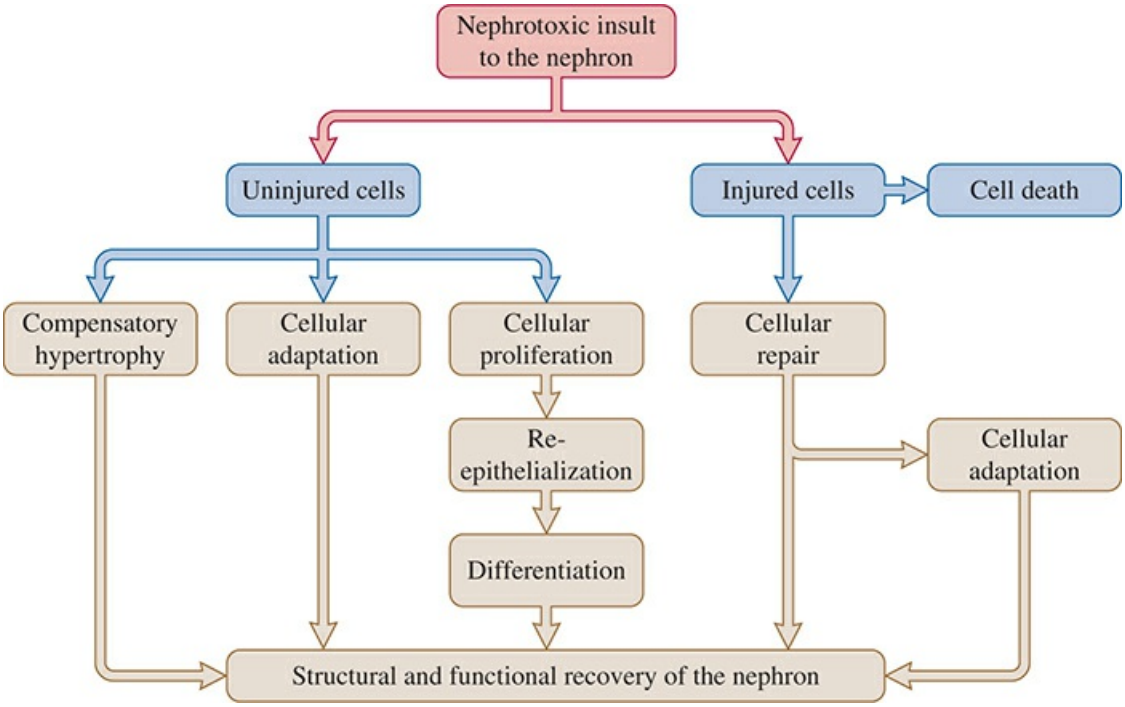

FIGURE 14–6 *The response of the nephron to a nephrotoxic insult.* After a population of cells is exposed to a nephrotoxicant, the cells respond; ultimately the nephron recovers function or, if cell death and loss are extensive, nephron function ceases. Terminally injured cells undergo cell death through oncosis or apoptosis. Cells injured sublethally undergo repair and adaptation in response to the nephrotoxicant. Cells not injured and adjacent to the injured area may undergo dedifferentiation, proliferation, migration or spreading, and differentiation.

Cells not injured may also undergo compensatory hypertrophy in response to the cell loss and injury. Finally, the uninjured cells also may undergo adaptation in response to a nephrotoxicant exposure. (Reprinted with permission from Schnellmann RG, Kelly KJ. Pathophysiology of nephrotoxic acute renal failure. In: Berl T, Bonventre JV, eds. *Atlas of Diseases of the Kidney*. Philadelphia, PA: Current Medicine; 1999.)

Two notable cellular adaptation responses are metallothionein induction and stress protein induction. Heat-shock proteins (HSPs) and glucose-regulated proteins are two examples of stress protein families that are induced in response to pathophysiologic states such as heat shock, anoxia, oxidative stress, toxicants, heavy metal exposure, and tissue trauma. These proteins are important in protein folding, protein translocation across organelle membranes, prevention of damaged protein aggregation, and repair and degradation of damaged proteins, and thereby provide a defense mechanism against toxicity and/or for the facilitation of recovery and repair.

Chronic Kidney Disease

Progression to chronic kidney disease (CKD) and end-stage renal failure involves secondary pathophysiologic processes triggered by the initial injury. Following nephron loss, adaptive increases in glomerular pressures and flows increase the single-nephron GFR of remnant viable nephrons. With time, compensatory alterations become maladaptive and foster the progression of renal failure. Focal glomerulosclerosis develops and may trigger further compensatory increases in the hemodynamics of less damaged nephrons, contributing to their eventual destruction. Although the underlying mechanisms are not precisely known, mechanical damage to the capillaries due to increased shear stress on the endothelium and damage to the glomerular capillary wall may lead to altered permeabilities and mesangial thickening.

SUSCEPTIBILITY OF THE KIDNEY TO TOXIC INJURY

Incidence and Severity of Toxic Nephropathy

Many drugs, environmental chemicals, and metals can cause nephrotoxicity (Table 14–1). Risk factors that may contribute to the incidence/severity of AKI include genetic/hereditary factors, volume depletion, septic shock, hypotension, multiple chemical insults, age, diabetes, and preexisting renal disease. The consequences of AKI can be profound, as permanent renal damage may result and dialysis or renal transplantation may be required.

Reasons for the Susceptibility of the Kidney to Toxicity

The kidneys receive about 20% to 25% of the resting cardiac output. Consequently, any drug or chemical in systemic circulation will circulate to these organs in relatively high amounts. The processes involved in forming concentrated urine also serve to concentrate potential toxicants in the tubular fluid. As water and electrolytes are reabsorbed from the glomerular filtrate, chemicals in the tubular fluid may be concentrated, thereby driving passive diffusion of toxicants into

tubular cells. Therefore, renal transport, accumulation, and metabolism of xenobiotics contribute significantly to the susceptibility of the kidney (and specific nephron segments) to toxic injury.

The incidence and/or severity of chemically induced nephrotoxicity may be related to the sensitivity of the kidney to circulating vasoactive substances, such as angiotensin II or vasopressin, whose actions are counterbalanced by the actions of increased vasodilatory prostaglandins. When prostaglandin synthesis is suppressed by NSAIDs, RBF declines markedly and AKI ensues, due to the unopposed actions of vasoconstrictors. Also, glomerular filtration pressure depends on angiotensin II-induced efferent arteriolar constriction. ACE inhibitors will block this vasoconstriction, resulting in a precipitous decline in filtration pressure and AKI.

Glomerular Injury

Many nephrotoxicants alter glomerular permeability to proteins. Cyclosporine, amphotericin B, and gentamicin are chemicals that impair glomerular ultrafiltration without significant loss of structural integrity. Amphotericin B decreases GFR by causing renal vasoconstriction and decreasing the glomerular capillary ultrafiltration coefficient (K_f). Gentamicin interacts with the anionic sites on the endothelial cells, decreasing K_f, and GFR. Finally, cyclosporine not only causes renal vasoconstriction and vascular damage but injures glomerular endothelial cells.

Chemically induced glomerular injury may be mediated by extrarenal factors. Circulating immune complexes may be trapped within the glomeruli; binding of complement, attraction of neutrophils, and phagocytosis may result. Neutrophils and macrophages are commonly observed within glomeruli in membranous glomerulonephritis, and the local release of cytokines and ROS may contribute to glomerular injury. Heavy metals, hydrocarbons, penicillamine, and captopril can produce this type of glomerular injury. A chemical may function as a hapten attached to some native protein or as a complete antigen and elicit an antibody response. Antibody reactions with cell-surface antigens (e.g., GBM) lead to glomerular tissue injury.

Proximal Tubular Injury

The proximal tubule with its leaky epithelium is the most common site of toxicant-induced renal injury owing to the selective accumulation of xenobiotics. Tubular transport of organic anions and cations, low-molecular-weight proteins and peptides, GSH conjugates, and heavy metals is greater in the proximal tubule than in other segments, resulting in proximal tubular accumulation and toxicity. Segmental differences in cytochrome P450 and cysteine conjugate β-lyase, which are localized in the proximal tubule, also contribute to the enhanced susceptibility of the proximal tubule. Finally, proximal tubular cells appear to be more susceptible to ischemic injury than distal tubular cells. The proximal tubule is the primary site of toxicity for chemicals that interfere with RBF, cellular energetics, and/or mitochondrial function.

Loop of Henle/Distal Tubule/Collecting Duct Injury

Functional abnormalities at these sites manifest primarily as impaired concentrating ability and/or acidification defects. Amphotericin B, cisplatin, and methoxyflurane induce an ADH-resistant polyuria, suggesting that the concentrating defect occurs at the level of the medullary thick ascending limb and/or the collecting duct.

Papillary Injury

The initial target to the chronic injurious effects of abusive consumption of analgesics is the medullary interstitial cells, followed by degenerative changes in the medullary capillaries, loops of Henle, and collecting ducts. High papillary concentrations of potential toxicants and inhibition of vasodilatory prostaglandins compromise RBF to the renal medulla/papilla and result in tissue ischemia.

ASSESSMENT OF RENAL FUNCTION

Both in vivo and in vitro methods are available to assess the effects of a chemical on renal function. Initially, nephrotoxicity is assessed by evaluating serum and urine chemistries following treatment with the test chemical. Noninvasive tests include measurement of urine volume and osmolality, pH, and urinary composition (e.g., electrolytes, glucose, and protein).

Chemically induced increases in urine volume accompanied by decreases in osmolality may suggest an impaired concentrating ability, possibly via a defect in ADH synthesis, release, and/or action. Glucosuria may reflect defects in proximal tubular reabsorption of sugars or may be secondary to hyperglycemia. Urinary excretion of high-molecular-weight proteins, such as albumin, is suggestive of glomerular damage, whereas excretion of low-molecular-weight proteins, such as β_2-microglobulin, suggests proximal tubular injury. Urinary excretion of enzymes localized in the brush border (e.g., alkaline phosphatase and γ-glutamyl transpeptidase) may reflect brush-border damage, whereas urinary excretion of other enzymes (e.g., lactate dehydrogenase) may reflect more generalized cell damage. Enzymuria is often a transient phenomenon, as chemically induced damage may result in an early loss of most of the enzyme available. Thus, the absence of enzymuria does not necessarily reflect an absence of damage. Simultaneous analysis of cellular metabolites in sera and urine using metabonomic techniques may provide an additional technology to identify and monitor nephrotoxicity.

GFR can be quantified directly by determining creatinine or inulin clearance. Creatinine is an endogenous compound that is completely filtered with limited secretion. Inulin is an exogenous compound that is completely filtered with no reabsorption or secretion. Creatinine or inulin clearance is determined by the following formula:

$$\text{Insulin clearance (mL/min)} = \frac{\text{Insulin concentration in urine (mg/L)} \times \text{Urine volume (mL/min)}}{\text{Inulin concentration in serum (mg/L)}}$$

Serial blood urea nitrogen (BUN) and serum creatinine concentrations are indirect markers of GFR, and a 50% to 70% decrease in GFR must occur before increases in serum creatinine and BUN develop. Increases in BUN and/or serum creatinine may not necessarily reflect renal damage but rather may be secondary to dehydration, hypovolemia, and/or protein catabolism.

Cystatin C, a 13-kDa endogenous protein that inhibits cysteine proteases, is a viable candidate to replace serum creatinine in the measurement of GFR. It is produced at a constant rate by all tissues, freely filtered by the glomerulus and catabolized by tubular epithelial cells. Serum cystatin C levels appear to be independent of height, gender, age, muscle mass, and coexisting diseases and are more sensitive than creatinine in mildly impaired GFR.

Site-specific biomarkers for common nephrotoxicants and mechanisms of injury are illustrated in Fig. 14–7. Kidney injury molecule-1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL), *N*-acetyl-β-glucosaminidase (NAG), fatty acid–binding protein (FABP), hepatocyte growth factor (HGF), and albumin are promising biomarkers of AKI.

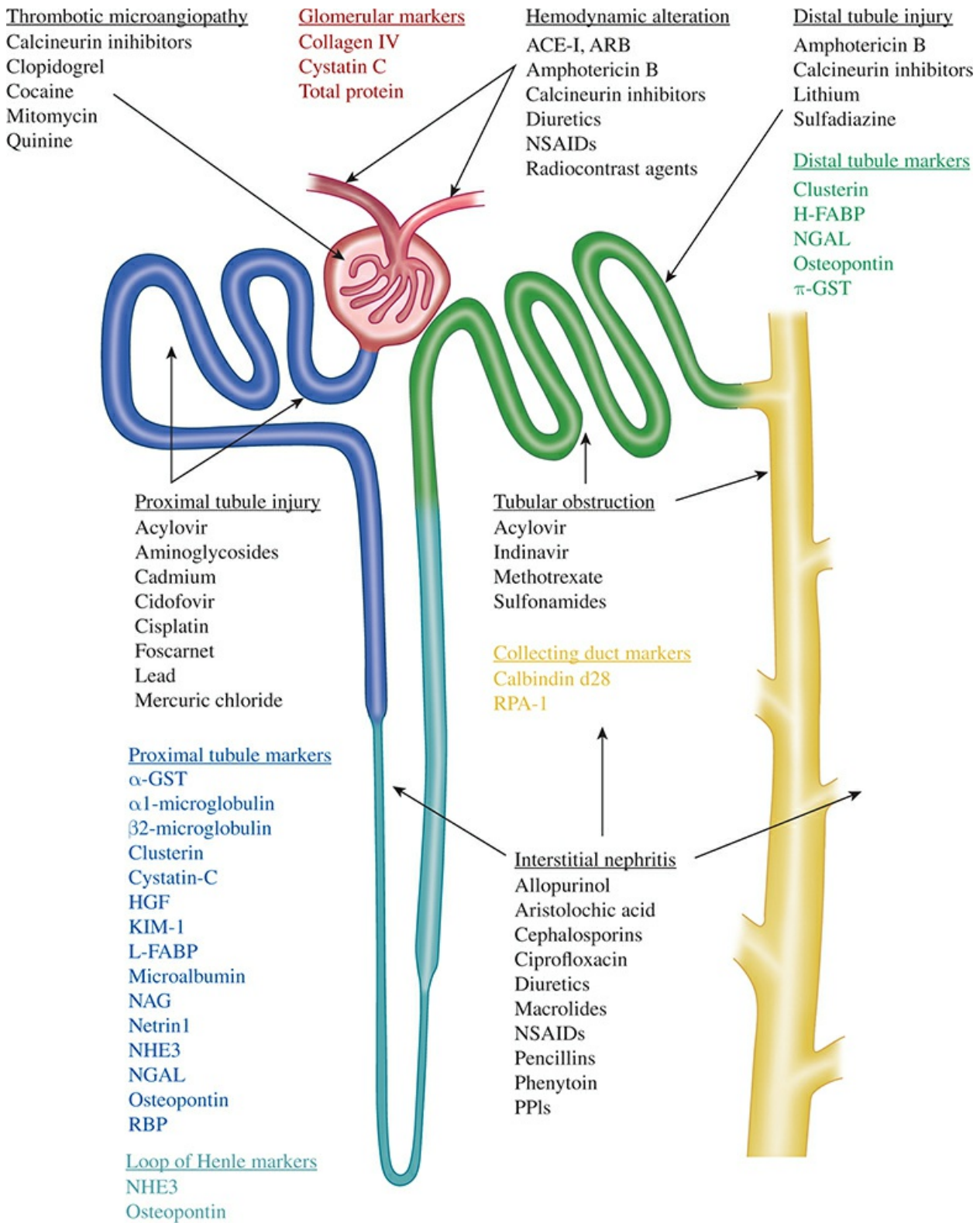

FIGURE 14–7 *Site-specific biomarkers, common nephrotoxicants, and mechanisms of injury.* (Reprinted from Vaidya VS, Bonventre JV, Ferguson MA. Biomarkers of acute kidney injury. In: McQueen CA, Schnellmann RG, eds. *Comprehensive Toxicology*. Vol 7. Oxford, UK:

2010:197–211.)

Histopathologic evaluation of the kidney following treatment is crucial in identifying the site, nature, and severity of a nephrotoxic lesion. Assessment of chemically induced nephrotoxicity, therefore, should include urinalysis, serum clinical chemistry, and histopathology to provide a reasonable profile of the functional and morphologic effects of a chemical on the kidney.

Various in vitro techniques may be used to elucidate underlying mechanisms. Freshly prepared isolated perfused kidneys, kidney slices, and renal tubular suspensions and cells exhibit the greatest degree of differentiated functions and similarity to the in vivo situation. However, these models have limited lifespans of 2 to 24 hours. In contrast, primary cultures of renal cells and established renal cell lines exhibit longer life spans (more than 2 weeks). Once a mechanism has been identified in vitro, the postulated mechanism must be tested in vivo. Thus, appropriately designed in vivo and in vitro studies should provide a complete characterization of the biochemical, functional, and morphologic effects of a chemical on the kidney and an understanding of the underlying mechanisms in the target cell population(s).

BIOCHEMICAL MECHANISMS/MEDIATORS OF RENAL CELL INJURY

Cell Death

Cell death may occur through either oncosis or apoptosis. Apoptosis is a tightly controlled, organized process that usually affects scattered individual cells, which break into fragments that are phagocytosed by adjacent cells or macrophages without producing an inflammatory response. In contrast, oncosis often affects many contiguous cells that rupture releasing cellular contents and inflammation follows. With many toxicants, lower injurious concentrations produce cell death through apoptosis, whereas increasing concentrations produce oncosis.

Mediators of Toxicity

A chemical may initiate toxicity due to its intrinsic reactivity with cellular macromolecules, may require renal or extrarenal biotransformation to a reactive intermediate, or may initiate injury indirectly by inducing oxidative stress via increased production of ROS, such as superoxide anion, hydrogen peroxide, and hydroxyl radicals. ROS and reactive nitrogen species from nitric oxide (peroxynitrite, $ONOO^-$) can attack proteins, lipids, and DNA to induce toxicity.

Cellular/Subcellular and Molecular Targets

Many cellular processes dependent on mitochondrial ATP become compromised simultaneously with inhibition of respiration. Mitochondrial dysfunction may be a consequence of some other cellular process altered by the toxicant. Whether toxicants target mitochondria directly or

indirectly, mitochondria play a critical role in determining whether cells die by apoptosis or oncosis. The mitochondrial permeability transition (MPT) is characterized by the opening of a high-conductance pore that allows solutes of less than 1500 molecular weight to pass. It is thought that the MPT occurs during cell injury and ultimately progresses to apoptosis if sufficient ATP is available, or oncosis if ATP is depleted.

Ca^{2+} is a second messenger that is involved in many cellular functions. Sustained elevations or abnormally large increases in cytosolic free Ca^{2+} can activate degradative Ca^{2+}-dependent enzymes, such as phospholipases and proteinases (e.g., calpains), and can produce aberrations in the structure and function of cytoskeletal elements.

Caspases are cysteine proteinases that play a role in the initiation and execution of renal cell apoptosis. Signaling kinases such as protein kinase C, mitogen-activated protein kinases (e.g., ERK1/2, p38, and JNK/SAPK), protein kinase B (Akt), src, and phosphoinositide-3-kinase phosphorylate other proteins and, thereby, alter their activity, expression, or localization. Numerous recent studies reveal critical roles for signaling kinases in renal cell death and in the recovery of renal cells after toxicant injury.

Cell volume and ion homeostasis are tightly regulated and are critical for the reabsorptive properties of the tubular epithelial cells. Toxicants can disrupt cell volume and ion homeostasis by interacting directly with the plasma membrane and increasing ion permeability or by inhibiting energy production. The loss of ATP during oncosis inhibits membrane transporters that maintain the internal ion balance and drive transmembrane ion movement. Following ATP depletion, Na^+, K^+-ATPase activity decreases, resulting in K^+ efflux, Na^+ and Cl^- influx, cell swelling, and ultimately cell membrane rupture. In contrast, the cell shrinkage that occurs during apoptosis is mediated by K^+ and Cl^- efflux.

SPECIFIC NEPHROTOXICANTS

Heavy Metals

Many metals, including cadmium, chromium, lead, mercury, platinum, and uranium, are nephrotoxic. The nature and severity of metal nephrotoxicity vary with respect to its form. Different metals have different primary targets within the kidney. Metals may cause renal cellular injury by binding sulfhydryl groups of critical proteins within cells, thereby inhibiting their normal function.

Mercury—The kidneys are the primary targets for accumulation of Hg^{2+}. Renal uptake of Hg^{2+} is rapid with as much as 50% of a nontoxic dose of Hg^{2+} found in the kidneys bound to an endogenous ligand, such as glutathione, cysteine, or albumin, or some plasma membrane Hg^{2+}-ligand complex. The acute nephrotoxicity induced by $HgCl_2$ is characterized by proximal tubular necrosis and AKI within 24 to 48 hours after administration. Early markers of $HgCl_2$-induced renal dysfunction include an increased urinary excretion of brush-border enzymes such as alkaline phosphatase and γ-GT. As injury progresses, tubular reabsorption of solutes and water decreases. A reduction in GFR results from the glomerular injury, tubular injury, and/or vasoconstriction.

Changes in mitochondria are early events following $HgCl_2$ administration, indicating that

mitochondrial dysfunction is an early and important contributor to inorganic mercury–induced cell death along the proximal tubule. Oxidative stress and dysregulation of Ca^{2+} homeostasis are also important in $HgCl_2$-induced renal injury. Also, an immunologically mediated membranous glomerular nephritis secondary to the production of antibodies against the GBM and the deposition of immune complexes may be involved.

Cadmium— Cadmium accumulates in the body over time and approximately 50% of the body burden of cadmium can be found in the kidney. Cadmium produces proximal tubule dysfunction (S_1 and S_2 segments) and injury characterized by increases in urinary excretion of glucose, amino acids, calcium, and cellular enzymes. This injury may progress to a chronic interstitial nephritis.

Following an oral exposure to $CdCl_2$, Cd^{2+} is thought to reach the kidneys both as Cd^{2+} and as a Cd^{2+}-metallothionein complex formed and released either from enterocytes or hepatocytes. The Cd^{2+}-metallothionein complex is freely filtered by the glomerulus and reabsorbed by the proximal tubule. Lysosomal degradation of the Cd^{2+}-metallothionein releases "free" Cd^{2+}, which initiates injury. Low concentrations of Cd^{2+} interfere with the normal function of several cellular signal transduction pathways.

Chemically Induced α_{2u}-Globulin Nephropathy

Unleaded gasoline, jet fuels, d-limonene, 1,4-dichlorobenzene, tetrachloroethylene, decalin, and lindane cause α_{2u}-globulin nephropathy or hyaline droplet nephropathy in male rats. The protein droplets accumulate in the S_2 segment of the proximal tubule and produce single-cell necrosis, the formation of granular casts at the junction of the proximal tubule and the thin loop of Henle, and cellular regeneration. Chronic exposure to these compounds results in progression of these lesions and ultimately in chronic nephropathy.

Humans are not at risk because (1) humans do not synthesize α_{2u}-globulin, (2) humans secrete fewer proteins in general and less low-molecular-weight proteins in urine than the rat, (3) the low-molecular-weight proteins in human urine either are not related structurally to α_{2u}-globulin, do not bind to compounds that bind to α_{2u}-globulin, or are similar to proteins in female rats, male Black Reiter rats, rabbits, or guinea pigs that do not exhibit α_{2u}-globulin nephropathy, and (4) mice excrete a low-molecular-weight urinary protein that is 90% homologous to α_{2u}-globulin, but they do not exhibit α_{2u}-globulin nephropathy and renal tumors following exposure to α_{2u}-globulin-nephropathy-inducing agents.

Halogenated Hydrocarbons

Halogenated hydrocarbons are a diverse class of compounds and are used extensively as chemical intermediates, solvents, and pesticides. Humans are exposed to these compounds not only in the workplace but also through the environment.

Chloroform—The primary cellular target is the proximal tubule, with no primary damage to the glomerulus or the distal tubule. Proteinuria, glucosuria, and increased BUN levels are all

characteristic of chloroform-induced nephrotoxicity. Chloroform is metabolized by renal cytochrome P450 to the reactive intermediate trichloromethanol, which is unstable and releases HCl to form phosgene. Phosgene can react with cellular macromolecules to initiate toxicity.

Tetrafluoroethylene—Tetrafluoroethylene is metabolized by hepatic GSH *S*-transferases to *S*-(1,1,2,2-tetrafluoroethyl)-glutathione, which is secreted into the bile and the small intestine where it is degraded to the cysteine *S*-conjugate (TFEC), reabsorbed, and transported to the kidney. The mercapturic acid may also be formed in the small intestine and reabsorbed. The cysteine *S*-conjugate is thought to be the penultimate nephrotoxic species. In the proximal tubule, the cysteine *S*-conjugate is a substrate for the cytosolic and mitochondrial forms of cysteine conjugate β-lyase. The products of the reaction are ammonia, pyruvate, and a reactive thiol that is capable of binding covalently to cellular macromolecules. The nephrotoxicity produced by haloalkenes is characterized morphologically as proximal tubular necrosis, and functionally by increases in urinary glucose, protein, cellular enzymes, and BUN.

Aristolochic Acid and Fungal Toxins

Mycotoxins are products of molds and fungi that include aflatoxin B_1, citrinin, ochratoxins, fumonisins, and patulin. Citrinin nephrotoxicity is characterized by decreased urine osmolality, GFR and RBF, glycosuria, and increased urinary enzyme excretion. Citrinin enters the cells through the organic anion transporter and causes mitochondrial dysfunction.

Fumonisins B_1 and B_2 are nephrotoxic, and cause disruption of the basolateral membrane, mitochondrial swelling, increased numbers of clear and electron-dense vacuoles, and apoptosis in proximal tubular cells at the corticomedullary junction. Changes in renal function include increased urine volume, decreased osmolality, and increased excretion of low and high-molecular-weight proteins. The fumonisins inhibit sphinganine (sphingosine) *N*-acyltransferase resulting in an increase in the ratio of free sphinganine to free sphingosine and a decrease in complex sphingolipids.

Aristolochic acids (AAs) and aristolactams are natural products found in the *Aristolochia* and *Asarum* genera that produce AA nephropathy. The renal dysfunction is characterized by tubular dysfunction, proteinuria, and interstitial fibrosis. AAs are a mixture of compounds that form covalent DNA adducts and are genotoxic and carcinogenic.

Therapeutic Agents

Acetaminophen—APAP nephrotoxicity is characterized by proximal tubular necrosis with increases in BUN and plasma creatinine; decreases in GFR and clearance of *para*-aminohippurate; increases in the fractional excretion of water, sodium, and potassium; and increases in urinary glucose, protein, and brush-border enzymes. Renal cytochrome P450 has been associated with APAP bioactivation to *N*-acetyl-*p*-amino-benzoquinoneimine, which arylates proteins in the proximal tubule and initiates cell death. However, glutathione conjugates of APAP may also contribute to APAP nephrotoxicity.

Nonsteroidal Anti-Inflammatory Drugs—At least three different types of nephrotoxicity have been associated with NSAID administration. AKI may occur within hours of a large dose of an

NSAID, is usually reversible upon withdrawal of the drug, and is characterized by decreased RBF and GFR and by oliguria. When the normal production of vasodilatory prostaglandins (e.g., PGE_2 and PGI_2) is inhibited by NSAIDs, vasoconstriction induced by circulating catecholamines and angiotensin II is unopposed, resulting in decreased RBF and ischemia. Risk factors (e.g., renal insufficiency, congestive heart failure, hepatic cirrhosis, hemorrhage, hypertension, sepsis, and diabetes) are known to facilitate the development of AKI after consumption of NSAIDs.

Chronic consumption of combinations of NSAIDs and/or APAP for more than 3 years can result in analgesic nephropathy. Impaired urinary concentration and acidification are the earliest clinical manifestations. The primary lesion in this nephropathy is papillary necrosis with chronic interstitial nephritis. The mechanism by which NSAIDs produce analgesic nephropathy is not known but may result from chronic medullary/papillary ischemia secondary to renal vasoconstriction, or may result from a reactive intermediate that initiates an oxidative stress, or binds covalently to critical cellular macromolecules.

The third, albeit rare, type of nephrotoxicity associated with NSAIDs is an interstitial nephritis, which is characterized by a diffuse interstitial edema with mild-to-moderate infiltration of inflammatory cells. Patients normally present with elevated serum creatinine, proteinuria, and nephritic syndrome. If NSAIDs are discontinued, renal function improves in 1 to 3 months.

Aminoglycosides—Renal dysfunction by aminoglycosides is characterized by a nonoliguric renal failure with reduced GFR, an increase in serum creatinine and BUN, and polyuria. Within 24 hours, increases in urinary brush-border enzymes, glucosuria, aminoaciduria, and proteinuria are observed. Histologically, lysosomal alterations are noted initially, followed by damage to the brush border, ER, mitochondria, and cytoplasm, ultimately leading to tubular cell necrosis. The earliest lesion observed following clinically relevant doses of aminoglycosides is an increase in the size and number of lysosomes that contain *myeloid bodies*, which are electron-dense lamellar structures containing undegraded phospholipids. This renal phospholipidosis may occur through their inhibition of lysosomal hydrolases, such as sphingomyelinase and phospholipases.

Amphotericin B—Amphotericin B is an antifungal drug whose clinical utility is limited by its nephrotoxicity. Amphotericin B nephrotoxicity is characterized by ADH-resistant polyuria, renal tubular acidosis, hypokalemia, and either acute or chronic renal failure. The functional integrity of the glomerulus and of the proximal and distal portions of the nephron is impaired. Some of the renal tubular cell effects of amphotericin B are due to the ability of this polyene to bind to cholesterol in the plasma membrane and form aqueous pores, which results in impaired proton excretion and renal tubular acidosis. The hypokalemia observed with amphotericin B may be due to an increase in luminal potassium ion permeability in the late distal tubule and the cortical collecting duct and the loss of potassium ions in the urine.

Cyclosporine—Cyclosporine nephrotoxicity may manifest as acute reversible renal dysfunction, acute vasculopathy, and chronic nephrotoxicity with interstitial fibrosis. Acute renal dysfunction is characterized by dose-related decreases in RBF and GFR and increases in BUN and serum creatinine. The decreased RBF and GFR are related to marked vasoconstriction induced by an imbalance in vasoconstrictor and vasodilatory prostaglandins, increased production of the vasoconstrictor thromboxane and endothelin, and activation of the renin-angiotensin system.

Acute vasculopathy or thrombotic microangiopathy is an unusual nephrotoxic lesion that affects arterioles and glomerular capillaries, without an inflammatory component, following

cyclosporine treatment. The lesion consists of fibrin-platelet thrombi and fragmented red blood cells occluding the vessels. Long-term treatment with cyclosporine can result in chronic nephropathy with interstitial fibrosis and tubular atrophy. Modest elevations in serum creatinine and decreases in GFR occur along with hypertension, proteinuria, and tubular dysfunction.

Cisplatin—Cisplatin nephrotoxicity includes acute and chronic renal failure, renal magnesium wasting, and polyuria, and patients treated with cisplatin regimens permanently lose 10% to 30% of their renal function. The nephrotoxicity of cisplatin can be grouped as (1) tubular toxicity, (2) vascular damage, (3) glomerular injury, and (4) interstitial injury. Early effects of cisplatin are decreases in RBF and polyuria that are concurrent with increased electrolyte excretion. The antineoplastic and perhaps the nephrotoxic effects of cisplatin may be due to its intracellular hydrolysis to the reactive mono-chloro-mono-aquodiammine-platinum or diaquo-diammine-platinum species and their ability to alkylate purine and pyrimidine bases. DNA synthesis, protein synthesis, glucose transport, Na^+, K^+-ATPase activity, and cell viability were all inhibited by cisplatin. These results suggest that cisplatin may produce acute nephrotoxicity through its ability to inhibit DNA synthesis as well as transport functions.

Radiocontrast Agents—Iodinated contrast media used for the imaging of tissues are (1) ionized at physiologic pH, (2) not significantly bound to protein, (3) restricted to the extracellular space, (4) almost entirely eliminated by the kidney, and (5) freely filtered by the glomerulus and neither secreted nor reabsorbed. These agents have a high osmolality (more than 1200 mOsm/L) and are potentially nephrotoxic, particularly in patients with existing renal impairment, diabetes, or heart failure or who are receiving other nephrotoxic drugs. Newer contrast agents (e.g., iotrol and iopamidol) are nonionic and less nephrotoxic. The nephrotoxicity of these agents is due to both hemodynamic alterations (vasoconstriction) and proximal tubular injury via ROS.

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QUESTIONS

1. The kidney is responsible for all of the following EXCEPT:
   - **a.** synthesis of renin.
   - **b.** acid–base balance.
   - **c.** reabsorption of electrolytes.
   - **d.** regulation of extracellular fluid.
   - **e.** release of angiotensin.

2. Which of the following does NOT contribute to filtrate formation in the nephron?
   a. capillary hydrostatic pressure.
   b. positive charge of glomerular capillary wall.
   c. hydraulic permeability of glomerular capillary wall.
   d. colloid oncotic pressure.
   e. size of filtration slits.

3. Which of the following is NOT a characteristic of the loop of Henle?
   a. There is reabsorption of filtered Na^+ and K^+.
   b. Tubular fluid in the thin descending limb is iso-osmotic to the renal interstitium.
   c. Water is freely permeable in the thin ascending limb.
   d. Na^+ and Cl^- are reabsorbed in the thin ascending limb.
   e. The thick ascending limb is impermeable to water.

4. The kidneys constitute 0.5% of total body mass and receive approximately how much of the resting cardiac output?
   a. 0.5% to 1 %.
   b. 5%.
   c. 10%.
   d. 20% to 25%.
   e. 50% to 60%.

5. Which of the following is most likely to occur after a toxic insult to the kidney?
   a. GFR will decrease in the unaffected kidney.
   b. Tight-junction integrity will increase in the nephron.
   c. The unaffected cells will undergo atrophy and proliferation.
   d. Clinical tests will likely show normal renal function.
   e. Glomerulotubular balance is lost.

6. Chronic renal failure does not typically result in:
   a. decrease in GFR of viable nephrons.
   b. glomerulosclerosis.
   c. tubular atrophy.
   d. increased glomerular pressures.
   e. altered capillary permeability.

7. All of the following statements regarding toxicity to the kidney are true EXCEPT:
   a. Concentration of toxins in tubular fluid increase the likelihood that the toxin will diffuse into tubular cells.
   b. Drugs in the systemic circulation are delivered to the kidneys at relatively high amounts.
   c. The distal convoluted tubule is the most common site of toxicant-induced renal injury.
   d. Immune complex deposition within the glomeruli can lead to glomerulonephritis.

e. Antibiotics and/or antifungal drugs affect the functioning of the nephron at multiple locations.

8. Which of the following test results is NOT correctly paired with the underlying kidney problem?
   a. increased urine volume—defect in ADH synthesis.
   b. glucosuria—defect in reabsorption in the proximal convoluted tubule.
   c. proteinuria—glomerular damage.
   d. proteinuria—proximal tubular injury.
   e. brush-border enzymuria—glomerulonephritis.

9. Renal cell injury is NOT commonly mediated by which of the following mechanisms?
   a. loss of membrane integrity.
   b. impairment of mitochondrial function.
   c. increased cytosolic Ca^{2+} concentration.
   d. increased Na^{+},K^{+}-ATPase activity.
   e. caspase activation.

10. Which of the following statements is FALSE with respect to nephrotoxicants?
    a. Mercury poisoning can lead to proximal tubular necrosis and acute renal failure.
    b. Cisplatin may cause nephrotoxicity because of its ability to inhibit DNA synthesis.
    c. Chronic consumption of NSAIDs results in nephrotoxicity that is reversible with time.
    d. Amphotericin B nephrotoxicity can result in ADH-resistant polyuria.
    e. Acetaminophen becomes nephrotoxic via activation by renal cytochrome P450.

CHAPTER 15

Toxic Responses of the Respiratory System

George D. Leikauf

KEY POINTS

- Inhaled xenobiotics can affect lung tissues directly or distant organs after absorption.
- Water solubility is a decisive factor in determining how deeply a given gas penetrates into the lung.
- Particle size is usually the critical factor that determines the region of the respiratory tract in which a particle or an aerosol will deposit.
- The lung contains most of the enzymes involved in xenobiotic biotransformation that have been identified in other tissues.
- Asthma is characterized by increased reactivity of the bronchial smooth muscle in response to exposure to irritants.
- In emphysema, destruction of the gas-exchanging surface area results in a distended, hyperinflated lung that no longer effectively exchanges oxygen and carbon dioxide.

RESPIRATORY TRACT STRUCTURE AND FUNCTION

Oronasal Passages

Structure—The respiratory tract is divided into the upper respiratory tract (extrathoracic airway passages above the neck) and lower respiratory tract (airway passages and lung parenchyma below the pharynx) (Fig. 15–1). The upper respiratory tract reaches from the nostril or mouth to the pharynx and functions to conduct, heat, humidify, filter, and chemosense incoming air. Leaving the nasal passage, air is warmed to about 33°C and humidified to about 98% water saturation. Air is filtered in the nasal passages with highly water-soluble gases being absorbed efficiently. The nasal passages also filter particles, which may be deposited by impaction or diffusion on the nasal mucosa.

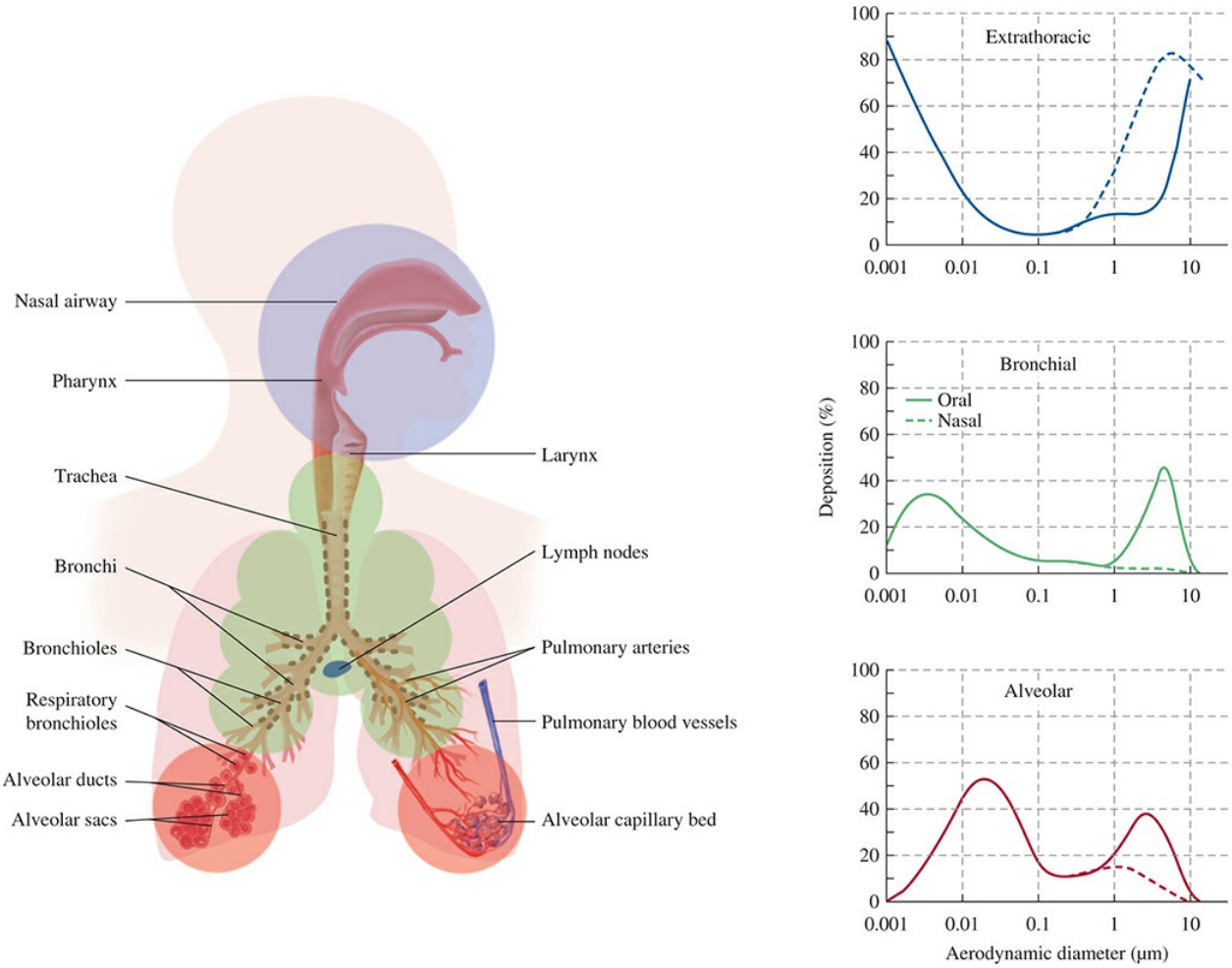


FIGURE 15–1 *Major regions of the respiratory tract and predicted fractional deposition of inhaled particles in the extrathoracic, bronchial, and alveolar region of the human respiratory tract during (solid line) oral or (dashed) nasal breathing.* (Adapted from Oberdörster G, Oberdörster E, Oberdörster J. Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. *Environ Health Perspect.* 2005;113:823–839.)

The airflow through the nasal passage is complex, and the resistance of this region limits the amount of air that can be inhaled through the nose. The nasal passages are lined with stratified squamous epithelium in the anterior vestibule, nonciliated cuboidal/columnar epithelium in the anterior chamber, and ciliated pseudostratified respiratory epithelium in the remainder of the passage including the turbinates. The turbinates also contain airflow pressure- and temperature-sensing neural receptors linked to the trigeminal nerve.

Sensory Functions—Humans can distinguish more than 5000 odors. Although the detection threshold concentrations can be low, a concentration only 10 to 50 times above the detection threshold value often is the maximum intensity that can be detected by humans. For this reason, smell often identifies the presence or absence of odor rather than quantifies concentration. In addition, odor thresholds vary greatly between individuals (>1000 fold) and can be altered by allergies or nasal infections, and individuals can acclimate to odors. The chemosensory functions of the nasal passages are accomplished by specialized receptors in major subtypes including (1)

olfactory, (2) trace amine–associated receptors (TAARs), (3) membrane guanylyl cyclase GC-D, (4) vomeronasal, and (5) formyl peptide receptors (FPRs).

Olfactory receptors are 7-transmembrane domain G-protein–coupled receptors that mediate transduction of odorant signals through formation of cyclic adenosine monophosphate (cAMP). TAARs detect amines (including 2-phenylethylamine, tyramine, tryptamine, and octopamine) and other substances. Low-molecular-weight amines have a fishy or putrid odor. Another olfactory sensory neuron receptor is the membrane guanylyl cyclase receptor, GUCY2D, which contains a cyclic guanosine monophosphate (cGMP)-dependent phosphodiesterase PDE2A and a cGMP-sensitive cyclic nucleotide-gated ion channel. Vomeronasal neurons respond to higher molecular weight stimuli including nonvolatile chemicals. In addition, the vomeronasal organ contains FPRs that are activated by bacterial or mitochondrial formylated peptides.

Irritant, Thermosensory, and Mechanosensory Functions—The detection of irritant chemicals, cold and hot temperatures, or mechanical stress can be a protective mechanism that may limit exposure. The chemical nociceptors perceive irritants, temperature, and mechanical stress. Two protein families, the transient receptor potential (TRP) channels and the taste (TAS) receptors, perform these functions in the upper respiratory tract.

TRP channels are ion channels that are permeable to cations, including calcium, magnesium, and sodium. The TRP ion channel proteins are divided into six subfamilies including TRPA (ANKTM1), TRPC (canonical), TRPM (melastatin), mucolipins (TRPML, also known as [aka] MCOLN), polycystic kidney disease (autosomal dominant) (PCK or TRPP), and TRPV (vanilloid) families. TRPA1 is responsive to allyl isothiocyanate (in mustard and wasabi), cinnamaldehyde (in cinnamon), allicin and allyl sulfides (in garlic and onion), carvacrol (in oregano), isovelleral (a fungal deterrent), and polygodial (in Dorrigo pepper). TRPA1 is also responsive to pain stimuli, cold (≤17°C), stretch, and a wide range of chemical irritants. TRPV1 is responsive to capsaicin (in chili pepper) or moderate heat (≥43°C), whereas TRPV2 is responsive to higher heat (≥52°C). TRPM8 is responsive to menthol (in peppermint and cigarettes) and cold (≤28°C).

Taste bud receptors determine salt, sour, sweet, umami (glutamates and nucleotides), and bitter. Sour also may be perceived by hydrogen ion channels and possibly a TRP channel (polycystic kidney disease 2-like 1). Sweet and umami are perceived by type 1 receptors, which consist of three members (TAS1R1, TAS1R2, and TAS1R3). Taste variety is achieved by formation heterodimers of these proteins. Bitter taste is detected by type 2 receptors (TAS2Rs).

Conducting Airways

Structure—At the beginning of the lower respiratory tract is the larynx, which is responsible for speech (phonation). The conducting airways of the lower respiratory tract can be divided into trachea, bronchi, and distal bronchioles. Conducting airways have a bifurcating structure, with successive airway generations containing about twice the number of bronchi progressively decreasing in internal diameter. Successive branching has two consequences—it increases total surface area of the airway epithelium, and it increases the cumulative cross-section diameter of the airways. Thus, airflow is faster in the larger diameter proximal airways but slower in the smaller distal airways.

The bifurcations of proximal airways are flow dividers. As airway bending points, they serve as sites of impaction for particles. The airflow is also altered by airway smooth muscle that

surrounds the airways. Vagal nerve endings release acetylcholine, which contracts smooth muscle and induces mucus secretion. Contraction of the smooth muscle leads to bronchoconstriction that increases particle deposition in the proximal airway upon inhalation and reduces airflow upon exhalation. The contraction of airway smooth muscle can be reversed by adrenergic stimulation either by circulating epinephrine or by inhaled bronchodilators that stimulate beta-2 adrenergic receptors on airway smooth muscle.

Cartilaginous airways (bronchi) give way to noncartilaginous airways (bronchioles), which in turn give way to gas exchange regions, respiratory bronchioles and alveoli. Although the airways are successively narrower in diameters, the cumulative diameter of the airways becomes large ultimately leading to slow or no net airflow. This favors the collection of gases and particles on airway walls by radial diffusion or sedimentation. In the bronchiolar epithelium, mucus-producing cells and glands give way to bronchiolar secretoglobin cells (BSCs).

Mucociliary Clearance and Antimicrobial Functions—In humans, the proximal airway and a portion of the nasal passage are covered by a pseudostratified respiratory epithelium that contains ciliated, mucous, and basal cells (Fig. 15–2). These cells work together to form a mucous layer that traps and removes inhaled material via mucociliary clearance. The epithelial cells are covered by an upper mucus layer (a gel-like polymer of high-molecular-weight mucins) and a lower periciliary liquid layer that separates the epithelial cell surface from the mucus layer. For mucociliary clearance in the airways to function optimally, regulation of ion transport, fluid, and mucus must be coordinated. To move fluid into the airway lumen, the large diameter airway epithelium can secrete chloride ions via chloride channels and the cystic fibrosis transmembrane regulator. To move water out of the lumen or alveolus, sodium ions are absorbed via sodium channels. These ionic gradients permit water movement that can travel pericellulary or through specialized proteins called aquaporins.

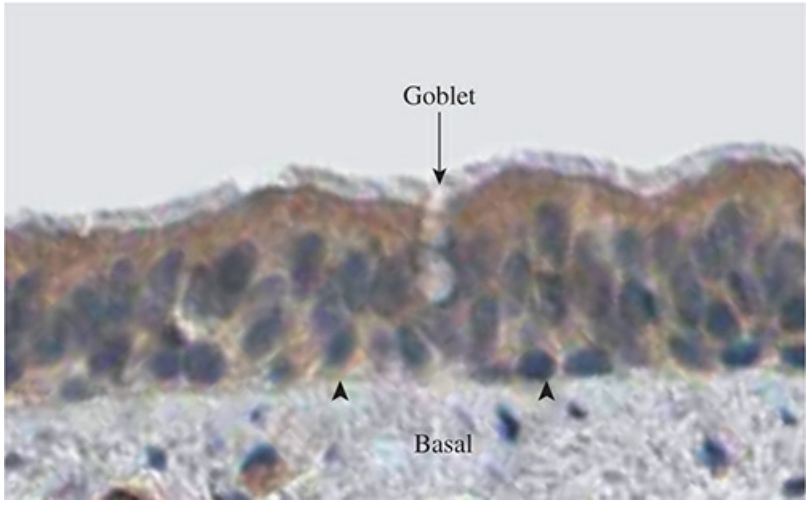


FIGURE 15–2 *Pseudo stratified respiratory epithelium lines the nasal cavity, trachea, and bronchi*. The surface includes mainly ciliated epithelial cells that may or may not touch the basement membrane, (arrow) surface mucous (goblet) cell, and (arrowhead) basal cells. (Photomicrograph modified from the Human Protein Atlas (www.proteinatlas.org). Orginal source: Uhlen M, Oksvold P, Fagerberg L, et al. Towards a knowledge-based Human Protein Atlas. *Nat Biotechnol.* 2010;28:1248–1250.)

Ciliated cells have cilia that are microtubule-based, apical membrane protrusions. Motile cilia exert mechanical force through continuous motion to propel harmful inhaled material out of the nose and lung. Primary cilia often serve as sensory organelles. Ciliary beat frequency is about 720 to 900 beats/minute, which can change in response to cholinergic (acetylcholine) or purinergic (adenosine or ATP) stimuli. In addition to controlling ciliary beat frequency, calcium is also involved in synchronizing the beat among cilia of a single cell and between cilia on different cells. Adenosine acts through the adenosine A2b receptor (ADORA2B). Motile cilia exhibit both mechanosensory (via TRPV4) and chemosensory (via TAS2Rs) functions. TRPV4 channels respond to mechanical stress, heat, acidic pH, endogenous and synthetic agonists, and activation leads to increases in intracellular calcium and ciliary beat frequency. In response to bitter compounds, TAS2Rs also increase the intracellular calcium and stimulate ciliary beat frequency.

Mucus cells also called goblet cells are full of lucid mucus granules. Mucus consists mainly of water (95%) combined with salts, lipids, proteins, and mucin glycoproteins. Mucin glycoproteins provide the gel-like viscoelastic properties of mucus. Serous cells contain and secrete a less viscous fluid and are enriched in antimicrobial proteins. Another airway secretory cell is the bronchiolar secretoglobin cell (BSC), previously called the Club or Clara cell. BSCs have an extensive endoplasmic reticulum and secretory granules containing secretoglobins. In humans, BSCs are found mainly in the distal airways and can act as tissue stem cells.

Neuroendocrine cells are contained in neuroepithelial bodies or separately in the proximal airways and contact can stimulate underlying sensory nerve fibers. They synthesize, store, and release bioactive substances including 5-hydroxytryptamine (aka serotonin), calcitonin-related polypeptide α (aka calcitonin), and gastrin-releasing peptide (aka bombesin). These cells express cholinergic, nicotinic, and α polypeptide 7 (CHRNA7) receptors and release serotonin in response to nicotine, and following hypoxia or mechanical strain. The release of these bioactive substances can redistribute pulmonary blood flow and alter bronchomotor tone and immune responses.

Gas Exchange Region

Structure—The gas exchange region consists of terminal bronchioles, respiratory bronchioles, alveolar ducts, alveoli, blood vessels, and lung interstitium (Fig. 15–3). A ventilatory unit is defined as an anatomical region that includes all alveolar ducts and alveoli distal to each bronchiolar–alveolar duct junction. Gas exchange occurs in the alveoli, which comprise ~85% of the total parenchymal lung volume. Adult human lungs contain an estimated 300 to 500 million alveoli. Capillaries, blood plasma, and formed blood elements are separated from the air space by a thin layer of tissue formed by epithelial, interstitial, and endothelial components.

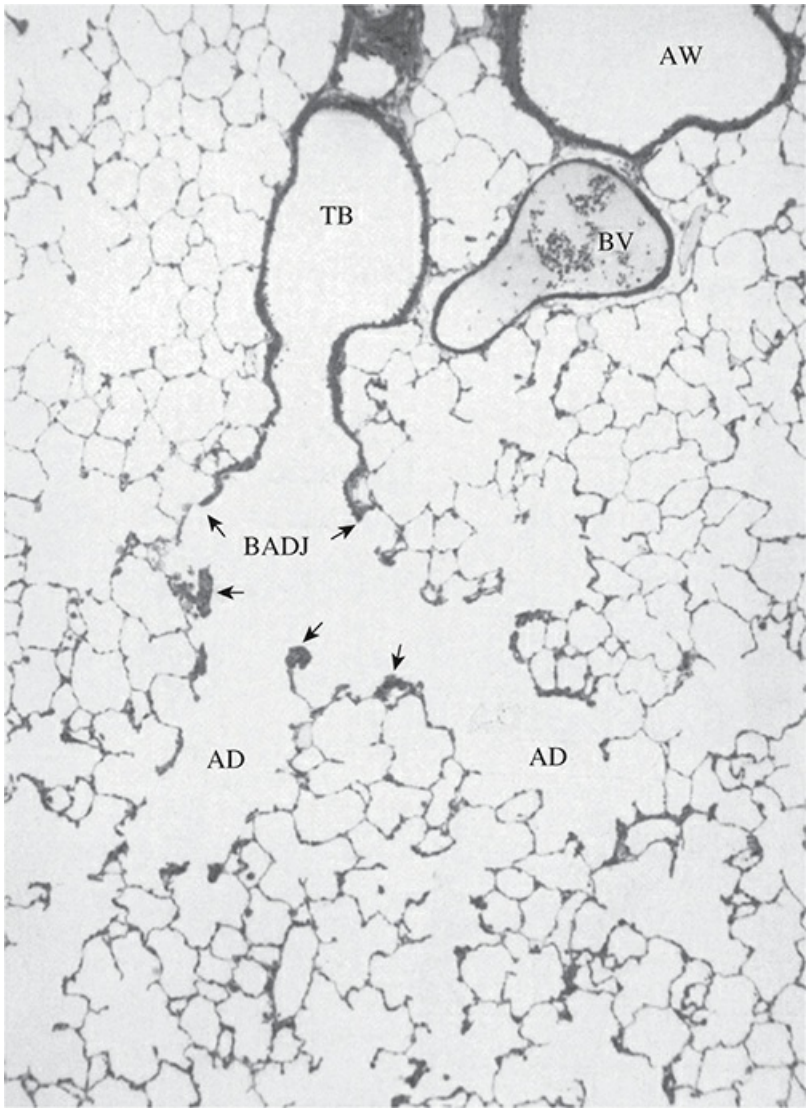


FIGURE 15–3 *Centriacinar region (ventilatory unit) of the lung*. An airway (AW) and a blood vessel (BV) (arteriole) are in close proximity to the terminal bronchiole (TB). The terminal bronchiole leads to the bronchiole–alveolar duct junction (BADJ) and the alveolar duct (AD). A number of the (arrows) alveolar septal tips close to the BADJ are thickened after a brief (4-hour) exposure to asbestos fibers, indicating localization of fiber deposition. Other inhalants, such as ozone, produce lesions in the same locations. (Used with permission from Dr. Kent E. Pinkerton, University of California, Davis.)

The alveolar epithelium consists of two cells, the alveolar type I and type II cells (Fig. 15–4). Alveolar type I cells cover ~95% of the alveolar surface, are susceptible to damage by noxious agents that penetrate to the alveolus, and have an attenuated cytoplasm to enhance gas exchange. Alveolar type II cells are cuboidal and have abundant perinuclear cytoplasm, extensive secretory capacity, and contain secretory vesicles called lamellar bodies. They produce surfactant, a mixture of lipids, and four surfactant associated proteins and can undergo mitotic division and replace damaged type I cells. The shape of type I and type II cells is independent of alveolar size and is remarkably similar in different species.

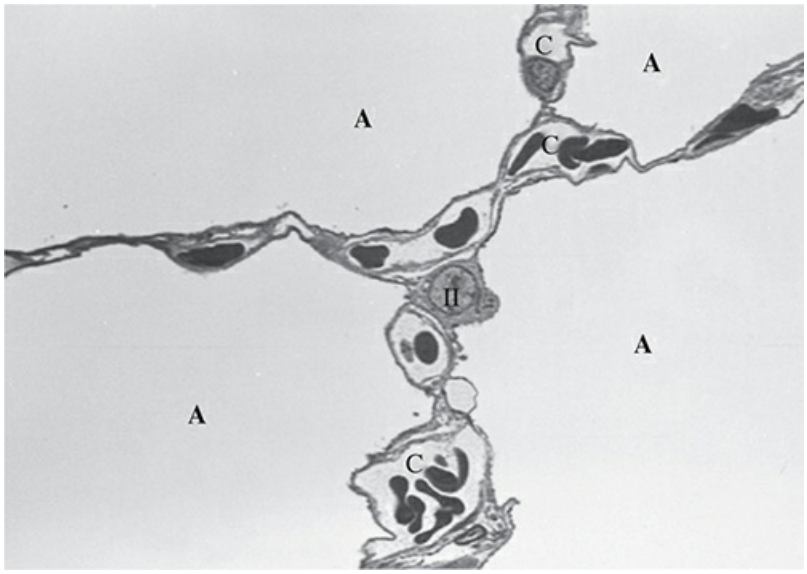


FIGURE 15–4 *Alveolar region of the lung*. The (A) alveolus is separated by the thin air-to-blood tissue barrier of the alveolar septal wall, which is composed of flat alveolar type I cells and occasional rounded (II) alveolar type II cells. A small interstitial space separates the epithelium and endothelium that form the (C) capillary wall. During lung injury the interstitial space enlarges and interferes with gas exchange. (Used with permission from Dr. Kent E. Pinkerton, University of California, Davis.)

The mesenchymal interstitial cell population consists of fibroblasts and myofibroblasts that produce collagen and elastin as well as other cell matrix components and various effector molecules. Pericytes, monocytes, and lymphocytes also reside in the interstitium, as do macrophages before they enter the alveoli. Endothelial cells have a thin cytoplasm and cover about one-fourth of the area covered by type I cells.

Ventilation—The principal function of the lung is gas exchange, which consists of ventilation, diffusion, and perfusion. During inhalation, fresh air moves into the lung through the upper respiratory tract and conducting airways and into the terminal respiratory units when the thoracic cage enlarges and the diaphragm moves downward; the lung passively follows this expansion. The thoracic cage enlarges mainly by the constriction of external intercostal and internal (interchondral part) intercostal muscles, which elevate the sternum and ribs and thus increase the width of the thoracic cavity. After diffusion of oxygen into the blood and that of CO_2 from the blood into the alveolar spaces, the air now enriched in CO_2 is exhaled. Relaxation of the chest wall and diaphragm diminishes the internal volume of the thoracic cage, the elastic fibers of the lung parenchyma recoil, and air is expelled from the alveolar zone through the airways. Any interference with the elastic properties of the lung adversely affects ventilation.

Lung function changes with age and disease and can be measured with a spirometer (Fig. 15–5). The total lung capacity (TLC) is the total volume of air in an inflated human lung, 4 to 5 L (women) and 6 to 7 L (men). After a maximum expiration, the lung retains 1.1 L (women) and 1.2 L (men), which is the residual volume (RV). The vital capacity (VC) is the air volume moved into and out of the lung during maximal inspiratory and expiratory movement and is about 3.1 L (women) and 4.8 L (men). Only a small fraction of the VC, the tidal volume (TV), is typically

moved into and out of the lung during quiet breathing. In resting humans, the TV measures ~0.5 L with each breath. The respiratory frequency is 12 to 20 breaths per minute, and the resting ventilation is about 6 to 8 L/min.

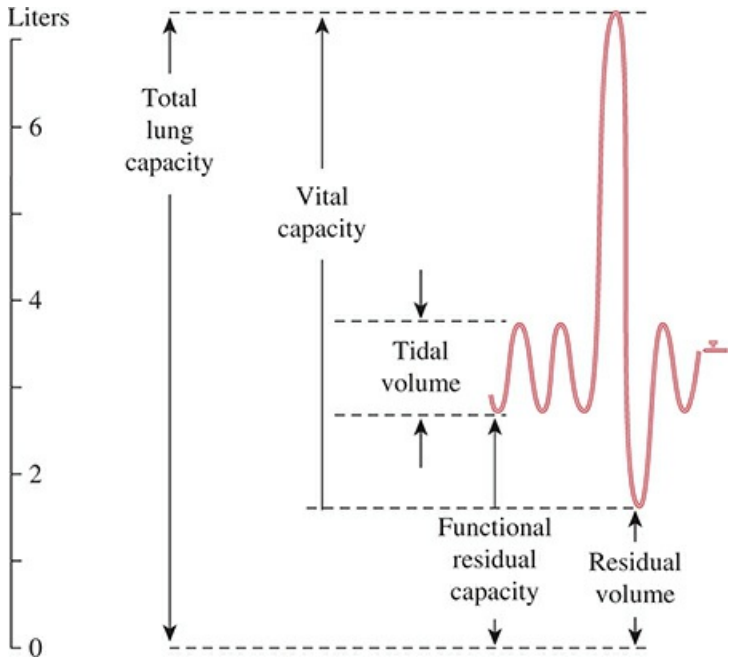


FIGURE 15–5 *A spirometer reading of lung volumes*. The total lung capacity is the total volume of air in an inflated human lung. After a maximum expiration, the lung retains a small volume of air, which is the residual volume. The vital capacity is the air volume moved into and out of the lung during maximal inspiratory and expiratory movement. The tidal volume is typically moved into and out of the lung during each breathe. The functional residual capacity and residual volume cannot be measured with a spirometer.

Lung function changes with age and disease and can be quantified by a forced expiratory maneuver with a spirometer. In this test, an individual first inhales maximally and then exhales as rapidly as possible. The volume of air expired in 1 second is the forced expiratory volume in 1 second (FEV1), and the total amount expired is the forced vital capacity (FVC). In a healthy individual the FEV1/FVC = ~80%.

Diffusion—Gas exchange takes place across the entire alveolar surface. Contact with an airborne toxic chemical thus occurs over a surface of ~140 m^2. Various abnormal processes may severely compromise the unhindered diffusion of oxygen to the erythrocytes. Acute events may include collection of liquid in the alveolar or interstitial space and disruption of the pulmonary surfactant system. Chronic toxicity can impair diffusion due to abnormal alveolar architecture or abnormal formation and deposition of extracellular substances such as collagen in the interstitium.

Perfusion—The lung receives the entire output from the right ventricle, ~75 mL of blood per heartbeat. Blood with high CO_2 and low O_2 travels to the lung via the pulmonary artery and leaves the lung with high O_2 and low CO_2 via the pulmonary vein. Substantial amounts of toxic chemicals carried in the blood can be delivered to the lung. A chemical entering the peripheral

venous system travels the pulmonary capillary bed before distribution to other organs or tissues in the body.

BIOTRANSFORMATION IN THE RESPIRATORY TRACT

The lung has substantial capabilities for biotransformation. Total lung cytochrome P450 (CYP) activity is roughly one-tenth to one-third of that in the liver. Metabolic competence in the lung and nasal tissues is concentrated in a few cell types that have a defined distribution in the respiratory tract. The balance of activation and inactivation is a critically important determinant of lung protection from injury. Protection from oxidation is another important function because oxygen concentrations in the respiratory tract are high relative to other organs.

BSCs have the most CYP, followed by alveolar type II cells. Other phase I enzymes found in lung tissue include epoxide hydrolases, flavin monooxygenases, prostaglandin-endoperoxide synthases, carbonyl reductases, and NAD(P)H:quinone oxidoreductase 1 (NQO1). There is a lack of uniformity in their expression by cell type and region throughout the lung and a tendency to concentrate in epithelia.

Phase II enzymes include glutathione-*S*-transferases (GSTs) (alpha, mu, and pi), glucuronosyl transferases, and sulfotransferases (SULTs). GSTs and glutathione play a major role in the modulation of both acute and chronic chemical toxicity in the lung. These enzyme systems work in concert with one another (i.e., a decrease in one enzyme may result in a concomitant increase in another) and it is the combined action of all enzymes, and their location, that determines toxicity. The regulation of many of these enzymes is under coordinated control of the transcription factor nuclear factor, erythroid derived 2, like 2 (NFE2L2, aka NRF2).

A major determinant of the potential for detoxification may also be the cellular localization of, and ability to synthesize, glutathione in the lung. The distribution of the isoforms of glutathione *S*-transferase varies by lung region with the alpha, mu, and pi isoforms. The alpha and pi classes are the predominate isoforms in the airway epithelium of human lung. In nasal tissue, glutathione *S*-transferases are found in the olfactory mucosa. Glucuronosyl transferase and sulfotransferase activities are found in nasal and lung tissue.

GENERAL PRINCIPLES IN THE PATHOGENESIS OF LUNG DAMAGE CAUSED BY CHEMICALS

Toxic Inhalants, Gases, and Dosimetry

The sites of deposition of gases in the respiratory tract define their pattern of toxicity. Solubility, diffusivity, and metabolism/reactivity in respiratory tissues and breathing rate are the critical factors in determining how deeply a given gas penetrates into the lung. Highly soluble gases such

as SO_2 do not penetrate farther than the nose and are relatively nontoxic to the lung of rats. Relatively insoluble gases such as ozone and NO_2 reach the smallest airways and the alveoli to elicit toxic responses. Mathematical models of gas entry and deposition in the lung predict sites of lung lesions fairly accurately. Insoluble gases such as CO and H_2S efficiently pass through the respiratory tract and are taken up by the pulmonary blood supply to be distributed throughout the body.

Regional Particle Deposition

Particle size is a critical determinant of where a particle will be deposited. The efficiency of particle deposition in various regions of the respiratory tract depends mainly on particle size. Particles in air are classified by particle size. Size controls particle shape and thus influences deposition by interception. Size also controls particle mass and thus influences the probability for coagulation, dispersion, sedimentation, and impaction. Particles can be monodispersed (essentially of one size like pollens) or, more typically, heterodispersed (many difference sizes). Particle surface area is of special importance when the surface is reactive with biomolecular targets or when toxic materials are adsorbed on particles and thus are carried into the lung.

In respiratory toxicology, aerosols (particles dispersed into air) include any of the following: (1) dusts (≥1.0-μm particles generated by mechanical division as in grinding), (2) fumes (≤0.1-μm particles generated by condensation of vapors as in heat metals or oils), (3) smoke (≤0.5-μm complex carbon particles generated condensation of products from combustion), (4) mists (2- to 50-μm water droplet or solutions generated by mechanical shearing of bulk liquid as in spraying), (5) fog (≤1.0-μm water droplets generated by water vapor condensation on atmospheric nuclei), or (6) smog (≥0.01- to 50-μm air pollution generated by stationary and mobile pollution source). Smaller aerosols include submicrometer particles ($0.1 \leq x \leq 1.0$ μm) and nanometer particles or nanoparticles (≤0.1 μm). All these distinguishing forms are included in the term “aerosol” or “particle.”

Atmospheric particles originate either as primary particles—by direct emission from a source—or as secondary particles—through atmospheric formation from the gas phase constituents (nucleation) (Fig. 15–6). Atmospheric particles are typically distributed into two modes and five submodes. The two modes are the accumulation and coarse modes, which dominate the particle volume (and therefore mass) distribution.

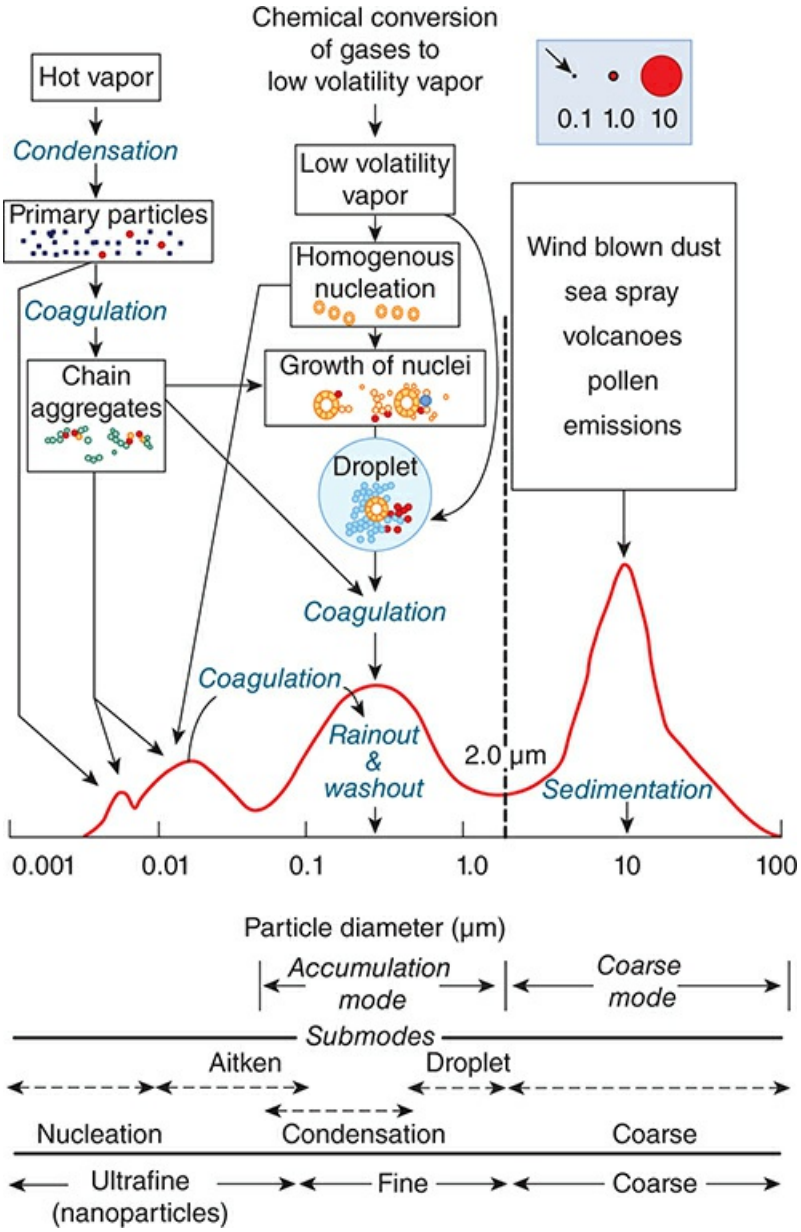


FIGURE 15–6 *Typical distribution of atmospheric particles consists of two major modes and five submodes*. The Accumulation and Coarse modes dominate the particle mass and Nucleation and Aitken submodes dominate the particle number. Nucleation particles are generated from gas phase emissions. Condensation can occur as plumes cool and particles and gases emitted together interact. Particles in the first two submodes typically have short half-lives as singlet primary particles. The condensation submode is formed from the coagulation of smaller solid particles and condensation of gases including sulfates, nitrates, and organics on the particles' surface. Particles move from the smaller submodes into the accumulation mode and these larger particles can have long half-lives (hours to days) in the atmosphere and can travel over long distances. When the atmospheric relative humidity is high (near 100%), particles in the accumulation mode seed rain droplets and are removed from the atmosphere. The coarse particles consist of particles >2.0 µm that are generated by mechanical processes or suspension of surface dust. Many of the particles in this range can be from natural sources

(e.g., windblown desert sand). Particles larger than 50 μm readily settle and are removed from the atmosphere within minutes. Human exposure to these large particles is typically occupational (e.g., dust from grinding wheels or wood sanding that is inhaled due to proximity to the source). Inset: The volume, and therefore mass, is to the cubed root of the radius. The size and the physical forces that maintain particles in the atmosphere are why the total mass is mainly in the accumulation and coarse modes.

Dominating the particle number distribution are the nucleation and Aitken submodes. The smallest nucleation submode particles are generated from gas phase emissions. This mode is dominated by a large number of nanoparticles ≤0.01 μm, but because they are so small they do not add much to the cumulative volume and therefore have little mass. The second submode is the Aitken nucleus submode, which also consists of nanoparticles ($0.01 \leq x \leq 0.1$ μm). Most Aitken particles are primary particles that have grown due to material condensing on their surface as they move through the atmosphere. Condensation can occur as plumes cool and particles and gases emitted together interact.

The next largest submode is the condensation submode ($0.05 \leq x \leq 0.5$ μm). These particles are formed from the coagulation of smaller solid particles and the condensation of gases including sulfates, nitrates, and organics on the particles' surface. They can be formed from chain aggregates of numerous particles of smaller diameter or also can be spherical with liquid surfaces. When these particles are hydroscopic and in a humid atmosphere, the size can increase to about 0.5 to 2.0 μm and become the droplet submode. Particles in the condensation and droplet submode contribute to the accumulation mode. These particles can have long half-lives (hours to days) in the atmosphere and can travel over long distances from sources because they are too few in number for rapid coagulation and too small for gravitational sedimentation. The second mode is the coarse particle and contains the coarse submode, which are particles >2.0 μm that are generated by mechanical processes or suspension of surface dust. Many of the particles in this range can be from natural sources (e.g., windblown desert sand). Particles larger than 50 μm settle readily and are removed from the atmosphere within minutes. Human exposure to these large particles is typically occupational (e.g., dust from grinding wheels or wood sanding that is inhaled due to proximity to the source).

The upper respiratory tract is efficient in removing particles that are large (>10 μm) or small (<0.01 μm) (Fig. 15–1). During nasal breathing, 1- to 10-μm particles are usually deposited in the upper nasopharyngeal region or the first five generations of large conducting airways. During oral breathing, deposition of these particles can increase in the tracheobronchial airways and alveolar region. Smaller particles (0.001 to 0.1 μm) can also be deposited in the tracheobronchial region. Particles ranging from 0.003 to 5 μm can be transported to the smaller airways and deposited in the alveolar region. Patterns of breathing can change the site of deposition of a particle of a given size. Materials that are hygroscopic, such as sodium chloride, sulfuric acid, and glycerol, take on water and enlarge in the warm, saturated atmosphere of the upper and lower respiratory tract.

Deposition Mechanisms

Particles deposit by impaction, interception, sedimentation, diffusion (Brownian movement), and electrostatic deposition (for positively charged particles only) (Fig. 15–7). Impaction occurs in the upper respiratory tract and large proximal airways where the airflow is faster than in the

small distal airways because the cumulative diameter is smaller in the proximal airways. In airstream bends, such as an airway bifurcation, larger diameter particles deviate from the airflow and impact on the surface. In humans, most >10 μm particles are deposited in the nose or oral pharynx and cannot penetrate tissues distal to the larynx. For 2.5- to 10-μm particles, impaction continues to be the mechanism of deposition in the first generations of the tracheobronchial region.

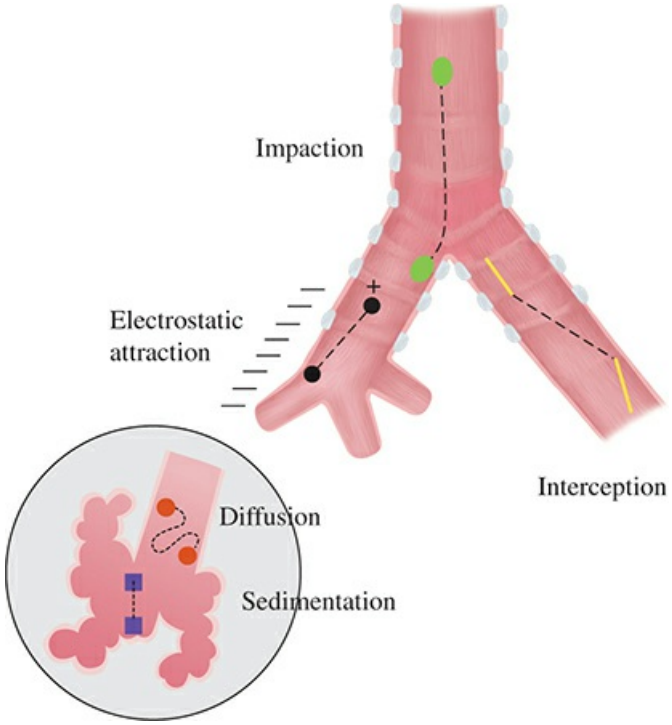


FIGURE 15–7 *Mechanism of particle deposition in the respiratory tract*. Impaction occurs in the upper respiratory tract and large proximal airways where fast airflow imparts momentum to the inhaled particle. The particle's inertia causes it to continue to travel along its original path and deposit on the airway surface. Interception occurs when the trajectory of a particle brings it near enough to a surface so that an edge of the particle contacts the airway surface. Sedimentation controls deposition in smaller bronchi, the bronchioles, and alveolar spaces, where the airways are small and the velocity of airflow is low. Diffusion is an important factor in the deposition of submicrometer particles. Electrostatic deposition is a minor deposition mechanism for positively charged particles because the negatively charged surface of the airways attracts positively charged particles. (Adapted from Lippmann M, Leikauf GD. Introduction and background (Chapter 1). In: Lippmann M, ed. *Environmental Toxicants Human Exposures and Their Health Effects*. New York: Wiley; 2009:3–18.)

Interception occurs when the trajectory of a particle brings it near enough to a surface so that an edge of the particle contacts the airway surface. Interception is important for all particles but is particularly important in the deposition of fibers. Although fiber diameter determines the probability of deposition by impaction and sedimentation, interception is dependent on fiber length. Thus, a fiber with a diameter of 1 μm and a length of 200 μm will be deposited in the bronchial tree primarily by interception rather than impaction. Interception is also important for submicrometer particles in the tracheobronchial region where inertial airflow directs a

disproportionately large fraction of the flow volume toward the surface of small airway bifurcations.

Sedimentation controls deposition in the smaller bronchi, the bronchioles, and the alveolar spaces, where the airways are small, and the velocity of airflow is low. Indeed, in the alveoli, there is no bulk airflow. As a particle moves through air, buoyancy and the resistance of air act on the particle in an upward direction while gravitational force acts on the particle in a downward direction. Eventually, the gravitational force equilibrates with the sum of the buoyancy and the air resistance, and the particle continues to settle with a constant velocity known as the terminal settling velocity.

Diffusion is an important factor in the deposition of submicrometer particles. A random motion is imparted to these particles by collisions with gas molecules. Diffusion is an important deposition mechanism in the nose, airways, and alveoli for particles ≤0.5 μm. Nanometer particles (0.1 μm and smaller) are also trapped relatively efficiently in the upper airways by diffusion.

During quiet breathing, in which the TV is only two to three times the volume of the anatomic dead space (i.e., the volume of the conducting airways where gas exchange does not occur), a large proportion of the inhaled particles may be exhaled. During exercise, when larger volumes are inhaled at higher velocities, impaction in the large airways and sedimentation and diffusion in the smaller airways and alveoli increase. Breath holding also increases deposition from sedimentation and diffusion.

Electrostatic deposition is a minor deposition mechanism for positively charged particles. The surface of the airways is negatively charged and attracts positively charged particles. Freshly fractured mineral dust particles and laboratory-generated aerosols from evaporation of aqueous droplets can have substantial electrostatic mobilities. Sonic jets (e.g., during wheezing and rales) formed by high air flowing through such partially occluded airways have the potential to increase the deposition of particles by impaction and diffusion in the small airways. Irritant materials that produce bronchoconstriction tend to increase the proximal tracheobronchial deposition of particles.

Particle Clearance

Lung defense is dependent on particle clearance. Once deposited in the lung, the adsorbed materials may dissolve from the surfaces of particles and enter the epithelium, endothelium, bloodstream, or lymphatics. Small particles (e.g., nanoparticles) may directly penetrate cell membranes and evade clearance. Moreover, particle clearance from the respiratory tract is not equivalent to clearance from the body. The only mechanisms by which deposited particles can be removed from the body are nasal wiping and coughing.

Nasal Clearance—Particles deposited in the nose are cleared depending on their site of deposition and solubility in mucus. Particles deposited in the anterior portion of the nose are removed by extrinsic actions such as wiping and blowing. Particles deposited in the posterior portion of the nose are entrapped in mucus and removed by mucociliary clearance that propels mucus toward the glottis, where the particles are swallowed. Insoluble particles are generally cleared from this region in healthy adults and swallowed within an hour of deposition.

Tracheobronchial Clearance—Particles deposited in the tracheobronchial tree are also

removed by mucociliary clearance. In addition to deposited particles, particle-laden macrophages are also moved upward to the oropharynx, where they are swallowed. Mucociliary clearance is relatively rapid in healthy individuals and is completed within 24 to 48 hours for particles deposited in the lower airways.

Alveolar Clearance—Particles deposited in the alveolar region are removed by the alveolar macrophage. Lung defense involves both the innate and adaptive immune systems. The innate immune system confers immediate recognition, phagocytosis, and killing of bacteria and microbes that are in the airway or alveolus. The adaptive immune system confers long-lasting or protective immunity to the host that is specific to a foreign microbe or material (antigen). Adaptive immunity involves dendritic cells that take up and present antigens to T lymphocytes (T cells) or antibody-producing B lymphocytes (B cells).

Macrophage phagocytosis depends on the recognition of foreign or damaged cells by macrophage surface macromolecules and receptors. Phagocytosis requires (1) particle binding to the membrane specifically via recognition molecule–receptor interactions or nonspecifically by electrostatic forces (inert materials), (2) receptor activation that initiates cell signaling, (3) actin polymerization and coordinated cytoskeletal movements that lead to extension of membranes, and (4) vesicular membrane closure closely apposed to the particle or the fiber ingested forming a phagosome shaped by the material ingested.

Following alveolar deposition, macrophages rapidly engulf particles (≥50% within 3 hours and nearly 100% by 24 hours). After several days or weeks, most macrophages then move to the airways and are removed mainly by mucociliary clearance. Some insoluble particles may be phagocytized by alveolar macrophages and removed via lymphatic drainage. Some deposited particles can be found in epithelial and interstitial cells or the lymphatic system with clearance time of months or years. Insoluble particles, especially long narrow fibers, may be sequestered in the lung for long periods, often in macrophages located in the interstitium.

ALVEOLAR MACROPHAGE RECEPTORS

Alveolar Macrophage Receptors and Innate Immunity

Several receptors are involved in the phagocytic uptake of micrometer-sized particles. Among these are Fc receptor and pattern-recognition receptors (PRRs) including complement, mannose, scavenger receptors, and other PRRs. These receptors differ in pathogen recognition motifs and are opsonin-dependent or -independent. Opsonins are binding enhancers (e.g., antibodies), which coat the negatively charged molecules, especially those on bacterial membranes. Molecules that activate the complement system also are considered opsonins.

Alveolar Macrophage Pattern-Recognition Receptors

Pattern-recognition receptors (PRRs) have various ectodomains that recognize pathogen-associated molecular patterns (PAMPs) present on microbial surfaces. PRRs include Toll-like receptors (TLRs), C-type lectin receptors (CLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and the RNA helicase retinoic acid-inducible gene I (RIG-I) (aka DExD/H-box helicase 58). TLR proteins contain three major domains, a leucine-rich repeat

motif containing ectodomain, a transmembrane region, and a cytosolic Toll-IL1 receptor (TIR) domain.

ACUTE RESPONSES OF THE RESPIRATORY TRACT TO INJURY

Trigeminally Mediated Airway Reflexes to Inhaled Chemicals

Inhaled toxic chemicals or particles can contact cells lining the respiratory tract from the nostrils to the gas-exchanging region. The sites of deposition in the respiratory tract have important implications in evaluating the risks posed by inhalants. Nasal and airway irritation represent a common response to inspired toxic compounds. Nasal irritation is mediated by irritant receptors (e.g., TRPA1) that trigger trigeminal nerve responses characterized by tickling, itching, and painful nasal sensations. When the concentration of an inhaled substance exceeds the biotransformation capacity of the nasal passages, it can penetrate to the lower respiratory tract.

Nasal irritation has been used as a basis for occupational exposure levels and is a common component in sick building syndrome resulting from poor indoor air quality. Nasal mucus secretion can dilute the irritant. Some chemicals can produce severe irritation at concentrations below those that induce a toxic response upon chronic exposure. Because the threshold dose of an irritant response can vary greatly among individuals (much like olfactory acuity), nasal irritation is not a reliable method for occupational safety.

Bronchoconstriction, Airway Hyperreactivity, and Neurogenic Inflammation

Bronchoconstriction can be evoked by irritants (acrolein, etc.), cigarette smoke, or air pollutants, and by cholinergic drugs such as acetylcholine. Bronchoconstriction decreases airway diameter with a corresponding increased airflow resistance. Bronchoconstriction can also be accompanied by an accumulation of thick mucus in the airway lumen. Characteristics of bronchoconstriction include the associated signs of coughing, wheezing, and rapid shallow breathing, and the associated symptoms of a sensation of chest tightness, substernal pain, and dyspnea (a feeling of breathlessness). Dyspnea is a normal consequence of exercise, which potentiates these problems as well as leads to more mouth breathing.

Bronchial smooth muscle tone is regulated by the autonomic nervous system. Postganglionic parasympathetic fibers, when stimulated, will release acetylcholine to the smooth muscle layer surrounding the bronchi. Acetylcholine binds muscarinic 3 cholinergic receptors to activate an intracellular G protein domain, which increases cGMP. The increase in cGMP in turn activates a phospholipase C (PLC) pathway that increases intracellular calcium concentrations $[Ca^{2+}]_i$. Increased $[Ca^{2+}]_i$ leads to contraction of the smooth muscle cells. The actions of cGMP can be antagonized by increased cAMP evoked by ligands that bind to β-adrenergic receptors on the cell surface, such as bronchodilators (β-adrenergic agonists such as albuterol) or by injected epinephrine.

Irritants can prime the autonomic response by lowering the threshold dose of acetylcholine

needed to induce bronchoconstriction causing airway hyperreactivity (or hyperresponsiveness). In bronchoprovocation testing, airway resistance is measured following inhalation of increasing doses of a methacholine aerosol. Methacholine is used because it is more stable than acetylcholine. Other bronchoconstrictive substances include histamine, various eicosanoids (including $PGF_2\alpha$ and PGD_2, thromboxane A_2, leukotrienes C_4 and D_4), and adenosine. The bronchial smooth muscles of individuals with asthma contract at a lower threshold dose during provocation than do those of individuals without asthma.

Irritants can stimulate TRP channels (especially TRPA1 and TRPV1) that cause neurogenic inflammation. Mediated by neuropeptides (including tachykinins) released from nociceptive nerve terminals, neurogenic inflammation also includes vasodilatation, plasma protein extravasation, and leukocyte adhesion to the vascular endothelium. Tachykinins including substance P (aka neurokinin 1) activate tachykinin receptors TACR2s and TACR1s to mediate bronchoconstriction, TACR1s to mediate mucin secretion, and cholinergic nerve TACR3s to mediate terminal stimulation.

Acute Lung Injury and Pulmonary Edema

Acute lung injury (aka adult or infant respiratory distress syndrome) is marked by alveolar epithelial and endothelial cell perturbation and inflammatory cell influx that leads to surfactant disruption, pulmonary edema, and atelectasis. Pulmonary edema produces a thickening of the alveolar capillary barrier and thereby limits O_2 and CO_2 exchange. Matching ventilation to vascular perfusion is critical to efficient gas exchange and is disrupted during acute lung injury. Alterations in coagulation and fibrinolysis accompany lung injury. Pulmonary edema may not only induce acute compromise of lung structure and function but also cause abnormalities that remain after resolution of the edematous process. During acute lung injury, profibrotic growth factors, TGFB1 and platelet-derived growth factor (PDGF), are activated and can initiate epithelial–mesenchymal transition (EMT).

Pulmonary edema is customarily quantified in experimental animals by measurement of lung water content. Lung water content can be expressed as the wet (undesiccated) weight of the whole lung or that of a single lung lobe. This value is often normalized to the weight of the animal or to the weight of the lung after complete drying in a desiccator or oven. The latter is typically expressed as lung wet weight:dry weight ratio.

CHRONIC RESPONSES OF THE LUNG TO INJURY

Asthma

Asthma is defined by sporadic bouts of airflow obstruction, which is measured as increased airway resistance (or decreased predicted FEV1). In asthma, resting levels of airway resistance can be normal or slightly increased, which typically is reversible with bronchodilators. The hallmark of asthma is a persistent or recurrent airway hyperreactivity that can be induced by exposure to a single known irritant or antigen or can result from a wide range of triggers

including irritants, cold-dry air, or exercise.

The pathogenesis of asthma involves the adaptive and innate immune systems. In allergic asthma, previous exposure to an antigen typically leads to the generation of IgE molecules that have molecular recognition sites specific to the antigen. Upon reexposure, the antigen causes cross-linking of IgE molecules and activation of lymphocytes, eosinophils, macrophages, and mast cells, with elaboration and release of an array of cytokines, chemokines, eicosanoids, histamine, tachykinins, and other mediators. Together, these mediators induce smooth muscle constriction, vascular leakage, mucus secretion, and inflammatory cell recruitment. Severe airway obstruction and ventilation-vascular perfusion mismatching lead to impaired gas exchange and hypoxemia. Persistent inflammation and epithelial damage contribute to airway hyperreactivity.

Occupational asthma can involve adaptive immunity induced by high-molecular-weight and some low-molecular-weight substances. High-molecular-weight agents, including flour-, latex-, cereals-, or animal-derived proteins and enzymes, cause sensitization through an IgE-mediated mechanism. The chemicals include metals (e.g., nickel, vanadium, chromium, cobalt, zinc, cadmium, or aluminum), diisocyanates (e.g., toluene diisocyanate), cleaning agents, wood dusts, soldering fluxes, pesticides, pharmaceuticals, and reactive dyes. Low-molecular-weight chemicals may act as haptens that combine with endogenous proteins to form a complex that is recognized as an antigen by the immune system.

Bronchiolitis Obliterans

Bronchiolitis obliterans is marked by fibrotic obstruction of the small diameter airways (bronchioles). The bronchiolar epithelium becomes damaged by inhaled chemicals or respiratory infections, particularly after organ transplants, leading to extensive fibro-proliferative, subepithelial thickening that blocks the airways. Chemicals associated with this condition include diacetyl, sulfur mustard, and chemicals and incinerator aerosol released during “burn pit” combustion. It also has been associated with rheumatoid arthritis and graft-versus-host disease following a lung or hematopoietic cell transplantation. The respiratory infections linked to this condition include cytomegalovirus, respiratory syncytial virus, adenovirus, *Pseudomonas aeruginosa* or *Mycoplasma pneumonia*.

Like COPD, bronchiolitis obliterans begins with a cough, wheezing, shortness of breath, and fatigue. These symptoms usually progress slowly, but severe symptoms can develop without warning. Other symptoms that appear in some individuals include fever, weight loss, and night sweats. The diagnosis of bronchiolitis obliterans is defined by a *sustained* (≥3 weeks) decline in FEV1 provided alternative causes of pulmonary dysfunction (e.g., COPD or asthma) have been excluded. The pathology is marked by subepithelial fibrosis causing partial or complete luminal occlusion of the bronchioles.

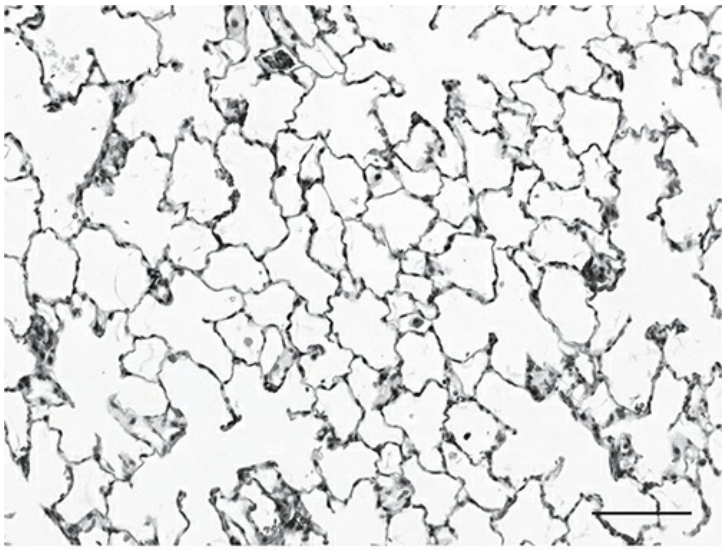
The fibrotic response is mediated by TGFB1, platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), and insulin-like growth factor-1 (IGF1). Chemokines and their interaction with specific cell receptors are essential components of inflammatory and immune responses via recruitment of specific leukocyte subpopulations.

Chronic Obstructive Pulmonary Disease

COPD involves airway (bronchitis) and alveolar (emphysema) pathology. Chronic bronchitis is defined by the presence of sputum production and cough for at least 3 months in each of 2 consecutive years. Bronchitis in COPD involves airway inflammation with excessive mucus production from surface epithelial (goblet) cells and submucosal glands. The number of goblet cells increases, the number of ciliated cells decreases, and the size of the submucosal glands increases markedly. The associated decreased mucociliary clearance and mucus retention may obstruct the airways and contribute to COPD exacerbations and possibly mortality.

COPD almost always is characterized by chronic cough. Frequent coughing in COPD can be exacerbated by respiratory infections, and many viruses and bacteria induce cough to move from host to host. Respiratory mechanical and ligand-gated cough receptors on rapidly adapting receptors, C fibers, and slowly adapting fibers provide input to the brainstem medullary central cough generator through the intermediary relay neurons in the nucleus tractus solitarius. Cough can be evoked by thermal, osmotic, and chemical (especially capsaicin) stimuli that engage the TRP channel (especially TRPV1) protein family. In addition, respiratory ligand-gated receptors are the degenerin/epithelial sodium channel proteins that include nonvoltage-gated sodium channels (e.g., SCNN1A) and amiloride-sensitive cation channels (ACCN2 aka acid-sensing ion channel 1). Cough can also be evoked by stimuli of other ligand-gated receptors including 5-hydroxytryptamine activation of 5-hydroxytryptamine (serotonin) receptor 3A (HTR3A), ATP activation of purinergic receptor P2X, ligand-gated ion channels, and nicotine activation of cholinergic receptors, nicotinic subtypes. Cough is effective in removing mucus in the first five to eight bronchial generations but as the cumulative diameter of the airways increases the acceleration of airflow is diminished.

Emphysema is physiologically defined by airflow obstruction that leads to dyspnea (especially on exertion) (Fig. 15–8) accompanied by diminished FEV1. The diagnosis of COPD is made by decreased FEV1:FVC ratio (which is not reversible by administration of a bronchodilator) and includes mild (<80% of normal), moderate (50% to 57%), severe (30% to 49%), and very severe (<30%, aka chronic respiratory failure.) Emphysema is pathologically defined by an abnormal enlargement of the airspaces distal to the terminal bronchiole accompanied by destruction of the walls without obvious fibrosis. Centriacinar emphysema begins in the respiratory bronchioles and spreads peripherally and is associated with cigarette smoking. Panacinar emphysema destroys the entire alveolus uniformly (typically in the lower half of the lungs).



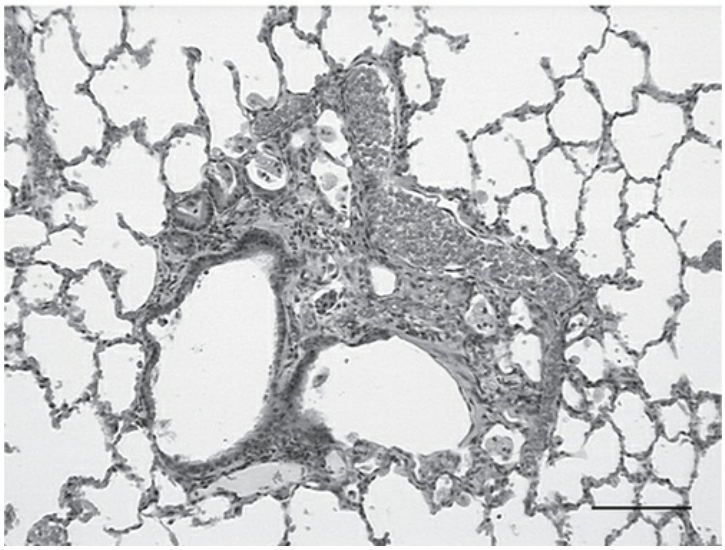


FIGURE 15–8 *Airspace enlargement induced by tobacco smoke and pulmonary fibrosis induced by asbestos in rat lung*. Top panel: Normal rat lung. Middle panel: Extensive distention of the alveoli (emphysema) in rat lung following inhalation of tobacco smoke (90 mg/m^3 of total suspended particulate material). Bottom panel: Lung of a rat 1 year after exposure to chrysotile asbestos. Note accumulation of connective tissue around blood vessel and airways (fibrosis). Bar length: 100 μm. (Used with permission from Dr. Kent E. Pinkerton, University of California, Davis.)

The pathogenesis of emphysema involves a proteinase—antiproteinase imbalance that leads to the remodeling of the supportive connective tissue in the parenchyma and separate lesions that coalesce to destroy lung tissue. This mechanism was proposed because of the association of serpin peptidase inhibitors, clade A (α-1 antiproteinase, antitrypsin), member 1 (SERPINA1) deficiency with early-onset emphysema. SERPINA1 inhibits serine proteinases including trypsin and ELANE. Macrophage matrix metalloproteinase 12 (MMP12 aka macrophage elastase), proteinase 3 (PRTN3), and plasminogen activator tissue (PLAT) can mediate alveolar enlargement.

Lung Cancer

Lung cancer in never-smokers is the seventh leading cause of cancer death worldwide. Environmental tobacco smoke increases the risk of developing lung cancer in nonsmokers. In addition, biomass cooking, high-temperature oil cooking, and coal heating especially in poorly-ventilated households are associated with lung cancer, COPD, and pneumonia, together leading to over 2 million deaths per year worldwide. Ambient $PM_{2.5}$ has been associated with increased lung cancer risk.

Human lung cancers may have a latency period of 20 to 40 years, making the relationship to specific exposures difficult to establish. The two major forms of lung cancer are non-small-cell lung cancer (NSCLC; which accounts for ~85% of all lung cancer cases) and SCLC (which accounts for ~15% of all lung cancer cases). NSCLC can be subdivided into three major histological subtypes: squamous-cell carcinoma, adenocarcinoma, and large-cell lung cancer. Smoking causes all types of lung cancer but is most strongly linked with SCLC and squamous-cell carcinoma. Adenocarcinoma is the most common type in patients who have never smoked. Large-cell lung cancer is rare and makes up less than 10% of the NSCLC cases.

Epithelial lung cancers develop in a sequence of distinct morphological changes. Initially, cell numbers in the epithelium lining the airways increase (hyperplasia) and eventually display abnormal nuclei and changes in shape (dysplasia), often assuming squamous-cell characteristics (squamous metaplasia). The lesions then progress to first carcinoma in situ, an accumulation of cancerous cells in small foci, and then into large tumor masses. Eventually tumor cells invade adjacent local tissues, blood vessels, and lymphatics, leading to the formation of distant metastases.

A key component is DNA damage induced by reactions with an activated carcinogen or its metabolic product. Persistence of O^6-alkyl-deoxyguanosine mutations in DNA appears to correlate with carcinogenicity. However, tumors do not always develop when adducts are present. DNA damage caused by reactive oxygen species is another potentially important mechanism. Ionizing radiation leads to the formation of superoxide, which is converted through the action of SOD to hydrogen peroxide. In the presence of Fe and other transition metals,

hydroxyl radicals may be formed, which then cause DNA strand breaks. Cigarette smoke contains high quantities of reactive oxygen species and acrolein. Additional oxidative stress may be placed on the lung tissue of smokers by the release of reactive oxygen/nitrogen species formed by activated macrophages and other inflammatory leukocytes.

Genomic instability is a hallmark of lung cancer. Critical genetic and epigenetic changes include DNA mutations, loss of heterozygosity, and promoter methylation and global transcriptome changes that can include stimulation of mitogenic pathways and suppression of apoptosis pathways. Genetic and epigenetic changes can persist over years and eventually lead to aberrant cellular function to produce premalignant changes, including dysplasia and clonal patches.

Lung cancers unrelated and related to smoking have different molecular profiles, mutation in EGFR being more common in never-smokers, and v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) being more common in smokers. Mutations in TP53 are found in both smokers and nonsmokers; however, they are more frequent in never-smokers. In addition, the TP53 mutational signature (i.e., relative amounts of specific transitions, transversions, or deletions), and spectrum (i.e., specific mutation within the gene) differ between smokers and never-smokers. Found in both NSCLCs and SCLCs, mutant TP53 protein fails to bind these sites, and hence lacks tumor suppressor activity. Acrolein and BaP adducts of TP53 are common in lung cancers. Mutation or amplification of *TP53*, *EGFR*, *ERBB2*, or *KRAS* thus provides a proliferative advantage to tumor cells.

Single-nucleotide polymorphisms (SNPs) located on chromosomal regions 5p15.33 and 15q25.1 have been consistently identified by genome-wide association studies (GWAS) as significant predictors of lung cancer risk. A SNP in 6p22.1 has been associated with survival time in small-cell lung cancer (SCLC) patients.

In toxicological testing, lung tumors or cancers in laboratory animals do not always mimic those in humans. Most lung tumors in mice are peripherally located adenomas and adenocarcinomas, even after tobacco or NNK exposure. Rarely do mice or rats develop bronchial squamous-cell carcinoma found in humans that smoke.

Pulmonary Fibrosis

The pathological hallmark of pulmonary fibrosis is increased focal staining of collagen fibers in the alveolar interstitium, throughout the centriacinar region, including the alveolar ducts and respiratory bronchioles (Fig. 15–8). These foci can contain the inducing material including asbestos, silica, or man-made mineral fiber (MMMF). The pleural surface of the lung may also become fibrotic and together with parenchymal stiffening prevent full lung inflation. Ultimately the lung is unable to inflate or deflate properly and thus is restricted. Restrictive lung disease can be detected by a decrease in the predicted FVC, with or without a change in the FEV1.

The pathogenesis of pulmonary fibrosis involves epithelial cell injury and macrophage activation produced by a wide range of toxic insults. For example, macrophages can be activated by phagocytosis of crystalline silica or crocidolite asbestos, which activate inflammasome receptor-mediated TNF and IL1B formation. Epithelial cells and macrophages also release chemokines that recruit and activate other inflammatory cells including neutrophils and T cells. These cells combine to produce excessive TGFB1, TNF, IL1B, IL13, and IL17. Of these, TNF and TGFB1 are major mediators in pulmonary fibrosis. In the lung, integrin α v β 6 (through cell–cell contact) and integrin α v β 8 (through a proteolytic process) can activate extracellular

latent TGFB1 and TGFB3.

The consequence of the combined TGFB1/3, cytokine release, and hypoxia leads to epithelial-to-mesenchymal transition in which lung epithelial cells transdifferentiate into fibroblast-like cells. These cells contribute to a larger population of myofibroblasts that arise from local mesenchymal cells, and bone marrow–derived fibrocytes. The migration, proliferation, and activation of myofibroblasts contribute to excessive extracellular matrix deposition that has altered collagen cross-linking. A feature of fibrogenesis is the oxidation of lysines in the extracellular matrix (ECM), mainly in collagen, to the aldehyde, allysine by lysyl-oxidase (LOX) enzymes. Allysine undergoes a series of condensation reactions with other amino acids on neighboring collagen molecules to form irreversible cross-links that stabilize the ECM. The consequence of this process is disorderly repair and substained fibrogenesis, which leads to progressive stiffness of the fibrotic lung.

AGENTS KNOWN TO PRODUCE RESPIRATORY DISEASE IN HUMANS

TLVs refer to occupational airborne concentrations of substances “that nearly all workers may be repeatedly exposed day after day without adverse health effects.” These values and other exposure limits have been developed because prevention of exposure is one of the most effective approaches to prevent lung injury and disease. Table 15-1 lists some respiratory toxicants that can produce acute and chronic lung injury in humans.

TABLE 15–1 Agents That Produce Lung Injury and Disease

Toxicant	Disease	Exposure	Acute Effects	Chronic Effects
Acrolein	Acute lung injury, chronic obstructive pulmonary disease	Biomass or hot oil cooking, firefighters, environmental tobacco smoke, biocide water treatment	Cough, shortness of breath, extreme oronasal irritation, pulmonary edema, airway hyperreactivity	Chronic obstructive pulmonary disease, possibly asthma or lung cancer
Aluminum abrasives	Shaver's disease, corundum smelter's lung, bauxite lung	Abrasives manufacturing, smelting	Alveolar edema	Interstitial fibrosis, emphysema
Aluminum dust	Aluminosis	Aluminum, firework, ceramic, paint, electrical good, and abrasive manufacturing	Cough, shortness of breath	Interstitial fibrosis
Ammonia		Farming, refrigeration operations, ammonia, fertilizer, chemical, and explosive manufacturing	Oronasal and bronchial irritation, pulmonary edema	Acute lung injury, chronic bronchitis
Arsenic		Pesticide, pigment, glass, and alloy manufacturing	Bronchitis	Laryngitis, bronchitis, and lung cancer
Asbestos	Asbestosis	Mining, construction, shipbuilding, brake repair, vermiculite contaminant		Fibrosis, pleural calcification, lung cancer, mesothelioma
Aspergillus (Mold)	Framer's lung, compost lung, malt worker's lung, aspergillosis	Working with moldy hay, compost, or barley	Bronchoconstriction, cough, chest tightness	Extrinsic allergic alveolitis (hypersensitivity pneumonitis)
Avian protein	Bird fancier's lung	Bird handling and farming with exposure to bird droppings	Bronchoconstriction, cough, chest tightness	Extrinsic allergic alveolitis (hypersensitivity pneumonitis)
Beryllium	Berylliosis	Mining, alloy, and ceramic manufacturing, milling beryllium	Pulmonary edema, pneumonia	Interstitial granulomatosis, progressive dyspnea, cor pulmonarle, fibrosis, and lung cancer
Cadmium		Welding, smelting, and electrical equipment, battery, alloy, and pigment manufacturing	Cough, pneumonia	Emphysema, cor pulmonale
Carbides of tungsten, titanium, or tantalum	Hard metal disease	Metal cutting and manufacturing	Bronchial epithelial hyper- and metaplasia	Peribronchial and perivascular fibrosis
Chlorine		Paper, plastics, chlorinated product manufacturing	Cough, hemoptysis, dyspnea, bronchitis, pneumonia	
Chromium (VI)		Chromium compound, paint, pigment, chromite ore reduction manufacturing	Oronasal and bronchial irritation	Fibrosis, lung cancer
Coal dust	Coal worker's pneumoconiosis	Coal mining		Fibrosis with emphysema
Cotton dust	Byssinosis	Textile manufacturing	Chest tightness, wheezing, dyspnea	Restrictive lung disease, chronic bronchitis
Diacetyl	Bronchiolitis obliterans ("Popcorn lung")	Manufacture of microwave popcorn, cookies, potato chip flavoring, and coffee roasting	Chest tightness, wheezing, dyspnea	Restrictive lung diseases with occlusion of bronchioles
Hydrogen fluoride		Chemical, photograph film, solvent and plastic manufacturing	Airway irritation, hemorrhagic pulmonary edema	

Iron oxides	Siderotic lung disease, silver finisher's lung, hematite miner's lung, arc welder's lung	Welding, steel and jewelry manufacturing, foundry work, hematite mining,	Cough	Silver finisher's lung with subpleural and perivascular macrophage aggregates; hematite miner's lung with diffuse fibrosis-like pneumoconiosis; arc welder's lung with bronchitis
Isocyanates		Auto painting, and plastic and chemical manufacturing	Airway irritation, cough, dyspnea	Asthma
Kaolin	Kaolinosis	Pottery making		Fibrosis
Manganese	Manganese pneumonia	Chemical and metal manufacturing	Acute pneumonia (often fatal)	Recurrent pneumonia
Nickel		Nickel mining, smelting, electroplating, battery manufacturing, fossil fuel combustion	Delayed pulmonary edema, skin allergy	Acute lung injury, chronic bronchitis, non-small cell lung cancer, nasal cancer
Nitrogen oxides	Silo-filler's diseases	Silo filling, welding, explosive manufacturing	Immediate or delayed pulmonary edema	Bronchiolitis obliterans, emphysema in experimental animals
Nontuberculous mycobacteria	Metalworking fluid hypersensitivity	Working with metal cutting fluid containing water and contaminated with mycobacteria	Bronchoconstriction, cough, chest tightness	Extrinsic allergic alveolitis (hypersensitivity pneumonitis)
Organic (sugar cane) dust (possibly contaminated with thermophilic *actinomycete*)	Bagassosis	Sugar cane and molasses manufacturing (bagasse is the fibrosis residue from sugar extraction)	Bronchoconstriction, cough, chest tightness	Extrinsic allergic alveolitis (hypersensitivity pneumonitis)
Ozone		Welding, photocopying, bleaching flour, water treatment, deodorizing	Substernal pain, exacerbation of asthma, bronchitis, pulmonary edema	Fibrosis (including airways)
Perchloroethylene (tetrachloroethene)		Dry cleaning, metal degreasing, grain fumigation	Edema	Hepatic and lung cancer
Phosgene		Plastic, pesticide, and chemical manufacturing	Severe pulmonary edema	Bronchitis and fibrosis
Silica	Silicosis, pneumoconiosis	Mining, stone cutting, sand blasting, farming, quarry mining, tunneling	Acute silicosis (inflammation)	Fibrosis, silicotuberculosis
Sulfur dioxide		Chemical manufacturing, refrigeration, bleaching, fumigation	Bronchoconstriction, cough, chest tightness	Chronic bronchitis
Talc	Talcosis	Mining, rubber manufacturing, cosmetics	Cough	Fibrosis
Thermophilic *actinomycete*	Farmer's lung, mushroom worker's lung, penguin humidifier lung	Farming (hay or grain degradation)	Bronchoconstriction, cough, chest tightness	Extrinsic allergic alveolitis (hypersensitivity pneumonitis)
Tin		Mining, tin processing		Widespread mottling in chest x-ray often without clinical impairment
Vanadium		Metal cutting and manufacturing, specialty steel manufacturing	Airway irritation and mucus production	Chronic bronchitis

Inhalation Hazards

Acrolein—As an α–β-unsaturated aldehyde, acrolein (2-propenal) is volatile at room

temperature and is highly irritating to the upper and lower respiratory tract. With a reactive carbonyl group and an electrophilic α-carbon, acrolein often forms cross-links with biological macromolecules and alters gene regulation, increases inflammation, decreases mucociliary transport, and diminishes alveolar-capillary barrier integrity. Susceptibility to acrolein exposure is associated with differential regulation of cell surface receptor, transcription factor, and ubiquitin–proteasome genes.

Asbestos—Asbestos refers to a group of silicate minerals in fiber form, including serpentine chrysotile asbestos and the amphiboles crocidolite, anthophyllite, amosite, actinolite, and tremolite asbestos. Asbestos causes three forms of lung disease: asbestosis, lung cancer, and malignant mesothelioma. Asbestosis is a form of pulmonary fibrosis with diffuse collagen foci and the presence of asbestos fibers, either free or coated with a proteinaceous material (asbestos bodies). Alveolar macrophage clearance is critical to the prevention of asbestosis and depends on fiber length, biopersistance, and dose. Asbestosis can produce characteristic nodular localized lesions of macrophages and asbestos fibers. Lung cancer develops in workers in the asbestos mining industry and smoking of cigarettes greatly enhances the risk. Malignant mesothelioma is a rare cancer that develops mainly in the pleural mesothelium, the protective lining that covers the lungs, diaphragm, and interior of the chest wall. Unlike lung cancer, mesothelioma is not associated with smoking history.

Health hazards associated with asbestos exposure depend on fiber shape, length, and surface properties. Chrysolite asbestos are curved fibers and tend to be less toxic than crocidolite fibers that are long and thin, straight fibers. Fibers 2 μm in length may produce asbestosis; mesothelioma is associated with fibers ≥5-μm long, and lung cancer with fibers ≥10 μm. Fiber diameter is another critical feature. Fibers with diameters larger than ~3 μm do not readily penetrate into the peripheral lung. Only fibers ≥0.15 μm in diameter are likely to produce asbestosis or lung cancer. Mesothelioma is more often associated with fiber diameter <0.5 μm. The surface properties of asbestos fibers also contribute to toxicity. Crocidolite contains more iron than chrysolite. The interaction of iron on the surface of asbestos fibers with oxygen can lead to the production of hydrogen peroxide and the highly reactive hydroxyl radical. Once asbestos fibers have deposited in the lung, they may be phagocytized by alveolar macrophages. Short fibers are completely ingested and subsequently removed via the mucociliary escalator. Longer fibers are incompletely ingested, and the macrophages become unable to leave the alveoli. Activated by the fibers, macrophages release mediators such as cytokine, chemokines, and growth factors, which in turn attract immunocompetent cells or stimulate collagen production. Asbestos-related lung disease thus may be mediated through the triggering of epithelial injury and macrophage activation, and inflammatory events or through the production of changes that eventually lead to the initiation or promotion of the carcinogenic process.

Coal Dust—Coal worker’s pneumoconiosis (CWP aka Black Lung Disease) results from the accumulation of coal dust in the lungs and can be simple coal worker’s pneumoconiosis (SCWP) and complicated coal worker’s pneumoconiosis (CCWP) also called progressive massive fibrosis (PMF). Normally, inhaled coal dust reaches the terminal bronchioles, and the carbon is engulfed by alveolar and interstitial macrophages. Phagocytosed coal particles are transported by macrophages up the mucociliary elevator and are expelled in the mucus or through the lymphatic system. However, when clearance mechanisms are overwhelmed, the dust-laden macrophages accumulate in the alveoli and may trigger an immune response.

Histological hallmarks of CWP are areas of focal deposition of coal dust and pigment-laden

macrophages, which are known as coal macules. When exposure is extended over a significant amount of time to dust particles 2 to 5 μm in diameter, activated fibroblasts secrete reticulin, which entraps the macrophages. If the dust-laden macrophages lyse, the fibroblastic response is augmented and more collagen is laid down in the area. In PMF, this involves the accumulation of necrotic macrophages, fibroblasts, reticulin, and collagen along the vascular tree. As the blood vessels become compromised, a progressive ischemic necrosis ensues. The lesion enlarges and appears as focal macules. The focal macules can extend, and join other macules in the vicinity, forming discrete areas of interstitial fibrosis. Coal mining often involves simultaneous silicon dioxide exposure due to silica-containing rock grinding, which exacerbates macrophage lysis stimulating more collagen deposition. This growing collagen network causes distention of the respiratory bronchioles, forming focal areas of emphysema. Coal dust exposure, cigarette smoking, age, and race are significant and additive predictors of the severity of emphysema among coal miners.

Diacetyl—Diacetyl is commonly used to give a buttery taste to foods. Industrial exposure to diacetyl has been associated with “popcorn lung,” which is a form of bronchiolitis obliterans. In the manufacture of microwave popcorn, cumulative diacetyl exposure >0.8 ppm-year was associated with increased risk of airflow obstruction. The latency period between time of first exposure and onset of symptoms is variable but is usually less than 10 years and may be as short as a few months. The mechanism of how diacetyl produces an effect localized to the bronchioles is yet to be determined.

Naphthalene—Naphthalene occurs in cigarette smoke, tars, petroleum and is a precursor in the chemical synthesis of tanning agents, phthalic acid anhydride, carbaryl, and 2-naphthol. The primary target in the surface epithelium is the bronchiolar secretoglobin cells. These cells are involved in the long-term maintenance and repair of airways.

Silica—Inhaled particles of silicon dioxide cause a characteristic human lung disease—silicosis. Mineral forms of silicon exist primarily as crystalline SiO_2 with a central silicon atom forming a tetrahedron with four shared oxygen atoms. The three principal crystalline isomeric forms are quartz, tridymite, and cristobalite. The tetrahedral structure is biopersistent and linked to fibrogenic potential. An occupational hazard ever since tools were cut from stone, silicosis is a significant industrial hazard in mining and quarrying, sandblasting, and foundry work.

Particle size, concentration, and surface properties affect the pathogenicity of silica both in vivo and in vitro. In humans, the most fibrogenic particle size appears to be about 1 μm (range 0.5 to 3 μm). Acute silicosis occurs only in subjects exposed to very high levels of silica (most often quartz or sand), small enough to be respirable (usually <5 μm) over a relatively short period, generally a few months or years. These patients have worsening dyspnea, fever, cough, and weight loss that can rapidly progress to respiratory failure and death within two years. No known therapeutic strategy controls the relentless course of acute silicosis.

Chronic silicosis has a long latency period, usually >10 years and can be divided into simple and complicated silicosis. Simple silicosis may progress into complicated silicosis, which is defined as the presence of conglomerate nodules larger than 1 cm in diameter. These nodules usually occur in the upper and mid-lung zones. In advanced stages, the nodules may be surrounded by emphysematous bullae.

Pulmonary alveolar macrophages phagocytose silica particles into phagosomes and

experience phagosomal destabilization which activates pathways including the NALP3 inflammasome, pattern-recognition receptors, and antiviral pathways to release inflammatory cytokines. Silica particles cannot be degraded and macrophages undergo cell death, releasing these particles that are engulfed by other macrophages, thus perpetuating the process of phagocytosis and cell death. This leads to elevated mediator release (TGFB1, TNF, etc.), epithelial-mesenchymal transition, and initiates and maintains myofibroblast collagen.

Blood-Borne Agents That Cause Pulmonary Toxicity in Humans

1,3 Bis (2-Chloroethyl)-1-Nitrosourea (BCNU)—Carmustine (BCNU) exerts its antitumor properties by reacting with cellular macromolecules to form inter- and intra-strand cross-links with DNA. In humans, a dose-related pulmonary toxicity can develop into fatal pulmonary fibrosis. BCNU inhibits pulmonary glutathione disulfide reductase, which may lead to a disturbed GSH/GSSG state in pulmonary cells leaving the cells unable to cope with oxidant stress. High concentrations of oxygen in inspired air may enhance the pulmonary toxicity of BCNU, bleomycin and cyclophosphamide.

Bleomycin—Bleomycin produces injury and necrosis of capillary endothelial cells and alveolar type I cells. This leads to edema formation and hemorrhage, delayed (after 1 to 2 weeks) proliferation, and apoptosis of alveolar type II cells. Eventually the alveolar walls are thickened by fibrotic changes. Bleomycin stimulates the production of collagen in the lung. Prior to increased collagen biosynthesis, steady-state levels of mRNA coding for fibronectin and procollagens are increased, subsequent to a bleomycin-mediated release of cytokines such as TGFB1 and TNF. Bleomycin can create reactive oxygen species leading to DNA single- and double-strand breaks.

Cyclophosphamide—The undesirable side effects include hemorrhagic cystitis and pulmonary fibrosis. Cyclophosphamide is metabolized by cytochrome P450 to two highly reactive metabolites: acrolein and phosphoramide mustard. In the lung, cooxidation with the PGH synthase system, which has high activity in the lung, is a possibility. Also, cyclophosphamide and its metabolite acrolein initiate lipid peroxidation.

EVALUATION OF TOXICANT-INDUCED LUNG DAMAGE

Humans Studies

Commonly used tests include measurement of FEV1, FVC, and airway resistance. Additional tests evaluate the maximal flow rates and different lung volumes, diffusion capacity, oxygen, and carbon dioxide content of the arterial and venous blood, distribution of ventilation, and lung and chest wall compliance. A reduced FEV1 is usually indicative of impaired ventilation such as that found in restrictive (increased lung stiffness) or obstructive (obstructed airflow) lung disease.

The structural properties that control gas exchange include the lung gas volume, the path length for diffusion in the gas phase, the thickness and area of the alveolar capillary membrane,

and the volume of blood in capillaries supplying ventilated alveoli. The functional properties that control gas exchange include the uniformity of ventilation and perfusion with respect to each other, the diffusion characteristics of the alveolar membrane, the binding properties of hemoglobin (Hb) in the alveolar capillaries, and the gas tensions in blood in the pulmonary vascular bed that exchanges gas with the alveoli. Gas exchange may be hindered by the accumulation of fluids or cellular elements in the alveoli (edema, pneumonic infiltrates), thickening of the alveolar wall (fibrosis), insufficient ventilation of the alveolar region (emphysema), ventilation–perfusion mismatching, or insufficient presence of oxygen transport elements (reduced alveolar blood volume or reduced amount of Hb in the blood). Gas exchange can be evaluated by measuring the arterial partial pressure of both oxygen and CO_2.

Proper lung function in humans can be evaluated with several additional techniques. Computed tomography provides detailed roentgenographic information of airways and lung parenchyma and has been useful in screening high-risk patients for lung cancer. Increased concentrations of nitric oxide are often found in exhaled air when inflammatory processes have led to induction of iNOS. Fiberoptic bronchoscopy allows direct visual inspection of the major lobar and segmental airways; the depth of penetration is limited by the external diameter of the bronchoscope, usually 5 mm. Bronchoscopy also allows the introduction and retrieval of saline solutions into the lung and subsequent analysis for cellular and molecular constituents (bronchoalveolar lavage). Excision of small tissue samples during bronchoscopy is helpful in the evaluation and staging of precancerous and cancerous lesions.

Animal Studies

Inhalation Exposure Systems—In inhalation studies, animals are kept within a chamber that is ventilated with a defined test atmosphere. Generation of such an atmosphere is comparatively easy for gases that are available in high purity in a compressed tank, for example, SO_2, O_2, or NO_2. Metering and dilution produce appropriate concentrations for exposure. More challenging is the generation of particles or complex mixtures (e.g., tobacco smoke, diesel, and gasoline exhaust or residual oil fly ash) because of the possibility of interactions between individual mixture constituents and the possibility of formation of artifacts.

Intratracheal and Intranasal Instillation—Alternative approaches are intratracheal and intranasal instillation of particles in mice or rats. These approaches have a major limitation in that the dose rate does not correspond to that by inhalation. In addition, these delivery methods produce a high focal dose that can trigger local response due to the dose intensity that may not be triggered following inhalation.

Pulmonary Function Tests in Experimental Animals—Conducting pulmonary function tests in experimental animals poses distinct challenges. Analysis of pressure–volume curves is a comparatively easy test to perform in animals. The test provides some indication of lung compliance. Compliance (volume/pressure) provides an indication of the intrinsic elastic properties of the lung parenchyma and, when measured in vivo, the thoracic cage. In emphysema, compliance of the lung increases because elastic recoil is decreased.

Analysis of airway resistance can be measured with video-assisted or acoustic plethysmography. An increase in airway resistance is a measure of bronchoconstriction inasmuch as airway smooth muscle contraction or airway mucosal edema narrows the airway lumen and

obstructs airflow. Airway resistance also can be measured after challenge doses of methacholine or acetylcholine. Analysis of breathing pattern may differentiate between upper airway and lower airway irritants.

Morphological Techniques—Acute and chronic injury may be examined by gross inspection and microscopically and should include the nasal passages, larynx, major bronchi, and the lung parenchyma. Paraffin sections of respiratory tract tissue are suitable for routine histopathological analysis of gross pathological changes, for example, inflammation, fibrosis, or lung tumors. The tissue can be stained with (a) hematoxylin and eosin stain for routine assessment, (b) periodic acid-Schiff's Alcian blue stain for glycoproteins (mucus cells), or (c) Masson trichrome stain for collagen. Plastic or Epon sections 1-μm thick are required for proper identification of additional cell types or recognition of cytoplasmic changes as in damaged BSCs. Transmission electron microscopy, scanning electron microscopy, and confocal microscopy have their uses. Additional tools include immunohistochemistry, in situ hybridization, analysis of cell kinetics, and profiling of the transcriptome, proteome, and metabolome. Other additional value tools to assess the lung in health and disease are available.

Pulmonary Lavage and Pulmonary Edema—The fluid lining the pulmonary epithelium can be recovered by bronchoalveolar lavage and analyzed to detect respiratory tract toxicity. Influx of neutrophils or other leukocytes such as lymphocytes or eosinophils into the lavage fluid is the most sensitive sign of inflammation.

In Vitro Studies

Isolated Perfused Lung—The isolated perfused lung method is applicable to lungs from many laboratory species (e.g., mouse, rat, guinea pig, or rabbit). The lung is perfused with blood or a blood substitute through the pulmonary arterial bed. At the same time, the lung is actively (through rhythmic inflation–deflation cycles with positive pressure) or passively (by creating negative pressure with an artificial thorax in which the lung is suspended) ventilated. Toxic chemicals can be introduced into the perfusate or the inspired air. Repeated sampling of the perfusate allows one to determine the rate of metabolism of drugs and the metabolic activity of the lung.

Airway Microdissection and Organotypic Tissue Culture Systems—Microdissection of the nasal passage and airways consists of stripping away surrounding tissue or parenchyma while maintaining the airway structure and exposing the epithelium. Tissue culture systems have been developed in which epithelial cells maintain their polarity, differentiation, and normal function similar to what is observed in vivo. Epithelial cell surfaces are exposed to air or a gas phase containing an airborne toxicant while the basal portion is bathed by a tissue culture medium.

Lung Cell Culture—Many lung-specific cell types can be maintained in cell culture. Maintenance of the epithelial cells at the air–liquid interface is important to maintain polarity and differentiation. Epithelial cells may be seeded on top of a suitable supporting material (e.g., collagen or nitrocellulose membranes) with mesenchymal cells seeded on the other side to observe epithelial cell–fibroblast interactions. Primary cell cultures have been transformed and/or immortalized and can be maintained for long periods in the laboratory. Cell lines established from lung tumors have been used extensively by investigators and have yielded many novel

insights into lung cancer.

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QUESTIONS

1. Which of the following statements is FALSE regarding the role of mucus in the conducting airways?
   - **a.** Pollutants trapped by mucus can be eliminated via expectoration or swallowing.
   - **b.** Mucus is of a basic pH.
   - **c.** The beating of cilia propels mucus out of the lungs.
   - **d.** Mucus plays a role promoting oxidative stress.
   - **e.** Free radical scavenging is believed to be a role of mucus.

2. Respiratory distress syndrome sometimes affects premature neonates due to lack of surfactant production by which of the following cell types?
   - **a.** lung fibroblasts.
   - **b.** type II pneumocytes.
   - **c.** endothelial cells.
   - **d.** alveolar macrophages.
   - **e.** type I pneumocytes.

3. In a situation where there is an increased metabolic demand for oxygen, which of the following volume measurements will greatly increase?
   - **a.** total lung capacity (TLC).
   - **b.** residual volume (RV).
   - **c.** functional residual capacity (FRC).
   - **d.** tidal volume (TV).
   - **e.** vital capacity (VC).

4. The free radicals that inflict oxidative damage on the lungs are generated by all of the following EXCEPT:
   - **a.** tobacco smoke.
   - **b.** neutrophils.
   - **c.** ozone.
   - **d.** monocytes.
   - **e.** SO_2.

5. Which of the following gases would most likely pass all the way through the respiratory

tract and diffuse into the pulmonary blood supply?

a. O_3 (ozone).
b. NO_2.
c. H_2O.
d. CO.
e. SO_2.

6. All of the following statements regarding particle deposition and clearance are true EXCEPT:

a. One of the main modes of particle clearance is via mucociliary escalation.
b. Diffusion is important in the deposition of particles in the bronchial regions.
c. Larger volumes of inspired air increase particle deposition in the airways.
d. Sedimentation results in deposition in the bronchioles.
e. Swallowing is an important mechanism of particle clearance.

7. Which of the following is not a common location to which particles are cleared?

a. stomach.
b. lymph nodes.
c. pulmonary vasculature.
d. liver.
e. GI tract.

8. Pulmonary fibrosis is marked by which of the following?

a. increased type I collagen.
b. decreased type III collagen.
c. increased compliance.
d. elastase activation.
e. decreased overall collagen levels.

9. Activation of what enzyme(s) is responsible for emphysema?

a. antitrypsin.
b. epoxide hydrolase.
c. elastase.
d. hyaluronidase.
e. nonspecific proteases.

10. Which of the following measurements would NOT be expected from a patient with restrictive lung disease?

a. decreased FRC.
b. decreased RV.
c. increased VC.
d. decreased FEV_1.
e. impaired ventilation.

CHAPTER 16

Toxic Responses of the Nervous System

Virginia C. Moser, Michael Aschner, Jason R. Richardson, Aaron B. Bowman, and Rudy J. Richardson

KEY POINTS

- The central nervous system (CNS) is protected from the adverse effects of many potential toxicants by an anatomical blood–brain barrier and by the action of membrane transporters.
- Neurons are highly dependent on aerobic metabolism because this energy is needed to maintain proper ion gradients.
- Individual neurotoxic compounds typically target the neuron, the axon, the myelinating cell, or the neurotransmitter system.
- Neuronopathy is the toxicant-induced irreversible loss of neurons, including its cytoplasmic extensions, dendrites and axons, and the myelin ensheathing the axon.
- Neurotoxicants that cause *axonopathies* cause axonal degeneration, and loss of the myelin surrounding that axon; however, the neuron cell body remains intact.
- Numerous naturally occurring toxins as well as synthetic chemicals may interrupt the transmission of impulses, block or accentuate transsynaptic communication, block reuptake of neurotransmitters, or interfere with second-messenger systems.

OVERVIEW OF THE NERVOUS SYSTEM

Many insights into the organization and function of the nervous system (NS) are based on observations derived from the action of neurotoxicants. Several general aspects modulate the NS response to chemicals, including (1) the maintenance of a biochemical barrier between the brain and the blood, (2) the importance of the high-energy requirements of the brain, (3) the spatial extensions of the NS as long cellular processes and the requirements of cells with such a complex geometry, (4) the maintenance of an environment rich in lipids, (5) the transmission of information across extracellular space at the synapse, (6) the distances over which electrical impulses must be transmitted, coordinated, and integrated, and (7) development and regenerative patterns of the NS. Each of these features of the NS carries with it specialized metabolic/physiological requirements and unique vulnerabilities to toxic compounds.

Blood–Brain Barrier

The NS is protected from the adverse effects of many potential toxicants by a functional and anatomic barrier between the blood and the brain, or a “blood–brain barrier.” Most of the brain, spinal cord, retina, and peripheral NS (PNS) maintain this barrier with the blood with specialized endothelial cells in the brain’s microvasculature, aided, in part, by interactions with glia. In addition to this interface with blood, the brain and spinal cord are covered with the meningeal surface and each fascicle of peripheral nerves is surrounded by perineurial cells. NS endothelial cells have tight junctions between cells. Thus, molecules must pass through membranes of endothelial cells, rather than between them, as they do in other tissues. The blood–brain barrier also contains transporters. If not actively transported into the brain, the penetration of toxicants is largely related to their lipid solubility and to their ability to pass through the plasma membranes of the cells forming the barrier. In the mature NS, the spinal and autonomic ganglia and other

sites within the brain called circumventricular organs do not contain specialized endothelial tight junctions and are not protected by blood–tissue barriers. It is this cellular anatomical arrangement that allows the endocrine-regulating components of the circumventricular organs to sense changes in blood hormone levels and respond accordingly. The blood–brain barrier is incompletely developed at birth and even less so in premature infants. This predisposes the premature infant to brain injury by toxicants.

Energy Requirements

Neurons and cardiac myocytes are critically dependent on aerobic respiration due to the high metabolic demand associated with the maintenance and repetitive reinstitution of ion gradients. To meet these high-energy requirements, the brain utilizes aerobic glycolysis and, therefore, is extremely sensitive to even brief interruptions in the supply of oxygen or glucose. Exposure to toxicants that inhibit aerobic respiration (e.g., cyanide) or to conditions that produce hypoxia (e.g., carbon monoxide poisoning) leads to the early signs of dysfunction in the myocardium and neurons. Damage to the NS under these conditions is a combination of direct toxic effects on neurons and secondary damage from systemic hypoxia or ischemia.

Axonal Transport

In the NS, impulses are conducted over great distances at rapid speed, providing information about the environment to the organism in a coordinated manner that allows an organized response to be carried out at a specific site. However, the intricate organization of such a complex network places an unparalleled demand on the cells of the NS. Single cells, rather than being spherical and a few micrometers in diameter, are elongated and may extend over a meter in length. Two immediate demands placed on the neuron are the maintenance of a larger cellular volume and the transport of intracellular materials over great distances.

In addition to the increased burden of protein synthesis, the neuron is dependent on the ability to distribute materials over the distances encompassed by its processes. Protein synthesis occurs in the cell body, and the products are then transported to the appropriate site through axonal transport. The assembly of the cytoskeleton at tremendous distances from their site of synthesis in the cell body represents a formidable challenge. Fast axonal transport carries numerous proteins, many of which are associated with vesicles, in an ATP-dependent process that reaches a rate of 400 mm/day (Fig. 16–1). This transport is dependent on microtubule-associated ATPase activity. Motor proteins, such as kinesin and dynein, provide both the mechanochemical force in the form of a microtubule-associated ATPase and the interface between microtubules as the track and vesicles as the cargo. Vesicles are transported rapidly in an anterograde direction by kinesin and in a retrograde direction by dynein.

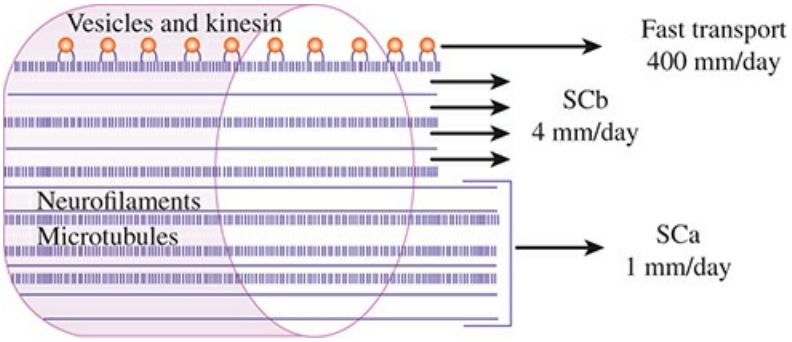

FIGURE 16–1 *Axonal transport.* Fast axonal transport is depicted as spherical vesicles moving along microtubules with intervening microtubule-associated motors. The slow component a (SCa) represents the movement of the cytoskeleton, composed of neurofilaments and microtubules. Slow component b (SCb) moves at a faster rate than SCa and includes soluble proteins, which are apparently moving between the more slowly moving cytoskeleton.

The transport of some organelles, including mitochondria, constitutes an intermediate component of axonal transport, moving at approximately 50 mm/day. The slowest component of axonal transport represents the movement of the cytoskeleton itself (Fig. 16–1), which is composed of structural elements, including microtubules formed by the association of tubulin subunits and neurofilaments formed by the association of three neurofilament protein subunits.

Neurofilaments and microtubules move at a rate of approximately 1 mm/day and make up the majority of slow component a (SCa), which is the slowest-moving component of axonal transport. Subunit structures appear to migrate and reassemble in a process that is dependent on nucleoside triphosphates, kinases, and phosphatases. Moving at only a slightly more rapid rate of 2 to 4 mm/day in an anterograde direction is slow component b (SCb), which includes several structural proteins, such as actin and several microfilament-associated proteins (M2 protein and fodrin), as well as clathrin and many soluble proteins.

This continual transport of proteins from the cell body through the various components of axonal transport provides the distal axon with its complement of functional and structural proteins. Some vesicles are also moving in a retrograde direction and provide the cell body with information concerning the status of the distal axon.

Axonal Degeneration

The sequence of events that occurs in the distal stump of an axon following transection is referred to as Wallerian degeneration. Following axotomy, there is degeneration of the distal nerve stump, followed by generation of a microenvironment supportive of regeneration and involving the distal axon, ensheathing glial cells and the blood nerve barrier.

Wallerian degeneration is an active process mediated by the axon itself, and it is possible to slow or even halt its progression. Moreover, although axonal degeneration can be initiated by many different means, including physical, genetic, or toxic, the mechanisms of degeneration converge into common regulated pathways that are potentially subject to pharmacological intervention.

The dynamic relationships between the neuronal cell body and its axon are important in understanding the basic pathological responses to some axonal and neuronal injuries caused by

neurotoxicants (Fig. 16–2). When the neuronal cell body has been lethally injured, it degenerates —in a process called neuronopathy. This is characterized by the loss of the cell body and its processes, with no potential for regeneration. When the injury is at the level of the axon, the axon may degenerate while the neuronal cell body continues to survive, a condition known as an axonopathy. In this setting, there is a potential for regeneration and recovery from the toxic injury as the axonal stump sprouts and regenerates.

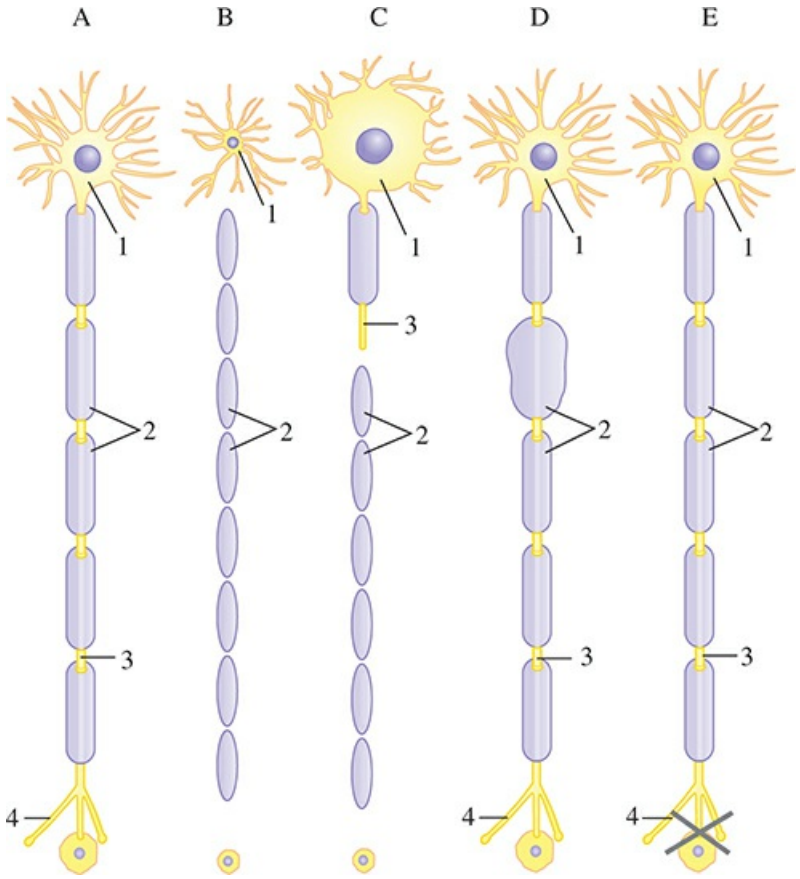

FIGURE 16–2 *Patterns of neurotoxic injury*. (**A**) Normal neuron showing (1) cell body and dendrites, (2) myelinating cells, encircling the (3) axon, and (4) synapse. (**B**) A neuronopathy resulting from the death of the entire neuron. Astrocytes often proliferate in response to the neuronal loss, creating both neuronal loss and gliosis. (**C**) An axonopathy occurs when the axon is the primary site of injury, the axon degenerates, and the surviving neuron shows only chromatolysis with margination of its Nissl substance and nucleus to the cell periphery. (**D**) Myelinopathy resulting from disruption of myelin or selective injury to the myelinating cells. To prevent cross talk between adjacent axons, myelinating cells divide and cover the denuded axon rapidly; however, the process of remyelination is much less effective in the CNS than in the PNS. (**E**) Some forms of toxicity are due to interruption of the process of neurotransmission, either through blocking excitation or by excessive stimulation, rather than actual cell death.

Terminating the period of survival is an active proteolysis that digests the axolemma and axoplasm, leaving only a myelin sheath surrounding a swollen degenerated axon. Digestion of the axon appears to be an all-or-none event effected through endogenous proteases that are

activated through increased levels of intracellular free Ca^{2}.

In the PNS, Schwann cells respond to loss of axons by decreasing synthesis of myelin lipids, downregulating genes encoding myelin proteins, and dedifferentiating to a premyelinating mitotic Schwann cell phenotype. In addition to providing physical guidance for regenerating axons, these cells provide trophic support from nerve growth factor, brain-derived nerve growth factor, insulin-like growth factor, and corresponding receptors produced by the associated Schwann cells. Resident and recruited hematogenous macrophages and denervated Schwann cells are involved in clearing myelin debris. In contrast to the proteolysis of the axon, myelin breakdown product processing proceeds in an established proximal-to-distal progression.

The degeneration of the distal axonal stump after transection is an active, synchronized process that can be delayed experimentally through decreasing temperature, preventing the entry of extracellular Ca^{2+}, or inhibiting calpain II–mediated proteolysis. Accompanying events in glial cells and macrophages direct and facilitate the sprouting neurite originating from the surviving proximal axon that also undergoes changes in protein expression resembling a less differentiated state. The facilitation of regeneration in the PNS by Schwann cells distinguishes it from the CNS, in which oligodendrocytes secrete inhibitory factors that impede neurite outgrowth. Eventually, though, even in the PNS, if axonal contact is not restored, Schwann cell numbers will decrease, bands of Bungner will disappear, and increased fibroblast collagen production will render regeneration increasingly unlikely.

Thus, a critical difference exists in the significance of axonal degeneration in the CNS compared with that in the PNS: peripheral axons can regenerate, whereas central axons cannot. In the PNS, glial cells and macrophages create an environment supportive of axonal regeneration, and Schwann cells transplanted to the CNS maintain this ability. In the CNS, release of inhibitory factors from damaged myelin and astrocyte scarring interferes with regeneration. The inability of the CNS to regenerate appears to be due to both unfavorable environmental glial factors and properties of the mature neuron. The clinical relevance of the disparity between the CNS and PNS is that partial recovery—or, in mild cases, complete recovery—can occur after axonal degeneration in the PNS, whereas the same event is irreversible in the CNS.

Myelin Formation and Maintenance

Myelin is formed in the CNS by oligodendrocytes and in the PNS by Schwann cells, which form concentric layers of lipid-rich myelin by the progressive wrapping of their cytoplasmic processes around the axon in successive loops (Fig. 16–3). These cells exclude cytoplasm from the inner surface of their membranes to form the major dense line of myelin. In a similar process, the extracellular space is reduced on the extracellular surface of the bilayers, and the lipid membranes stack together, separated only by a proteinaceous intraperiod line existing between successive layers.

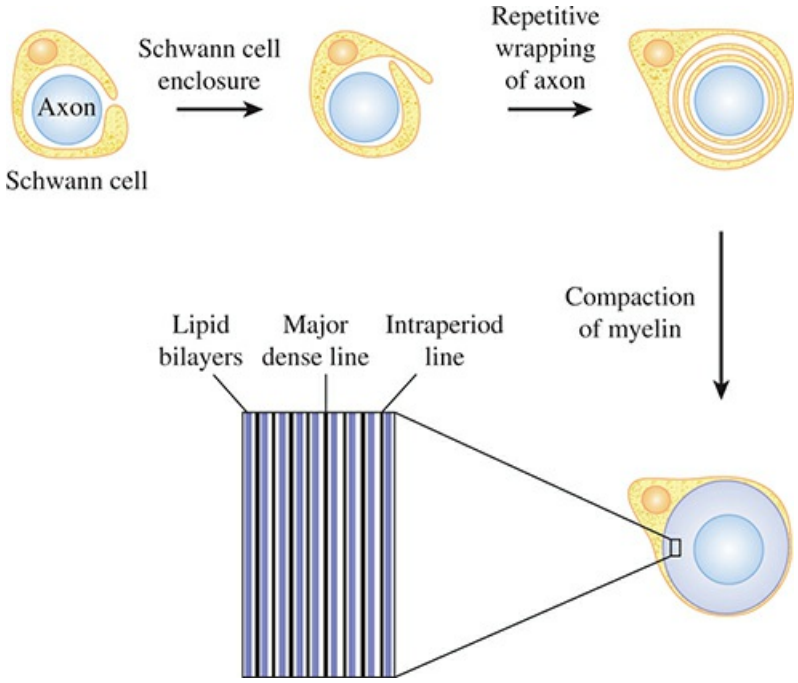

FIGURE 16–3 *Process of myelination.* Myelination begins when a myelinating cell encircles an axon, either Schwann cells in the peripheral nervous system or oligodendrocytes in the CNS. Simple enclosure of the axon persists in unmyelinated axons. Myelin formation proceeds by a progressive wrapping of multiple layers of the myelinating cell around the axon, with extrusion of the cytoplasm and extracellular space to bring the lipid bilayers into close proximity. The intracellular space is compressed to form the major dense line of myelin, and the extracellular space is compressed to form the intraperiod line.

The formation and maintenance of myelin requires metabolic and structural proteins that are unique to the NS. Myelin basic protein, an integral protein of CNS myelin, is closely associated with the intracellular space and an analogous protein, P1, is located in the PNS. On the extracellular surface of the lipid bilayers is the CNS proteolipid protein at the intraperiod line of myelin. It is now known that the maintenance of myelin is dependent on several membrane-associated proteins and on metabolism of specific lipids present in myelin bilayers. Some toxic compounds interfere with this complex process of the maintenance of myelin and result in myelinopathies (Fig. 16–2). The loss of myelin with the preservation of axons is referred to as primary demyelination, whereas degeneration of myelin accompanied by removal of the myelin sheath resulting from axonal loss is known as secondary demyelination.

Neurotransmission

Intercellular communication is achieved in the NS through the synapse. Neurotransmitter released from one axon to another neuron acts as the first messenger. Binding of the transmitter to the postsynaptic receptor is followed by modulation of an ion channel or activation of a second messenger system, leading to changes in the responding cell. Many therapeutic and toxic compounds impact neurotransmission by interrupting the transmission of impulses, blocking or accentuating transsynaptic communication, blocking reuptake of neurotransmitters or precursors,

or interfering with second-messenger systems.

Development of the Nervous System

Proliferation, migration, differentiation, synaptogenesis, apoptosis, and myelination are the basic processes that underlie development of the NS, and these occur in a tightly choreographed sequence that depends on the region, cell type, and neurotrophic signals. Neuronal and glial precursors replicate in a discrete zone near the inner surface of the neural tube. The proliferation and migration of these cells occur in waves that are specific for brain regions. During differentiation (phenotype expression) and synaptogenesis (formation of functional synaptic connections), the circuitry of the NS is established. Chemicals such as nerve growth factors, adhesive molecules, and neurotransmitters serve as morphogenic signals. The glial supportive cells develop last, and myelination is protracted. The period of rapid proliferation of glial cells is known as the brain growth spurt, during which time it is particularly vulnerable to insult.

The immature NS is especially vulnerable to certain chemicals. There are several factors that make the developing NS uniquely susceptible. Cell sensitivity differs with the developmental stage, leading to critical windows of vulnerability. While synaptogenesis can continue throughout life, proliferation cannot; therefore, the CNS is unique in that damaged neural cells are not readily replaced. Finally, slow formation of the blood–brain barrier and lack of key metabolic enzymes to protect the brain and eliminate toxicants influence NS sensitivity.

Although the developing NS is often more sensitive than the mature NS to insult (depending on the stage of development), the high rate of proliferation and regeneration in the developing NS may also lead to greater recovery or plasticity (an ability of one portion of the NS to assume the function of an injured area), which could attenuate some injuries. Some developmental changes may appear transient due to this plasticity and compensation, but underlying changes in NS development could become manifest with aging or some form of challenge.

In evaluating developmental neurotoxicity, chemical exposure or treatment may occur during critical windows of susceptibility or may cover the entire developmental process (i.e., during gestation, lactation, and adolescence). In general, injurious exposures early in gestation impact development of major brain regions, whereas later exposures alter biochemical, morphological, or functional features of the neural systems.

FUNCTIONAL MANIFESTATIONS OF NEUROTOXICITY

Functional assessment may be described using a series, or battery, of tests. Specific behavioral methods include observational assessments, tests of motor activity, and expanded clinical observations. These tests have the advantage over biochemical and pathological measures in that they permit evaluation of a single animal over time to determine the onset, progression, duration, and reversibility of a neurotoxic injury. Electrophysiological tests provide sensory-specific information on nerve conduction velocity and neuromotor integrity, and have been used to complement behavioral evaluations. Characterization of the effects of a test compound on sensory, motor, autonomic, and cognitive functions may include a wide range of tests. Deficits in

cognitive function, especially in the context of developmental toxicity, represent an endpoint of great public concern and rhetoric.

MECHANISMS OF NEUROTOXICITY

Individual neurotoxic compounds often have one of four targets: the neuron cell body, the axon, the myelinating cell, or the neurotransmitter system. As a result, neurotoxic compounds that cause neuronopathies, axonopathies, myelinopathies, or neurotransmitter-associated toxicity may be identified (Fig. 16–2).

Neuronopathies

Certain toxicants are specific for neurons, resulting in their injury or death. Neuron loss is irreversible and includes degeneration of its cytoplasmic extensions, dendrites and axons, and of the myelin ensheathing the axon (Fig. 16–2). Some features of the neuron that place it at risk for the action of cellular toxicants include a high metabolic rate, a long cellular process that is supported by the cell body, and an excitable membrane that is rapidly depolarized and repolarized.

Although many compounds are known to produce toxic neuronopathies (Table 16–1), all of these toxicants share certain features. Each toxic condition is the result of a cellular toxicant that has a predilection for neurons. The initial injury to neurons is followed by apoptosis or necrosis, leading to permanent loss of the neuron. These chemicals tend to have diffuse actions, although they may show some selectivity in the degree of injury of different neuronal subpopulations. The expression of these cellular events is often a diffuse encephalopathy, with global dysfunctions.

TABLE 16–1 Compounds associated with neuronal injury (neuronopathies).

Neurotoxicant	Neurologic Findings	Cellular Basis of Neurotoxicity
Aluminum	Dementia, encephalopathy (humans), learning deficits	Spongiosis cortex, neurofibrillary aggregates, degenerative changes in cortex
6-Amino-nicotinamide	Not reported in humans; hind limb paralysis (experimental animals)	Spongy (vacuolar) degeneration in spinal cord, brainstem, cerebellum; axonal degeneration of the peripheral nervous system (PNS)
Arsenic	Encephalopathy (acute), peripheral neuropathy (chronic)	Brain swelling and hemorrhage (acute); axonal degeneration in PNS (chronic)
Azide	Insufficient data (humans); convulsions, ataxia (primates)	Neuronal loss in cerebellum and cortex
Bismuth	Emotional disturbances, encephalopathy, myoclonus	Neuronal loss, basal ganglia, and Purkinje cells of cerebellum
Carbon monoxide	Encephalopathy, delayed parkinsonism/dystonia	Neuronal loss in cortex, necrosis of globus pallidus, focal demyelination; blocks oxygen-binding site of hemoglobin and iron-binding sites of brain
Carbon tetrachloride	Encephalopathy (secondary to liver failure)	Enlarged astrocytes in striatum, globus pallidus
Chloramphenicol	Optic neuritis, peripheral neuropathy	Neuronal loss (retina), axonal degeneration (PNS)
Cyanide	Coma, convulsions, rapid death; delayed parkinsonism/dystonia	Neuronal degeneration, cerebellum, and globus pallidus; focal demyelination; blocks cytochrome oxidase/ATP production
Doxorubicin	Insufficient data (humans); progressive ataxia (experimental animals)	Degeneration of dorsal root ganglion cells, axonal degeneration (PNS)
Ethanol	Mental retardation, hearing deficits (prenatal exposure)	Microcephaly, cerebral malformations
Lead	Encephalopathy (acute), learning deficits (children), neuropathy with demyelination (rats)	Brain swelling, hemorrhages (acute), axonal loss in PNS (humans)
Manganese	Emotional disturbances, parkinsonism/dystonia	Degeneration of striatum, globus pallidus
Mercury, inorganic	Emotional disturbances, tremor, fatigue	Insufficient data in humans (may affect spinal tracts; cerebellum)
Methanol	Headache, visual loss or blindness, coma (severe)	Necrosis of putamen, degeneration of retinal ganglion cells
Methylazoxymethanol acetate (MAM)	Microcephaly, retarded development (rats)	Developmental abnormalities of fetal brain (rats)
Methyl bromide	Visual and speech impairment; peripheral neuropathy	Insufficient data
Methyl mercury (organic mercury)	Ataxia, constriction of visual fields, paresthesias (adult)	Neuronal degeneration, visual cortex, cerebellum, ganglia
	Psychomotor retardation (fetal exposure)	Spongy disruption, cortex, and cerebellum
1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)	Parkinsonism, dystonia (acute exposure)	Neuronal degeneration in substantia nigra
	Early onset parkinsonism (late effect of acute exposure)	Neuronal degeneration in substantia nigra
3-Nitropropionic acid	Seizures, delayed dystonia/grimacing	Necrosis in basal ganglia
Phenytoin (diphenyl-hydantoin)	Nystagmus, ataxia, dizziness	Degeneration of Purkinje cells (cerebellum)
Quinine	Constriction of visual fields	Vacuolization of retinal ganglion cells
Streptomycin (aminoglycosides)	Hearing loss	Degeneration of inner ear (organ of Corti)
Thallium	Emotional disturbances, ataxia, peripheral neuropathy	Brain swelling (acute), axonal degeneration in PNS
Trimethyltin	Tremors, hyperexcitability (experimental animals)	Loss of hippocampal neurons, amygdala pyriform cortex

Doxorubicin—Doxorubicin (Adriamycin), a cardiotoxic quinone-containing anthracycline antibiotic, injures neurons in the PNS, specifically those of the dorsal root ganglia and autonomic

ganglia, by intercalating into grooves of DNA and interfering with transcription, by interaction with topoisomerase II, and by generation of reactive oxygen species (ROS). The vulnerability of sensory and autonomic neurons appears to reflect the lack of protection of these neurons by a blood–tissue barrier within ganglia.

Methyl Mercury—The most dramatic sites of injury are the neurons of the visual cortex and the small internal granular cell neurons of the cerebellar cortex, whose massive degeneration results in blindness and marked ataxia. In children, developmental disabilities, retardation, and cognitive deficits occur. Such age-related differences are seen also in other mammals, although the specific areas damaged may differ. These differences may be caused by an immature blood–brain barrier causing a more generalized distribution of mercury in the developing brain.

MeHg exposure may cause impaired glycolysis, nucleic acid biosynthesis, aerobic respiration, protein synthesis, and neurotransmitter release. In addition, there is evidence for enhanced oxidative injury and altered calcium homeostasis. Exposure to MeHg leads to widespread neuronal injury and subsequently to a diffuse encephalopathy.

Trimethyltin—Trimethyltin exposure leads to diffuse neuronal injury. Trimethyltin triggers selective apoptosis in specific subregions of the mammalian CNS and specific subsets of immune system cells. The hippocampus is particularly vulnerable to this process. Trimethyltin may cause energy deprivation and excitotoxic damage. Organotins, such as trimethyltin, interact with the CXC region of stannin, which is a highly conserved, 88 amino acid membrane-bound protein that interacts with other regulatory proteins to alter growth and apoptosis. Trimethyltin exposure results in tumor necrosis factor-α (TNF-α) production, which, in turn, induces stannin gene expression.

Axonopathies

The neurotoxic disorders in which the primary site of toxicity is the axon are called axonopathies. The axon degenerates, including the myelin surrounding that axon; however, the neuron cell body remains intact (Fig. 16–2). The toxicant causes a “chemical transection” of the axon at some point along its length, and the axon distal to the transection, biologically separated from its cell body, degenerates.

The number of axonal toxicants is large and increasing in number (Table 16–2); however, they all result in the pathological loss of distal axons with the survival of the cell body. Because the axonopathies pathologically resemble the actual physical transection of the axon, axonal transport appears to be a likely target in many of the toxic axonopathies. As these axons degenerate, sensations and motor strength are first impaired in the most distal extent of the axonal processes, the feet and hands, resulting in a “glove and stocking” distribution. With time and continued injury, the deficit progresses to involve more proximal areas of the body and the long axons of the spinal cord.

TABLE 16–2 Compounds associated with axonal injury (axonopathies).

Neurotoxicant	Neurologic Findings	Basis of Neurotoxicity
Acrylamide	Peripheral neuropathy (often sensory)	Axonal degeneration, axon terminal affected in earliest stages
p-Bromophenylacetyl urea	Peripheral neuropathy	Axonal degeneration in the peripheral nervous system (PNS) and central nervous system (CNS)
Carbon disulfide	Psychosis (acute), peripheral neuropathy (chronic)	Axonal degeneration, early stages include neurofilamentous swelling
Chlordecone (Kepone)	Tremors, in coordination (experimental animals)	Insufficient data (humans); axonal swelling and degeneration
Chloroquine	Peripheral neuropathy, weakness	Axonal degeneration, inclusions in dorsal root ganglion cells; also vacuolar myopathy
Clioquinol	Encephalopathy (acute), subacute myelooptic neuropathy (subacute)	Axonal degeneration, spinal cord, PNS, optic tracts
Colchicine	Peripheral neuropathy	Axonal degeneration, neuronal perikaryal filamentous aggregates; vacuolar myopathy
Dapsone	Peripheral neuropathy, predominantly motor	Axonal degeneration (both myelinated and unmyelinated axons)
Dichlorophenoxyacetate	Peripheral neuropathy (delayed)	Insufficient data
Dimethylaminopropionitrile	Peripheral neuropathy, urinary retention	Axonal degeneration (both myelinated and unmyelinated axons)
Ethylene oxide	Peripheral neuropathy	Axonal degeneration
Glutethimide	Peripheral neuropathy (predominantly sensory)	Insufficient data
Gold	Peripheral neuropathy (may have psychiatric problems)	Axonal degeneration, some segmental demyelination
n-Hexane	Peripheral neuropathy, severe cases have spasticity	Axonal degeneration, early neurofilamentous swelling, PNS, and spinal cord
Hydralazine	Peripheral neuropathy	Insufficient data
β,β′-Iminodipropionitrile	No data in humans; excitatory movement disorder (rats)	Proximal axonal swellings, degeneration of olfactory epithelial cells, vestibular hair cells
Isoniazid	Peripheral neuropathy (sensory), ataxia (high doses)	Axonal degeneration
Lithium	Lethargy, tremor, ataxia (reversible)	Insufficient data
Methyl *n*-butyl ketone	Peripheral neuropathy	Axonal degeneration, early neurofilamentous swelling, PNS, and spinal cord
Metronidazole	Sensory peripheral neuropathy, ataxia, seizures	Axonal degeneration, mostly affecting myelinated fibers; lesions of cerebellar nuclei
Misonidazole	Peripheral neuropathy	Axonal degeneration
Nitrofurantoin	Peripheral neuropathy	Axonal degeneration
Organophosphorus compounds (NTE inhibitors)	Abdominal pain (acute); peripheral neuropathy	Axonal degeneration
Paclitaxel (taxoids)	Delayed peripheral neuropathy (motor), spasticity	Axonal degeneration (delayed after single exposure), PNS, and spinal cord
Platinum (cisplatin)	Peripheral neuropathy	Axonal degeneration; microtubule accumulation in early stages
Pyridinethione (pyrithione)	Movement disorders (tremor, choreoathetosis)	Axonal degeneration (variable)
Vincristine (vinca alkaloids)	Cranial (most often trigeminal) neuropathy	Insufficient data
	Peripheral neuropathy, variable autonomic symptoms	Axonal degeneration (PNS), neurofibrillary changes (spinal cord, intrathecal route)

Hexane and Gamma-Diketones—Humans develop a progressive sensorimotor distal axonopathy when exposed to high concentrations of *n*-hexane and methyl *n*-butyl ketone (2-

hexanone). The carbon chain undergoes ω-1 oxidation, resulting in 2,5-hexanedione (HD), which is ultimately the toxic species produced from *n*-hexane and 2-hexanone. γ-Diketones, including HD, react with amino groups on all proteins, forming pyrroles that are oxidized and cross-link between neurofilament subunits. Neurofilaments accumulate in the distal axon, usually just proximal to a node of Ranvier, and form massive axonal swellings leading to retraction of myelin from the nodes and axonal atrophy, which is the pathological change that leads to nerve dysfunction and behavioral changes.

Carbon Disulfide—Significant exposures of humans to carbon disulfide (CS_2) cause a distal axonopathy that is identical pathologically to that caused by *n*-hexane. Covalent cross-linking of neurofilaments occurs, and CS_2 is the ultimate toxicant. Neurofilament cross-linking involves all three subunits and demonstrates a cumulative dose–response and temporal relationship consistent with it being a contributing event in the development of the axonal neurofilamentous swellings.

The clinical effects of exposure to CS_2 in the chronic setting are very similar to those of *n*-hexane exposure, with the development of sensory and motor symptoms occurring initially in a stocking-and-glove distribution. In addition to this chronic axonopathy, CS_2 can lead to aberrations in mood and indications of diffuse encephalopathic disease.

β,β′-Iminodipropionitrile—β,β′-Iminodipropionitrile (IDPN) is a synthetic, bifunctional nitrile that causes a “waltzing syndrome” in rats and other mammals, although human exposure has never been documented. Features of this waltzing syndrome include excitement, circling, head twitching, and overalertness, which may result from degeneration of vestibular sensory hair cells.

Accumulation of neurofilaments in the proximal axon occurs, leading to swelling without degeneration in most animals. Repeated exposure to IDPN leads to demyelination and onion bulb formation, and eventually can produce distal axonal atrophy due to a reduction in anterograde neurofilament transport to the distal axon. This impairment of axonal transport results from the disruption of the association between microtubules and neurofilaments by IDPN, causing neurofilament accumulation. This leads to complete disturbance of the cytoskeleton of the axon.

Acrylamide—Acrylamide is a man-made vinyl monomer used widely in water purification, paper manufacturing, mining, and waterproofing. It is also used extensively in biochemical laboratories, and is present in smaller amounts in many foods prepared at high temperatures. Studies of acrylamide neuropathy revealed a distal axonopathy characterized by multiple axonal swellings. Repeated dosing results in a more proximal axonopathy, in a “dying back” process. These changes are caused by accumulations of neurofilaments at the nerve terminal. It has also been observed that nerve terminal degeneration occurs prior to development of axonopathy, suggesting that this degeneration is the primary lesion.

Organophosphorus Compounds—OP insecticides and nerve agents are designed to inhibit AChE, thereby causing accumulation of acetylcholine in cholinergic synapses resulting in cholinergic toxicity and death. Some OP compounds, such as tri-*o*-cresyl phosphate (TOCP), can cause a severe sensorimotor central peripheral distal axonopathy called OP–induced delayed neurotoxicity (OPIDN) without inducing cholinergic poisoning. This condition is also referred to as a delayed neuropathy or delayed polyneuropathy (OPIDP).

Many OP compounds are lipophilic and readily enter the NS, where the parent compound or a metabolite can phosphorylate neural target proteins, such as various serine hydrolases. When the

principal target is AChE, cholinergic toxicity can ensue, either because of suprathreshold levels of inhibition or inhibition plus aging. When aging of inhibited AChE also occurs (net loss of a ligand from the phosphorus of the OP-enzyme conjugate, leaving a negatively charged phosphoryl moiety attached to the active site), the qualitative nature of the toxicity does not change. Instead, the inhibited AChE becomes intractable to reactivation with oximes, such as 2-pralidoxime methiodide (2-PAM).

When the principal target is neuropathy target esterase (neurotoxic esterase [NTE], also known as patatin-like phospholipase domain containing 6 [PNPLA6]), OPIDN can result only if both suprathreshold (more than 70%) inhibition occurs and the inhibited enzyme undergoes aging. Thus, in the case of NTE and OPIDN, inhibition alone is insufficient to precipitate toxicity. It appears that the biochemical lesion is not simply a blockade of the active site. Instead, axonopathy is triggered by specific chemical modification of the NTE protein. Aging inhibitors of NTE include compounds from the phosphate, phosphonate, and phosphoramidate classes of OP compounds.

Axonal degeneration does not commence immediately after acute exposure to a neuropathic OP compound but is delayed for at least 8 days between the acute high-dose exposure and clinical signs of axonopathy. Some effective regeneration of axons occurs in the PNS. In contrast, axonal degeneration is progressive and persistent in long tracts of the spinal cord.

Recently, concerns about the neurotoxicity of TOCP have resurfaced in the guise of aerotoxic syndrome, a constellation of neurobehavioral symptoms attributed to breathing aircraft cabin air containing a variety of contaminants including TOCP from jet engine lubricants. Binding of the cresyl saligenin phosphate (CBDP) toxic metabolite of TOCP to NTE and other proteins has been detected.

Pyridinethione—Zinc pyridinethione or zinc pyrithione's (ZPT) intended uses as an antidandruff shampoo can lead to human exposures through direct dermal contact and potential exposure to biota through leaching into marine and freshwater environments. Pyridinethione chelates zinc, copper, and other metal ions and, once oxidized to the disulfide, may lead to the formation of protein–pyridinethione mixed disulfides. Pyridinethione appears to interfere with the fast axonal transport systems and impairs the turnaround of rapidly transported vesicles and slows their retrograde transport. As materials accumulate in one region of the axon, they distend the axonal diameter, and the axon degenerates in its more distal regions beyond the accumulated structures. The earliest signs are diminished grip strength and electrophysiological changes of the axon terminal.

Microtubule-Associated Neurotoxicity—Plant alkaloids, including vinca alkaloids and colchicine, alter the assembly and depolymerization of microtubules in nerve axons, causing neurotoxicity. Colchicine, an alkaloidal pharmaceutical used in the treatment of gout, familial Mediterranean fever, and other disorders, commonly causes a mild peripheral axonal neuropathy that is often accompanied by a disabling myopathy that can lead to the inability to walk. Vincristine and vinblastine produce a peripheral axonopathy very similar to that induced by colchicine. Vincristine, commonly used to treat leukemias and lymphomas, binds to tubulin subunits and prevents the polymerization into microtubules. Parasthesias of the fingers, general weakness and clumsiness occur. Paclitaxel (Taxol), another plant alkaloid used to treat a variety of neoplasms, causes a predominantly sensory neuropathy, beginning in the hands and feet. Paclitaxel binds to tubulin promoting the formation and stabilization of microtubules.

Myelinopathies

Myelin provides electrical insulation of neuronal processes, and its absence leads to a slowing of and/or aberrant conduction of impulses between adjacent processes, so-called ephaptic transmission. Toxicants exist that result in the separation of the myelin lamellae, termed intramyelinic edema, and in the selective loss of myelin, termed demyelination. Intramyelinic edema may be caused by alterations in the transcript levels of myelin basic protein-mRNA. However, the initial stages may progress to demyelination, with loss of myelin from the axon. Remyelination in the CNS occurs to only a limited extent after demyelination. However, Schwann cells in the PNS are capable of remyelinating the axon after a demyelinating injury.

The compounds in Table 16–3 lead to a myelinopathy. The functional consequences of demyelination depend on the extent of the demyelination and whether it is localized within the CNS or the PNS or is more diffuse in its distribution. Those toxic myelinopathies in which the disruption of myelin is diffused generate a global neurological deficit, whereas those that are limited to the PNS produce the symptoms of peripheral neuropathy.

TABLE 16–3 Compounds associated with injury of myelin (myelinopathies).

Neurotoxicant	Neurologic Findings	Basis of Neurotoxicity
Acetylethyltetramethyl tetralin (AETT)	Not reported in humans; hyperexcitability, tremors (rats)	Intramyelinic edema; pigment accumulation in neurons
Amiodarone	Peripheral neuropathy	Axonal degeneration and demyelination; lipid-laden lysosomes in Schwann cells
Cuprizone	Not reported in humans; encephalopathy (experimental animals)	Status spongiosis of white matter, intramyelinic edema (early stages); gliosis (late)
Disulfiram	Peripheral neuropathy, predominantly sensory	Axonal degeneration, swellings in distal axons
Ethidium bromide	Insufficient data (humans)	Intramyelinic edema, status spongiosis of white matter
Hexachlorophene	Irritability, confusion, seizures	Brain swelling, intramyelinic edema in CNS and PNS, late axonal degeneration
Lysolecithin	Effects only on direct injection into PNS or CNS (experimental animals)	Selective demyelination
Perhexilene	Peripheral neuropathy	Demyelinating neuropathy, membrane-bound inclusions in Schwann cells
Tellurium	Hydrocephalus, hind limb paralysis (experimental animals)	Demyelinating neuropathy, lipofuscinosis (experimental animals)
Triethyltin	Headache, photophobia, vomiting, paraplegia (irreversible)	Brain swelling (acute) with intramyelinic edema, spongiosis of white matter

Hexachlorophene—Following dermal absorption, hexachlorophene (methylene 2,2′-methylene-bis(3,4,6-trichlorophenol)) enters the NS and results in intramyelinic edema, splitting the intraperiod line of myelin in both the CNS and the PNS. The intramyelinic edema leads to the formation of vacuoles, creating a “spongiosis” of the brain. Hexachlorophene binds tightly to cell membranes, resulting in the loss of ion gradients across the membrane, which leads to water and ion entry, which separates the myelin layers as “edema.” Intramyelinic edema is reversible in the early stages, but with increasing exposure, hexachlorophene causes segmental demyelination. Humans exposed acutely to hexachlorophene may have generalized weakness, confusion, and seizures. Progression to include coma and death may occur.

Tellurium—Although human exposures have not been reported, neurotoxicity of tellurium has been demonstrated in animals. Tellurium or one of its derivatives may interfere with the normal conversion of squalene to cholesterol. Squalene epoxidase, a microsomal monooxygenase that utilizes NAPDH cytochrome P450 reductase, has been strongly implicated as the target of tellurium. Lipids accumulate in Schwann cells within intracytoplasmic vacuoles; shortly afterward, these Schwann cells lose their ability to maintain myelin. Axons and the myelin of the CNS are impervious to the effects of tellurium. However, individual Schwann cells in the PNS that encompass the greatest distances appear to be affected.

Lead—The neurotoxicity of lead has been appreciated for centuries. Chronic lead intoxication in adults results in peripheral neuropathy, often accompanied by manifestations outside the NS, such as gastritis, colicky abdominal pain, anemia, and the prominent deposition of lead in particular anatomic sites, creating lead lines in the gums and in the epiphyses of long bones in children. The effects of lead on the peripheral nerve of humans (lead neuropathy) are not entirely understood. Electrophysiological studies have demonstrated a slowing of nerve conduction.

Astrocytes

Astrocytes perform and regulate a wide range of physiological functions in the CNS. The astrocyte appears to be a primary means of defense in the CNS following exposure to neurotoxicants, as a spatial buffering system for osmotically active ions and as a depot for the sequestration and metabolic processing of endogenous molecules and xenobiotics.

Ammonia—At high CNS concentrations, ammonia produces seizures, resulting from its depolarizing action on cell membranes, whereas at lower concentrations, ammonia produces stupor and coma, consistent with its hyperpolarizing effects. Ammonia intoxication is associated with astrocytic swelling and morphological changes. Brain detoxification of glutamate requires ATP-dependent amidation of glutamate to glutamine mediated by the astrocyte-specific enzyme, glutamine synthetase (GS), and catalyzed by ammonia. Increased intracellular ammonia concentrations have also been implicated in the inhibition of neuronal glutamate precursor synthesis, resulting in diminished glutamatergic neurotransmission, changes in neurotransmitter uptake (glutamate), and changes in receptor-mediated metabolic responses of astrocytes to neuronal signals.

Nitrochemicals—The neurotoxic compound, 1,3-dinitrobenzene (DNB), produces gliovascular lesions that specifically target astrocytes in the periaqueductal gray matter of the brainstem and deep cerebellar roof nuclei. It has been proposed that bioactivation of DNB by NADPH-

dependent cytochrome *c* reductase and subsequent induction of oxidative stress underlie its toxicity. Brainstem nuclei with high glucose requirements, such as the vestibular and deep cerebellar roof nuclei, are affected more severely than forebrain and mesencephalic structures that have similar or higher requirements for glucose and oxygen. Differences in mitochondrial respiratory capacity, cellular antioxidant levels, and the expression of proteins that regulate the mitochondrial permeability transition pore (mtPTP) contribute to the observed regional and cellular differences in susceptibility.

The antimicrobial, antiprotozoal drug metronidazole (1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole) is associated with a peripheral neuropathy characterized by paraesthesias, dysesthesias, headaches, glossitis, urticaria, pruritus, and other somatosensory disorders. Long-term administration of metronidazole produces an irreversible sensorimotor deficit in the lower extremities of humans. Metronidazole is readily reduced to the highly reactive and toxic hydroxylamine intermediate and binds to cellular macromolecules including proteins and DNA. Use of high intravenous doses for an extended period results in the expression of epileptiform seizures, hallucination, and attendant encephalopathy. Metronidazole and its reduced metabolites bear close structural resemblance to the antineuritic nutrient, thiamine. Thiamine triphosphate (vitamin B1) is an essential coenzyme in the mitochondrial metabolism of α-ketoglutarate and pyruvate, and also modulates the activity of sodium channels.

Methionine Sulfoximine—Methionine sulfoximine (MSO) is an irreversible inhibitor of the astrocyte-specific enzyme, glutamine synthetase (GS). Ingestion of large amounts of MSO leads to neuronal cell loss in the hippocampal fascia dentata and pyramidal cell layer, in the short association fibers and lower layers of the cerebral cortex, and in cerebellar Purkinje cells. MSO also leads to large increases of glycogen levels, primarily within astrocytic cell bodies, as well as swollen and damaged astrocytic mitochondria. The relationship between inhibition of GS by MSO and seizure generation is not well understood.

Fluoroacetate and Fluorocitrate—The Krebs cycle inhibitor fluorocitrate (FC) and its precursor fluoroacetate (FA) are preferentially taken up by glia. FA occurs naturally in a number of plants and is available commercially as a rodenticide (Compound 1080). Ingestion of large amounts of FA results in tonic convulsions. Animals consuming FA commonly seize within minutes, and those surviving these episodes frequently die later due to respiratory arrest or heart failure. The actions of FC and FA have been attributed both to the disruption of carbon flux through the Krebs cycle and to impairment of ATP production.

Neurotransmission-Associated Neurotoxicity

A wide variety of naturally occurring toxins, as well as synthetic chemicals, alter specific mechanisms of intercellular communication (Table 16–4). Multiple neurotransmitter targets may be more common than was once expected.

TABLE 16–4 Compounds associated with neurotransmitter-associated toxicity.

Neurotoxicant	Neurologic Findings	Basis of Neurotoxicity
Amphetamine and methamphetamine	Tremor, restlessness (acute); cerebral infarction and hemorrhage; neuropsychiatric disturbances	Bilateral infarcts of globus pallidus, abnormalities in dopaminergic, serotonergic, cholinergic systems
		Acts at adrenergic receptors (PNS)
Atropine	Restlessness, irritability, hallucinations	Blocks cholinergic receptors (anticholinergic)
Cocaine	Increased risk of stroke and cerebral atrophy (chronic users); increased risk of sudden cardiac death; movement and psychiatric abnormalities, especially during withdrawal	Infarcts and hemorrhages; alteration in striatal dopamine neurotransmission
	Decreased head circumference (fetal exposure)	Structural malformations in newborns
Domoic acid	Headache, memory loss, hemiparesis, disorientation, seizures	Neuronal loss, hippocampus and amygdala, layers 5 and 6 of neocortex
		Kainate-like pattern of excitotoxicity
Kainate	Insufficient data in humans; seizures in animals (selective lesioning compound in neuroscience)	Degeneration of neurons in hippocampus, olfactory cortex, amygdala, thalamus
		Binds AMPA/ kainate receptors
β-*N*-Methylamino-L-alanine (BMAA)	Weakness, movement disorder (monkeys)	Degenerative changes in motor neurons (monkeys)
		Excitotoxic probably via NMDA receptors
Muscarine (mushrooms)	Nausea, vomiting, headache	Binds muscarinic receptors (cholinergic)
Nicotine	Nausea, vomiting, convulsions	Binds nicotinic receptors (cholinergic) low-dose stimulation; high-dose blocking
β-*N*-Oxalylamino-L-alanine (BOAA)	Seizures	Excitotoxic probably via AMPA class of glutamate receptors

Nicotine—Nicotine exerts its effects by binding to nicotinic receptors located in ganglia, at the neuromuscular junction, and within the CNS, where the psychoactive and addictive properties most likely reside. Smoking and "pharmacological" doses of nicotine accelerate heart rate, elevate blood pressure, and constrict blood vessels within the skin. Because most of these effects may be prevented by the administration of α- and β-adrenergic blockade, these consequences may be viewed as the result of stimulation of the ganglionic sympathetic NS. The nicotine sensation of "relaxation" is probably related to the binding of nicotine with nicotinic receptors within the CNS.

The rapid rise in circulating levels of nicotine leads to excessive stimulation of nicotinic receptors, a process that is followed rapidly by ganglionic paralysis. Initial nausea, rapid heart rate, and perspiration are followed shortly by marked slowing of heart rate with a fall in blood pressure. Somnolence and confusion may occur, followed by coma; if death results, it is often the result of paralysis of the muscles of respiration.

In humans, it has been difficult to separate the effects of nicotine from those of other components of cigarette smoke. The complications of smoking include cardiovascular disease, cancers (especially malignancies of the lung and upper airway), chronic pulmonary disease, and attention deficit disorders in children of women who smoke during pregnancy. Nicotine may be a factor in some of these problems.

Cocaine and Amphetamines—Cocaine blocks the reuptake of dopamine (DA), norepinephrine, and serotonin at nerve terminals in the CNS, and causes release of DA from storage vesicles. The primary event responsible for the addictive properties and euphoric feeling when intoxicated is a block of the DA reuptake transporter (DAT). This leads to enhanced DAergic transmission and can result in a variety of symptoms in the user. Many individuals report an euphoric feeling and increased self-confidence, in addition to racing thoughts and a feeling of pressure. In other users, a period of paranoid psychosis ensues. Cocaine abuse places individuals at risk for cerebrovascular defects, a greater degree of cerebral atrophy, and they are more at risk of stroke and intracranial hemorrhage. In chronic cocaine users, neurodegenerative disorders have been observed.

Amphetamines affect catecholamine neurotransmission in the CNS by competing for uptake via plasma membrane transporters and disruption of vesicular storage. Amphetamines increase energy and sensation in adults. Serious side effects may be due to affecting DAergic neurons, and damage to 5-HT axons and axon terminals. The result is a distal axotomy of DA and 5-HT neurons.

Excitatory Amino Acids—Glutamate and certain other acidic amino acids are excitatory neurotransmitters. The toxicity of excitatory amino acids may be related to such divergent conditions as hypoxia, epilepsy, and neurodegenerative diseases.

Glutamate is the main excitatory neurotransmitter of the brain, and its effects are mediated by several subtypes of receptors (Fig. 16–4) called excitatory amino acid receptors (EAARs). The two major subtypes of glutamate receptors are those that are ligand-gated directly to ion channels (ionotropic) and those that are coupled with G proteins (metabotropic). Ionotropic receptors may be further subdivided by their specificity for binding kainate, quisqualate, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), and *N*-methyl-d-aspartate (NMDA). The entry of glutamate into the CNS is regulated at the blood–brain barrier. Glutamate injures neurons, apparently by opening glutamate-dependent ion channels, ultimately leading to neuronal

swelling and neuronal cell death. The toxicity affects the dendrites and neuronal cell bodies but seems to spare axons. The only known related human condition is the “Chinese restaurant syndrome,” in which consumption of large amounts of monosodium glutamate may lead to a burning sensation in the face, neck, and chest in sensitive individuals.

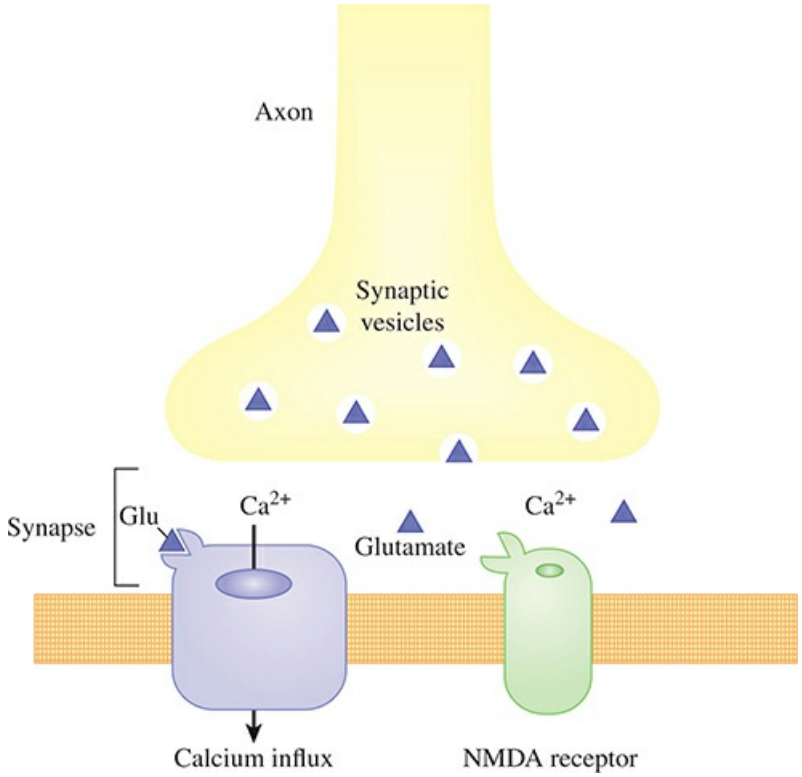

FIGURE 16–4 *Excitatory synapse*. Synaptic vesicles are transported to the axonal terminus and released across the synaptic cleft to bind to the postsynaptic receptors. Glutamate, as an excitatory neurotransmitter, binds to its receptor and opens a calcium channel, leading to the excitation of the postsynaptic cell.

The cyclic glutamate analog kainate, initially isolated from seaweed, is extremely potent as an excitotoxin, being 100-fold more toxic than glutamate and is selective at a molecular level for the kainate receptor. Like glutamate, kainate selectively injures dendrites and neurons and shows no substantial effect on glia or axons. As a result, neurobiologists, with the help of this neurotoxic scalpel, can study the role of neurons in a particular area independent of the axonal injuries that occur when similar lesioning experiments are performed by mechanical cutting.

Development of permanent neurological deficits in individuals accidentally exposed to high doses of an EAAR agonist has underscored the potential importance of excitatory amino acids in disease. Domoic acid, an analog of glutamate, causes an acute illness that commonly presents as gastrointestinal disturbance, severe headache, and short-term memory loss. A subset of the more severely afflicted patients was subsequently shown to have chronic memory deficits and motor neuropathy. Neuropathological investigation of patients who died within 4 months of intoxication showed neurodegeneration that was most prominent in the hippocampus and amygdala, but also affected regions of the thalamus and cerebral cortex.

Toxicant-Based Models of Neurodegenerative Disease

1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine (MPTP)—Over hours to days after injection with MTPT, dozens of patients developed the signs and symptoms of irreversible Parkinson disease (PD). The symptomatology of each reflects a disruption of the nigrostriatal pathway: masked facies, difficulties in initiating and terminating movements, resting "pill-rolling" tremors, rigidity, and bradykinesias (Fig. 16–5). Autopsy studies demonstrated marked degeneration of DAergic neurons in the substantia nigra (SN).

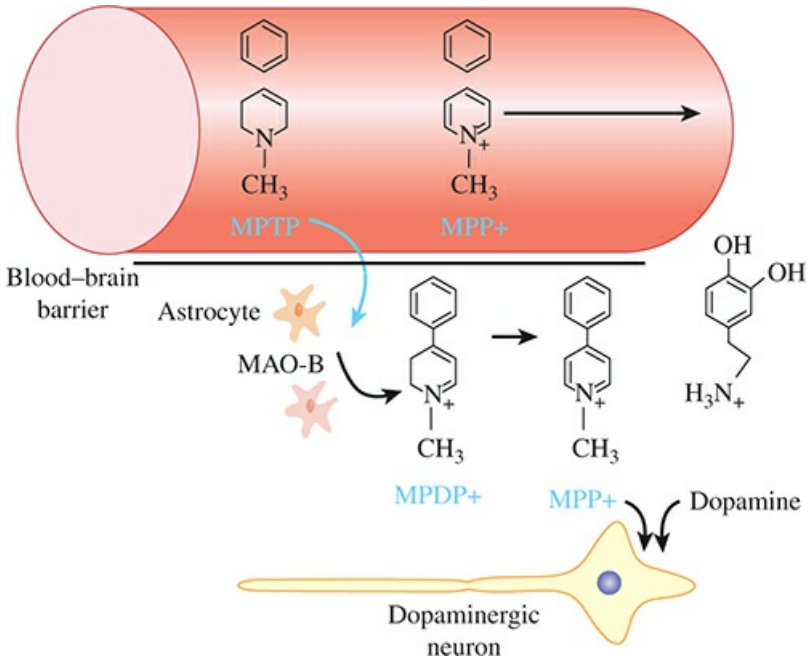

FIGURE 16–5 *MPTP toxicity*. MPP^+, either formed elsewhere in the body following exposure to MPTP or injected directly into the blood, is unable to cross the blood–brain barrier. In contrast, MPTP gains access and is oxidized in situ to $MPDP^+$ and MPP^+. The same transport system that carries dopamine into the dopaminergic neurons also transports the cytotoxic MPP^+.

MPTP is neurotoxic and a substrate for the B isozyme of monoamine oxidase (MAO-B). MPTP, an uncharged species at physiological pH, crosses the blood–brain barrier (BBB) and diffuses into cells, including astrocytes. The MAO-B in astrocytes catalyzes a two-electron oxidation to yield $MPDP^+$, the corresponding dihydropyridinium ion. A further two-electron oxidation yields the pyridinium ion MPP^+ (Fig. 16–5). MPP^+ enters DAergic neurons of the substantia nigra (SN) via the DA uptake system, resulting in injury or death of the neuron. Noradrenergic neurons of the locus ceruleus are also vulnerable to repeated exposures of MPTP, although they are less affected by single exposures than are the DAergic neurons. Once inside neurons, MPP^+ acts as a general mitochondrial toxicant, blocking respiration at complex I. MPP^+ may also lead to the production of ROS and the release of DA from vesicles due to the higher pH environment of the cytosol, where the neurotransmitter undergoes autoxidation.

Manganese—As an essential metal that is found in all tissues, Mn is required for normal

metabolism of amino acids, proteins, lipids, and carbohydrates. Mn acts as a cofactor for enzymes, such as manganese metalloenzymes, which include arginase, glutamine synthetase (GS), phosphoenolpyruvate decarboxylase, and manganese-superoxide dismutase (Mn-SOD). Mn-dependent enzymes include oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases. In rare Mn deficiencies, clinical manifestations can include seizures, impaired growth, skeletal abnormalities, and impaired reproductive function.

Excessive exposure to Mn causes neurotoxicity as Mn accumulates in the basal ganglia and targets DAergic neurons and perhaps other neurotransmitter systems. Occupational exposure to toxic levels of Mn in industrial workers results in psychological and neurological disturbances, including delusions, hallucinations, depression, disturbed equilibrium, compulsive or violent behavior, weakness, and apathy, followed by extrapyramidal motor system defects such as tremors, muscle rigidity, ataxia, bradykinesia, and dystonia. Mn toxicity causes a loss of DA neurons in the SN, and as in PD, oxidative stress appears to play a significant role in the disorder. The brain area, most susceptible to Mn injury, is also highly sensitive to oxidative stress. Mn accumulates in the SN, globus pallidus (GP), and striatum and interferes with ATP synthesis, analogous to effects seen with mitochondrial inhibitors or ischemia.

DAergic neurodegeneration associated with both PD and Mn exposure shares multiple common mechanisms, namely mitochondrial dysfunction, aberrant signal transduction, oxidative stress, protein aggregation, and the activation of cell death pathways. The accumulation of Mn in the striatum causes damage to the SN, reduction in tyrosine hydroxylase (TH) activity, and loss of DA neurons. The specificity for Mn accumulation in GP and striatum likely correlates with Mn transporter distribution and the metabolic activity of these basal ganglia nuclei.

Guamanian Cycad–Induced Parkinsonism/Amyotrophic Lateral Sclerosis Syndrome— Native Guam Chamorros have an unusually high incidence of amyotrophic lateral sclerosis (ALS) and parkinsonism–dementia complex (PDC). Guam ALS is clinically indistinguishable from ALS that occurs elsewhere in the world. Guam PDC is a distinct neurodegenerative disorder where the main clinical features include mental deterioration, parkinsonism, and evidence of motor neuron involvement. Macroscopic neuropathological features are the presence of cortical atrophy and depigmentation of the substantia nigra, and microscopic evaluations revealed widespread ganglion cell degeneration and neurofibrillary tangles throughout the CNS.

Seeds of Cycas circinalis contain several toxins including β-N-methylamino-l-alanine (BMAA). Direct or indirect consumption of BMAA, a glutamate excitotoxic amino acid, causes neurodegeneration by an excitotoxic mechanism involving the mitochondrial permeability transition pore (mtPTP). BMAA increases the intracellular calcium concentration through the formation of a β-carbamate intermediate that can act as a glutamate agonist to induce glutamate excitotoxicity.

Environmental Factors Relevant to Neurodegenerative Diseases

The relationship between MPTP intoxication and parkinsonism stimulated investigations into the role that environmental and occupational exposures may play in the pathogenesis of neurodegenerative diseases. Epidemiological studies implicate environmental and occupational exposure to pesticides, metals, and solvents as risk factors for PD. Studies of agricultural workers and communities have associated higher PD incidence with exposure to specific pesticides such as organochlorines, paraquat, rotenone, and OP compounds. Converging

evidence in PD research suggests that mitochondrial damage and oxidative stress are the primary causes of DAergic neuronal death.

Developmentally Neurotoxic Chemicals

Replication, migration, differentiation, apoptosis, myelination, and synapse formation are basic processes that occur in specific spatial and temporal patterns and underlie development of the NS. Many insults are known to disrupt NS development, including exposures to certain metals, solvents, antimetabolites, persistent organic pollutants, pesticides, pharmaceuticals, and ionizing radiation. Multiple mechanisms of action may be present, producing a wide array of effects in the offspring. The impact on the developing NS may be different, and often cannot be predicted, from effects observed in adults. Several neurodevelopmental disorders have been, at least partially, attributed to exposures to neurotoxicological chemicals during the fetal, infant, or childhood periods. In recent years, the concept has emerged that extremely low levels of exposure to some substances in “asymptomatic” children may actually affect their behavioral and cognitive development. This has led to the suspicion that the number of chemically induced neurological disorders is larger than generally assumed.

Chemicals That Induce Depression of Nervous System Function

The CNS maintains balance via interplay between inhibitory and excitatory influences. With general depressant chemicals, initial suppression of inhibitory systems at low doses produces excitation, such as intoxication observed with ethanol. Thus, acutely, CNS depressants produce a continuum of effects from excitation to sedation, motor impairment, coma, and ultimately death by depression of respiratory centers. Therapeutic indications of drugs causing CNS depression include anxiety, insomnia, epilepsy, and muscle relaxation. Generalized depression of CNS function is produced by a variety of volatile solvents. Human exposures range from chronic low levels in the general environment or occupational settings to high acute or chronic levels arising from household uses or solvent abuse.

IN VITRO AND OTHER ALTERNATIVE APPROACHES TO NEUROTOXICOLOGY

High-Throughput Analysis

The use of high-throughput cell-based assays and quantitative structure–activity relationships (QSARs) to predict adverse outcomes will replace standard in vivo neurotoxicity assessments in due time to assess the backlog of thousands of untested chemicals. Tiered testing schemes have been proposed, where the first tiers rely on high-throughput methods that test for chemical actions on critical biological receptors that initiate pathways of changes that lead to adverse outcomes, in order to identify chemicals for future testing. Second-tier tests could involve the use of alternative species, such as small fish or invertebrates, that will allow more moderate throughput, but in an intact or developing NS. Chemicals identified as having neurotoxic

properties could then be tested in intact mammalian models as necessary.

New approaches for screening and characterizing the neurotoxic potential of chemicals must be established. Critical to this process are complementary approaches that will allow assessment of the impact of chemical-induced alterations on crucial processes in intact multicellular organisms. The extraordinary conservation of both genomic/epigenomic elements and differentiation processes between mammals and nonmammalians makes more feasible the use of these alternative models. Finally, each method of analysis will contribute unique information to the overall picture of neurotoxicity and inform the process and interpretation of the other avenues of investigation.

Cell Culture and Model Organism Approaches

Cell-based assays can provide critical new information regarding toxicant effects on intracellular signaling and cell lifecycle processes. Evaluation of cytotoxicity, particularly in a heterogeneous system such as the brain, is difficult in the intact animal because numerous factors (neural, hormonal, and hemodynamic) are not under experimental control. Neural cultures offer numerous advantages over in vivo techniques. Cell morphology, protein synthesis and release, energy metabolism, receptor interaction, neurotransmitter uptake and release, as well as electrolyte and nonelectrolyte uptake and release can be directly studied. The culture model also makes it possible to study regional specialization, and it can be extended to study cellular interactions by co-culturing various cell types. Limitations of the culture systems include the fact that cells can undergo varying degrees of differentiation, lose heterogeneous cell–cell interactions, and hence lose autocrine and paracrine signaling processes that modulate form and function of the cell. Different, sometimes competing, processes can influence the ability of a toxicant to damage specific cells. The ability of a cell to repair or replace damaged organelles or enzymes can also be critical in determining cell survival. This effect may also be dependent on neighboring cells and physical barriers, which may altogether be absent in a culture system.

Emerging alternative test species, such as *Caenorhabditis elegans*, Zebrafish (*Danio rerio*), and *Drosophila melanogaster*, are making it possible to assess the effects of small molecules rapidly, inexpensively, and on a miniaturized scale. Such model systems provide an approach to study toxicity in an intact organism where cell–cell interactions and complex metabolic milieus influence and modify xenobiotic-induced neurotoxic potential, not possible in in vitro systems. Embryonic stages of many of these species are optically transparent, allowing for easy real-time examination of the neuronal morphology and direct viewing of protein expression patterns. High-throughput approaches include genome-wide screening for molecular targets or mediators of toxicity and rapid, high-content chemical screens to detect potential toxicants by RNA interference (RNAi), DNA microarrays, and gene expression analysis.

Stem Cell–Based Approaches

Advances in human and mammalian stem cell technology has increasingly enabled in vitro study of neurotoxic properties of chemicals spanning effects from early development through maturity. Such analysis can permit the prioritization of compounds for further developmental and adult neurotoxicity testing by in vivo and other approaches, thereby speeding up efforts to identify neurotoxicants for public health research and minimize or reduce animal testing. Thus, stem cell

models of neuronal differentiation have proved to be valuable model systems for the exploration of mechanisms of neurotoxicity and identification of neurotoxicants.

Systems and Computational Approaches

Emerging advances in analytical techniques, imaging, sensing, and massively parallel computational capabilities will permit new insights into the function and chemically induced dysfunction of the brain, spinal cord, and PNS. Systems approaches that scale from the molecular level to the whole body are currently under development and may soon revolutionize the science of neurotoxicology. Considerations of dose, pharmacokinetics and dynamics, absorption, distribution, metabolism, and excretion must not be lost in the estimation of hazard and risk from exposure to neurotoxic chemicals. Computational approaches to these considerations are improving alongside advances in computing technology.

EPIGENETICS IN NEUROTOXICOLOGY

Epigenetics can be defined broadly as transmission of heritable changes in gene expression that are not the result of changes in DNA sequence. Epigenetic changes are central to the idea that early life exposures to toxicants may lead to disease and dysfunction later in life is rapidly becoming a focus in toxicology. There are data demonstrating that this concept may also apply to neurological disorders. Alterations of the epigenetic machinery can have a powerful impact on the proper expression of genes, and potentially lead to significant alterations in development that result in disease and dysfunction. Emerging evidence has identified epigenetic alterations as contributors to neurodegenerative diseases, including AD and PD, both of which may be influenced by exposure to environmental neurotoxicants. As such, there is increasing recognition for the potential of xenobiotic-induced neurotoxicity to occur through epigenetic mechanisms.

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QUESTIONS

1. Which of the following statements regarding axons and/or axonal transport is FALSE?
   - **a.** Single nerve cells can be over 1 m in length.
   - **b.** Fast axonal transport is responsible for movement of proteins from the cell body to the axon.
   - **c.** Anterograde transport is accomplished by the protein kinesin.

d. The motor proteins, kinesin and dynein, are associated with microtubules.
e. A majority of the ATP in nerve cells is used for axonal transport.

2. Which of the following statements is not characteristic of Schwann cells in Wallerian degeneration?
   a. Schwann cells provide physical guidance needed for the regrowth of the axon.
   b. Schwann cells release trophic factors that stimulate growth.
   c. Schwann cells act to clear the myelin debris with the help of macrophages.
   d. Schwann cells increase synthesis of myelin lipids in response to axonal damage.
   e. Schwann cells are responsible for myelination of axons in the peripheral nervous system.

3. Prenatal exposure to ethanol can result in mental retardation and hearing deficits in the newborn. What is the cellular basis of the neurotoxicity?
   a. neuronal loss in cerebellum.
   b. acute cortical hemorrhage.
   c. microcephaly.
   d. loss of hippocampal neurons.
   e. degeneration of the basal ganglia.

4. Which of the following characteristics is LEAST likely to place a neuron at risk of toxic damage?
   a. high metabolic rate.
   b. ability to release neurotransmitters.
   c. long neuronal processes supported by the soma.
   d. excitable membranes.
   e. large surface area.

5. The use of meperidine contaminated with MPTP will result in a Parkinson's disease-like neurotoxicity. Where is the most likely site in the brain that MPTP exerts its toxic effects?
   a. cerebellum.
   b. cerebral cortex.
   c. brainstem.
   d. substantia nigra.
   e. hippocampus.

6. Which of the following statements regarding the PNS and the CNS is TRUE?
   a. Nerve impulse transduction is much faster in the CNS than in the PNS.
   b. PNS axons can regenerate, whereas CNS axons cannot.
   c. Remyelination does not occur in the CNS.
   d. Oligodendrocytes perform remyelination in the PNS.
   e. In the CNS, oligodendrocyte scarring interferes with axonal regeneration.

7. Platinum (cisplatin) results in which of the following neurologic problems?

a. peripheral neuropathy.
b. trigeminal neuralgia.
c. spasticity.
d. gait ataxia.
e. tremor.

8. Which of the following is NOT characteristic of axonopathies?
a. There is degeneration of the axon.
b. The cell body of the neuron remains intact.
c. Axonopathies result from chemical transaction of the axon.
d. A majority of axonal toxicants cause motor deficits.
e. Sensory and motor deficits are first noticed in the hands and feet following axonal degeneration.

9. All of the following statements regarding lead exposure are true EXCEPT:
a. Lead exposure results in peripheral neuropathy.
b. Lead slows peripheral nerve conduction in humans.
c. Lead causes the transection of peripheral axons.
d. Segmental demyelination is a common result of lead ingestion.
e. Lead toxicity can result in anemia.

10. Regarding excitatory amino acids, which of the following statements is FALSE?
a. Glutamate is the most common excitatory amino acid in the CNS.
b. Excitotoxicity has been linked to conditions such as epilepsy.
c. Overconsumption of monosodium glutamate (MSG) can result in a tingling or burning sensation in the face and neck.
d. An ionotropic glutamate receptor is coupled to a G protein.
e. Glutamate is toxic to neurons.

CHAPTER 17

Toxic Responses of the Cornea, Retina, and Central Visual System

Donald A. Fox and William K. Boyes

KEY POINTS

- Toxic chemicals and systemic drugs can affect all parts of the eye, including cornea, iris, ciliary body, lens, retina, and optic nerve.
- Ophthalmologic procedures for evaluating the health of the eye include routine clinical screening evaluations using a slit-lamp biomicroscope and ophthalmoscope, and an examination of the pupillary light reflex.
- Most electrophysiologic or neurophysiologic procedures for testing visual function after

toxicant exposure involve stimulating the eyes with visual stimuli and electrically recording potentials generated by visually responsive neurons.

INTRODUCTION TO OCULAR AND VISUAL SYSTEM TOXICOLOGY

Environmental and occupational exposure to chemicals, toxicants, gases, and vapors as well as systemic and ocular therapeutic drugs may result in structural and functional alterations to the eye and central visual system. The retina and central visual system may be especially vulnerable to toxic insult. Subtle alterations in visual processing of information (e.g., visual perception, visual motor) can have profound immediate, long-term, and delayed effects on the mental, social, and physical health and performance of an individual.

This chapter will provide essential information on ocular pharmacodynamics, pharmacokinetics, and drug metabolism; review the procedures for testing visual function; and evaluate and review the structural and functional alterations in the mammalian eye and central visual system produced by environmental and workplace chemicals, toxicants, gases and vapors, and off-target effects of major therapeutic drugs. The adverse effects of these chemicals on the different compartments of the eye (i.e., cornea, lens, retina, and retinal pigment epithelium [RPE]), central visual pathway (i.e., optic nerve and optic tract), and central processing areas (i.e., lateral geniculate nucleus [LGN] and visual cortex) are listed in Table 17–1. The distribution of phase I and phase II drug metabolizing enzymes, and xenobiotic uptake and efflux transporters in ocular tissues are presented in Tables 17–2 and 17–3, respectively. Table 17–4 provides examples of common signs, symptoms, and potential pathophysiological mechanisms of visual dysfunction associated with acute or chronic exposure to toxicants and selected drugs.

TABLE 17–1 Ocular, Retinal, and Central Visual System Sites of Action of Selected Xenobiotics Following Systemic Exposure

Xenobiotic	Cornea	Lens	RPE	Outer Retina: Rods and Cones	Inner Retina: BCS, ACS, IPCS	RGCS, OPTIC Nerve or Tract	LGN, Visual Cortex
Acrylamide				–	–	++	++
Amiodarone	+	+				+	
Carbon disulfide				+	–	++	+
Chloroquine	+		+	+		+	
Chlorpromazine	+	+	+	+			
Corticosteroids		++				+	
Digoxin and digitoxin	+	+	+	++		+	+
Ethambutol				+		++	
Hexachlorophene				+			++
Indomethacin	+		+	+			
Isotretinoin	+						
Lead	+		+	++	+	+	+
Methanol			+	++	–	++	+
Methyl mercury, mercury			+	–	–	++	+
n-Hexane			+	+		+	
Naphthalene		+		+			
Organic solvents				+			+
Organophosphates		+		+		+	+
Sildenafil	–	–	–	+	+	+	–
Styrene				+			
Tamoxifen	+			+		+	
Vigabatrin	–	–	–	+	+	+	–

RPE, retinal pigment epithelium; BC, bipolar cell; AC, amacrine cell; IPC, interplexiform cell; RGC, retinal ganglion cell; LGN, lateral geniculate nucleus. "+" indicates that this site of action was cited in one or more case reports, review articles, clinical or animal studies. "–" indicates that this site of action showed no adverse effect as cited in one or more case reports, review articles, clinical or animal studies, productivity, and a distinct decrease in the overall quality of life.

TABLE 17–2 Distribution of Ocular Xenobiotic-Biotransforming Phase I and Phase II Enzymes

	Enzymes	Tears	Cornea	Iris/Ciliary	Lens	Retina	Choroid
Phase I enzymes	Acetylcholinesterase (AChE)	+		+		+	+
	Alcohol dehydrogenase		+		−	+	+
	Aldehyde dehydrogenase		+		+	+	+
	Aldehyde reductase		+	+	+	+	+
	Aldose reductase		+		+	+	
	Carboxylesterase	+	+	+		+	+
	Catalase	−	+	+	+	+	+
	Cu/Zn superoxide dismutase	+	+	−	±	+	
	MAO-A or MAO-B	+		+		+	+
	CYP1A1 or CYP1A2	+	+	+	−	+	+
	CYP1B1		+	+	+	+	
	CYP2B1 or CYP2B2				+	+	
	CYP2C11				+		
	CYP3A1				+	+	
	CYP4A1 or CYP4B2		+	+		+	
	CYP27A1					+	
Phase II enzymes	Glutathione peroxidase	−	+	+	+	+	+
	Glutathione reductase		+		+	+	
	Glutathione-*S*-transferase		+	+	+	+	
	Sulfotransferases				+	+	
	UDP-glucuronosyl transferases				+	+	
	N-Acetyltransferase		+	+	+	+	+

"+" and "−" indicate that the enzyme was present (localized by immunohistochemistry, immunogold electron microscopy, Western blot, or gene expression) or absent, respectively, in human, monkey, or rodent tissues.

TABLE 17–3 Distribution of Ocular Xenobiotic Uptake and Efflux Transporters (TPs)

	Xenobiotic Transporter	Cornea: Epithelium	Cornea: Endothelium	RPE	Rods & Cones	Inner Retina	NFL
TPs	SLC22A7 (OAT2)	+	−	+	−	+	+
	SLC22A3 (OCT3)	+	+	+	+	−	−
	ABCB1 (MDR1; P-gp)	−	−	−	−	−	−
	ABCC1 (MRP1)	−	−	−	−	−	−
	ABCC4 (MRP4)	+	−	−	−	−	−
	ABCC5 (MRP5)	−	−	−	−	−	−
	ABCG2 (BCRP)	+	−	−	−	+	+

SLC, solute carrier; OAT, organic anion transporter; ABC, ATP binding cassette; MDR1, multidrug resistance protein 1; P-gp, permeability glycoprotein; MRP, multidrug resistance-associated protein efflux pump; BCRP, breast resistance protein; RPE, retinal pigment epithelium; NFL, nerve fiber layer. "+" and "−" indicate that the enzyme was present (localized by immunohistochemistry, Western blot, or gene expression) or absent, respectively, in human tissues.

TABLE 17–4 Common Signs and Symptoms of Visual System Dysfunction

Common Signs and Symptoms	Possible Pathophysiological Basis	Examples of Chemicals Producing this Effect
Cornea		
Pigment deposits in corneal epithelium (verticillate keratopathy)	Intralysosomal accumulation of lipids	Amphiphilic medications such as amiodarone, chloroquine, clofazimine, phenothiazines, suramin
Lens		
Cataracts anterior cortical (AC), posterior subcapsular (PSC)	Chemical deposition, photochemical oxidation	Long-term systemic use of phenothiazine (AC), corticosteroids (PSC)
Pupil		
Pupil constriction (miosis)	Anticholinesterases	Organophosphate and carbamate insecticides, nerve gas agents such as sarin, soman, and tabun
Pupil dilation (mydriasis) and photophobia	Cholinergic antagonists	Atropine or belladonna
	Adrenergic agonists	Amphetamine, cocaine, phenylephrine
Ocular motility		
Diplopia (double vision), nystagmus	Oculomotor impairment, damage or dysfunction in vestibular/oculomotor reflex pathways	Acute alcohol intoxication, barbiturate toxicity, acute solvent exposure
Retinal pigment epithelium		
Loss of central vision (central scotoma)	Degeneration of retinal pigment epithelium and underlying photoreceptors	Carbon disulfide, chloroquine
Retina		
Poor night (scotopic) vision and impaired dark adaptation	Damage to and apoptosis of rod photoreceptors	Lead, methyl mercury, vigabatrin
	Acetylcholinesterase inhibitors	Organophosphate and carbamate insecticides, nerve gas agents
Altered color perception, central scotoma	Inhibition of cone photoreceptor sodium-pumps	Digitalis/digitoxin
Altered color perception	Inhibition of cone photoreceptor cGMP-phosphodiesterase	Sildenafil and tadalafil
Impaired color discrimination (blue/yellow)	Damage to cone photoreceptors and inner retina	Chronic exposure to styrene and organic solvents, trimethadione, chronic high dose antibiotics
Impaired color discrimination (red/green)	Acquired damage to cone photoreceptors, neural retina and/or afferent visual pathway	Higher level chronic exposure to organic solvents, carbon disulfide or hexane, chronic carbon monoxide, chronic alcoholism, ethambutol
Loss of peripheral vision (tunnel vision, peripheral scotoma, visual field constriction)	Degeneration of peripheral retina and nerve fiber layer	Methyl mercury, vigabatrin
Reduced contrast sensitivity and visual acuity	Degeneration of the retinal ganglion cells and optic tract, microaneurysms and retinal vasculopathy	Acrylamide, carbon disulfide
Optic nerve and optic tract		
Reduced contrast sensitivity and visual acuity	Optic neuritis and/or degeneration of the optic tract, generally affecting mitochondrial ATP production	Higher level chronic exposure to organic solvents such as carbon disulfide or hexane, ethambutol, ethylene glycol, isoniazid, linezolid and chloramphenicol, methanol, vigabatrin
Monocular and/or binocular visual loss	Nonarteritic anterior ischemic optic neuropathy	Amiodarone, sildenafil, and tadalafil
Lateral geniculate nucleus and visual cortex		
Central scotoma	Degeneration of calcarine fissure of visual cortex	Methyl mercury
Visuomotor deficits and reduced contrast sensitivity	Visual and motor cortex dysfunction	Lead, chronic exposure to carbon disulfide, hexane and other solvents, toluene

Inclusion in this table indicates that this drug, chemical, or toxicant was cited in one or more case reports, review articles, clinical or animal studies. The pathophysiological causes and chemicals listed are provided as examples and are not exhaustive.

Many chemicals initially appear to affect a single site and, by inference, mechanism of action, whereas others have several sites and corresponding mechanisms of action. However, depending upon the dose (concentration), many of these chemicals have multiple sites of action. For example, carbon disulfide produces optic nerve/tract degeneration and also adversely affects the neurons and vasculature of the retina, resulting in photoreceptor and retinal ganglion cell (RGC) structural and functional alterations. Gestational and postnatal exposure to inorganic lead affects rod photoreceptors in developing and adult mammals, resulting in rod-mediated (scotopic) vision deficits; however, structural and functional deficits at the level of the RGCs, visual cortex, and oculomotor system also are observed. Although both gestational and postnatal lead exposure produce scotopic flash electroretinographic (ERG) deficits, the amplitude changes are in opposite directions and their underlying mechanisms are distinctly different. Some neurotoxicants (e.g., acrylamide or lead) have been utilized for in vivo and in vitro animal models to examine the pathogenesis of different retinal, neuronal, or axonal diseases; the basic functions of the retinocortical pathways; and/or the molecular mechanisms of apoptosis.

EXPOSURE TO THE EYE AND VISUAL SYSTEM

Ocular Pharmacodynamics and Pharmacokinetics

Toxic chemicals and systemic drugs can affect all parts of the eye (Fig. 17–1; Table 17–1). Factors determining whether a chemical reaches a particular ocular site of action include the physiochemical properties of the chemical, concentration and duration of exposure/treatment, route of exposure, and the movement of the chemical into and across the different ocular barriers and compartments. The cornea, conjunctiva, and eyelids are often exposed directly to chemicals, gases, particles, and drugs. The first site of action is the tear film: a four-layered structure with both hydrophobic and hydrophilic properties. The outermost thin tear film layer (0.1 μm) is hydrophobic and is secreted by the meibomian (sebaceous) glands. This superficial lipid layer helps stabilize the tear film by retarding evaporation and protects the underlying thicker (7 μm) aqueous layer that is produced by the lacrimal glands. The third layer with both hydrophobic and hydrophilic properties is the very thin (0.02 to 0.05 μm) mucoid layer that is secreted by the goblet cells of the conjunctiva and that acts as an interface between the hydrophilic layer of the tears and the hydrophobic layer of the corneal epithelial cells. The fourth glycocalyx layer consists of a meshwork of membrane-bound mucins. Water-soluble chemicals more readily mix with the tears and gain access to the cornea.

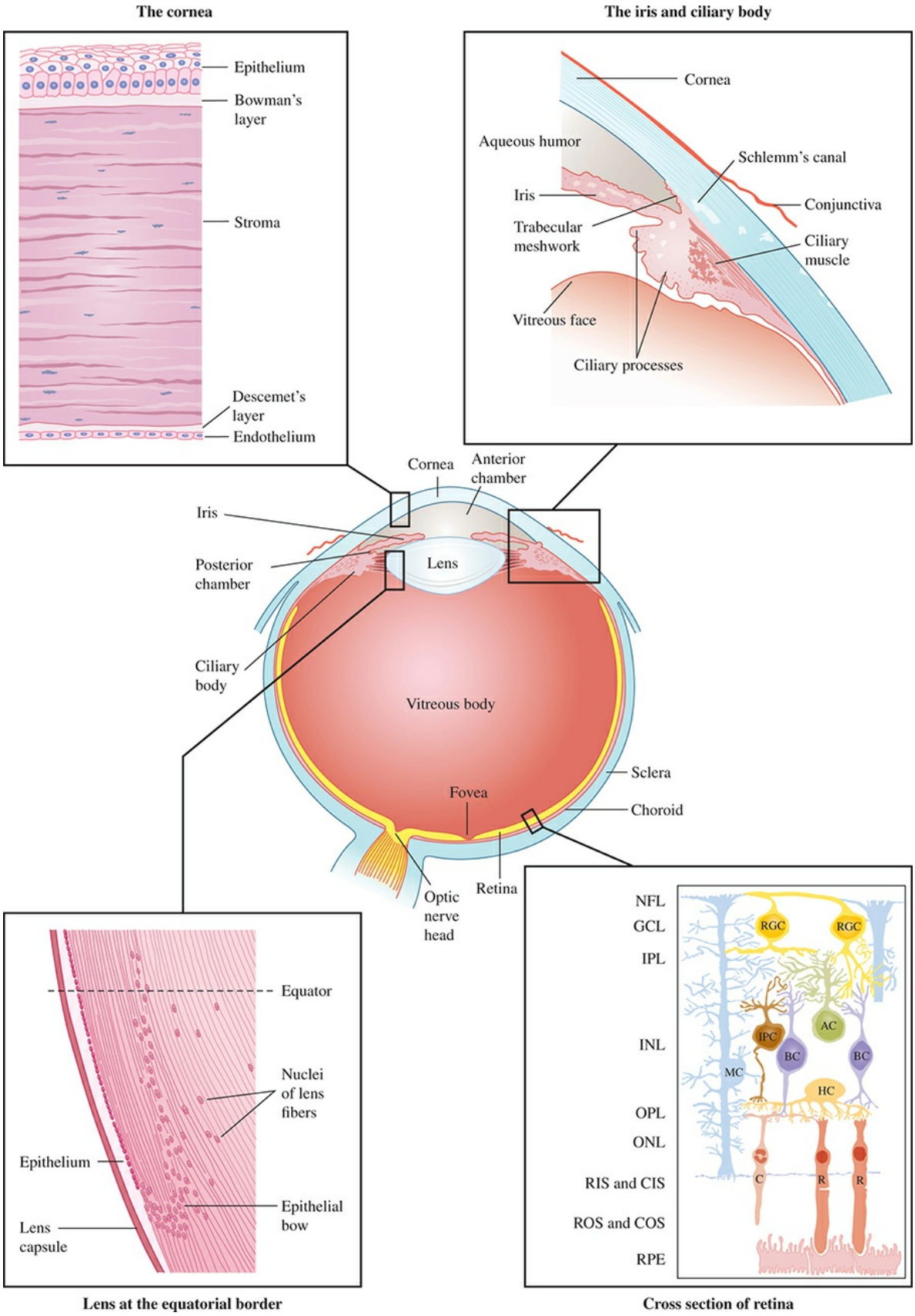

FIGURE 17–1 *Diagrammatic horizontal cross section of the eye, with medium-power enlargement of details for the cornea, iris and ciliary body, lens, and retina.* The morphological features, their role in ocular pharmacodynamics, pharmacokinetics, drug metabolism, and the adverse effects of drugs and chemicals on these sites are discussed in the text.

The avascular cornea is the external barrier to the internal ocular structures. Greater systemic absorption and higher blood concentration occurs through contact with the vascularized conjunctiva (Fig. 17–2). The human cornea has several distinct layers, through which a chemical must pass in order to reach the anterior chamber. The first is the corneal epithelium: stratified squamous, non-keratinized cells with tight junctions. The permeability of the corneal epithelium is low and only lipid-soluble chemicals readily pass through this layer. Bowman's membrane separates the epithelium from the stroma. The corneal stroma makes up 90% of the corneal thickness and is composed of water, collagen, and glycosaminoglycans, which permit hydrophilic chemicals to easily dissolve in this thick layer. The inner edge of the corneal stroma is bounded by a thin, limiting basement membrane, called Descemet's membrane, which is secreted by the corneal endothelium. The innermost layer of the cornea, the corneal endothelium, is composed of a single layer of cells surrounded by lipid membranes. The permeability of the corneal endothelial cells to ionized chemicals is relatively low.

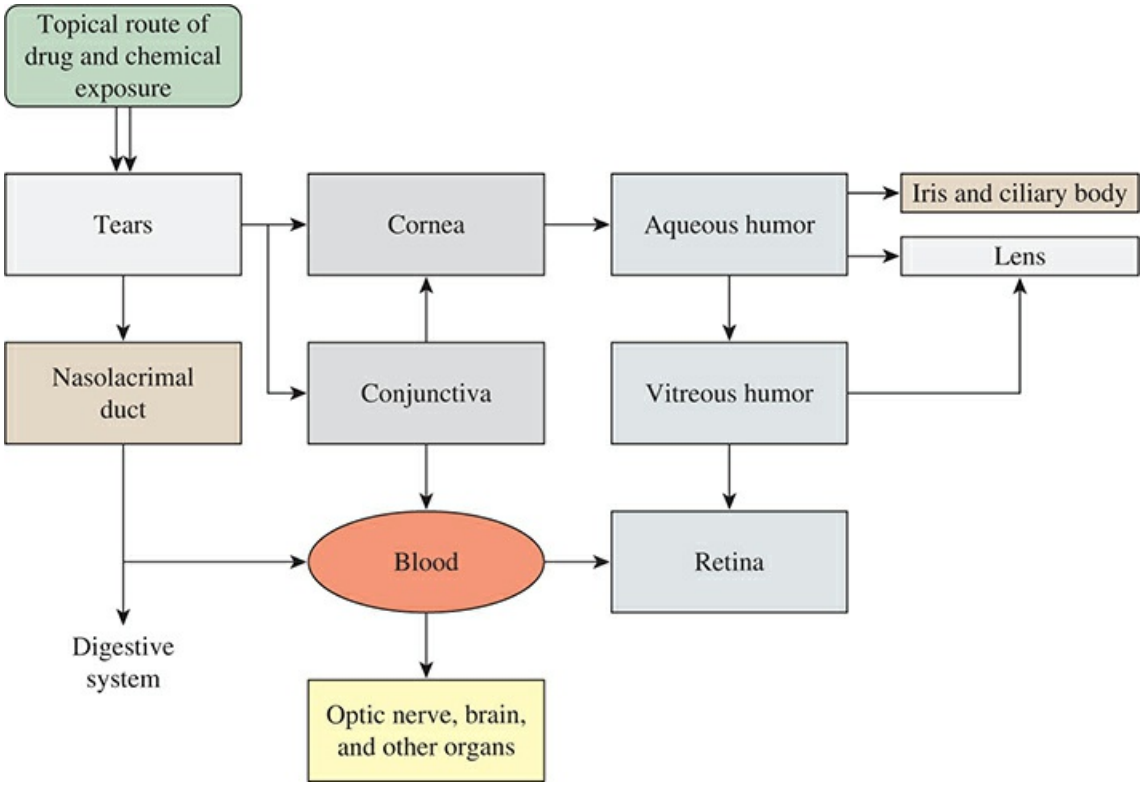

FIGURE 17–2 *Ocular absorption and distribution of drugs and chemicals following the topical route of exposure.* The details for movement of drugs and chemicals between compartments of the eye and subsequently to the optic nerve, brain, and other organs are discussed in the text.

The two separate arterial systems in the human eye are (1) uveal arteries, which supply the vascular beds of the iris, ciliary body, and choroid, and (2) retinal arteries. In the anterior segment of the eye, there is a blood–aqueous barrier that has relatively tight junctions between the endothelial cells of the iris capillaries and nonpigmented cells of the ciliary epithelium. The major function of the ciliary epithelium is the production of aqueous humor from the plasma filtrate present in the stroma of the ciliary processes.

The retina of humans, monkeys, pigs, dogs, rats, and mice has a dual circulatory supply: choroidal and retinal. The outer or distal retina, which consists of the RPE, rod, and cone photoreceptor outer segments (ROS, COS) and inner segments (RIS, CIS), and the photoreceptor outer nuclear layer (ONL), are supplied by the choriocapillaris: a dense, one-layered network of fenestrated vessels formed by the short posterior ciliary arteries and located next to the RPE. These capillaries with loose endothelial junctions and abundant fenestrae are permeable to large proteins. The retinal blood vessels are distributed within the inner or proximal portion of the retina, which consists of the outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), and ganglion cell layer (GCL). The endothelial cells of capillaries of the retinal vessels have tight junctions that form the blood–brain barrier in the cerebral capillaries. The optic disc lacks these tight-junction types of capillaries and thus hydrophilic molecules can enter the optic nerve head by diffusion from the extravascular space and cause selective damage at this site of action.

Following systemic exposure to drugs and chemicals, these compounds are distributed to all parts of the eye by the blood in the uveal and retinal blood vessels (Fig. 17–3). Most drugs and chemicals equilibrate rapidly with the extravascular space of the choroid where they are separated from the retina and vitreous body by the retinal pigment epithelium (RPE) and endothelial cells of the retinal capillaries, respectively. Hydrophilic molecules with molecular weights less than 200 to 300 Da can cross the ciliary epithelium and iris capillaries and enter the aqueous humor. The corneal endothelium, which maintains normal hydration and transparency of the corneal stroma, could be exposed to chemical compounds by the aqueous humor and limbal capillaries. Similarly, the anterior surface of the lens can also be exposed as a result of its contact with the aqueous humor. The most likely retinal target sites following systemic drug and chemical exposure appear to be the RPE and photoreceptors in the distal retina because the endothelial cells of the choriocapillaris are permeable to proteins smaller than 50 to 70 kDa. However, the cells of the RPE are joined on their basolateral surface by tight junctions, zonula occludens, which limit the passive penetration of large molecules into the neural retina.

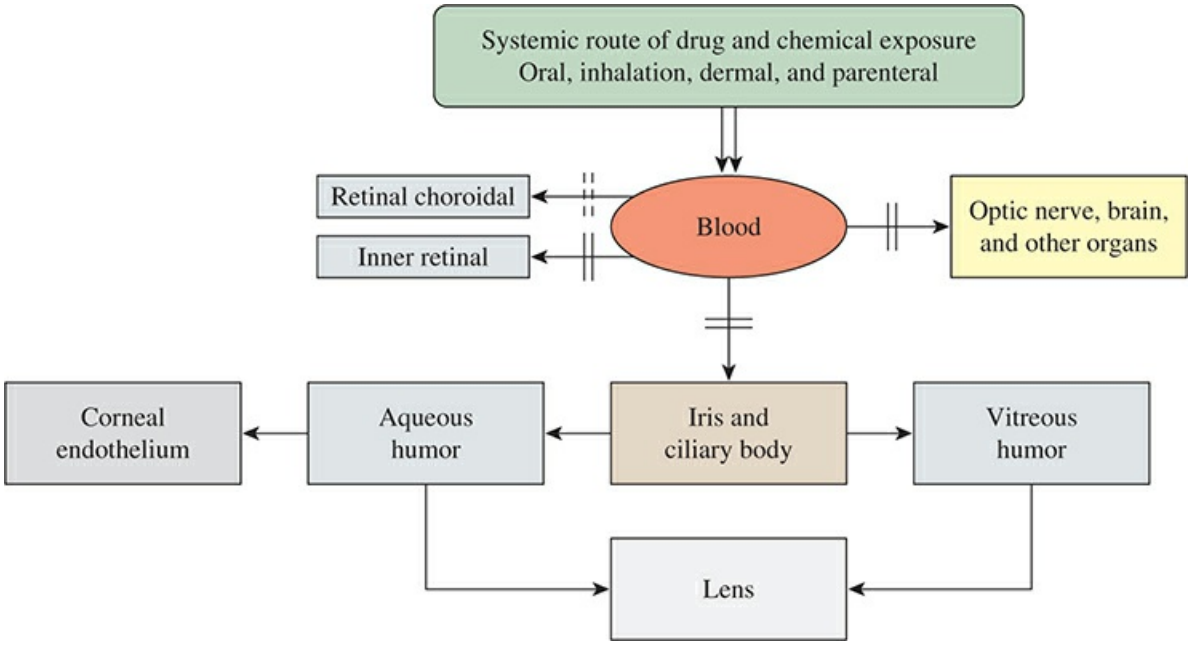

FIGURE 17–3 *Distribution of drugs and chemicals in the anterior and posterior segments of the eye, optic nerve, brain, and other organs following the systemic route of exposure.* The details for movement of drugs and chemicals between compartments of the eye are discussed in the text. The solid and dotted double lines represent the different blood–tissue barriers present in the anterior segment of the eye, retina, optic nerve, and brain. The solid double lines represent tight endothelial junctions, whereas the dotted double lines represent loose endothelial junctions.

Intraocular melanin, found in several different locations in the eye (pigmented cells of the iris, ciliary body, RPE, and uveal tract), has a high binding affinity for polycyclic aromatic hydrocarbons, electrophiles, calcium, and toxic heavy metals such as aluminum, iron, lead, and mercury. Although this initially may play a protective role, it can also result in the excessive accumulation, long-term storage, and slow release of numerous drugs and chemicals from melanin.

Ocular Drug Delivery and Engineered Nanoparticles

The main ocular target sites of importance for disease treatment and neuroprotection are the anterior segment, retinal neurons (i.e., photoreceptors, retinal ganglion cells: RGCs), retinal vasculature, and RPE. Numerous superficial barriers, blood–retina barriers, transporters, and depot sites restrict bioavailability, decrease therapeutic efficacy, and increase side effects. The goal of nanoscale formulations for topical drug delivery is to enhance penetration from the cornea, deliver a wide variety of drugs and molecules, and increase the concentration and contact time of drugs with these tissues. New intravitreal nanoparticles as drug carriers have been developed, including chitosan-coated, compacted DNA, drug-coated, lipid-based, liposome, magnetic, unimolecular micelles, etc. All vehicles for topical and intravitreal ocular drug delivery need to have low toxicity to ocular tissues. Because nanomaterials are readily transformed in environmental or biological media, it is difficult to know the final active form of

nano-enabled ocular drugs or the effects of nanomaterials engineered for other purposes that might inadvertently contact ocular tissues.

Ocular Drug Metabolism and Transport

Metabolism and excretion of xenobiotics occurs in all compartments of the eye by phase I and II xenobiotic-biotransforming enzymes and drug transporters in ocular tissues. Drug metabolizing enzymes are present in the tears, iris–ciliary body, choroid, and retina of many different species (Table 17–2). The activity of these enzymes varies between species and ocular tissues. The whole lens appears to have low biotransformational activity, except for glutathione-S-transferase activity found in the lens epithelium. The presence and need for a competent glutathione conjugation system is clearly understandable in ocular tissues that directly interact with UV radiation, light, and xenobiotics.

Several ATP-binding cassette (ABC) families of transporters, solute carrier (SLC) transporters, and organic-anion and cation transporters (OATs; OCTs) have been identified in the cornea, retina, and blood–retinal barriers (Table 17–3). Their cellular and subcellular roles in normal homeostatic function, chemical/drug/toxicant exposures, diseases, and pathophysiological responses in the retina and other tissues are important areas of investigation.

Light, UV Radiation, and Photoinduced Toxicity

The most important oxidizing agents are visible light and UV radiation, particularly UV-A (320 to 400 nm) and UV-B (290 to 320 nm). Light- and UV-induced photo-oxidation can generate reactive oxygen species that produce oxidative damage over time. Absorption of light energy in the lens can trigger various photoreactions, including generation of fluorophores and pigments that lead to the yellow–brown coloration of the lens.

Drugs, diagnostic dyes, dietary supplements, nanoparticles, and other chemicals can serve as mediators of photo-induced toxicity in the cornea, lens, or retina when the chemical structure allows absorption of light energy in the UV or visible spectrum and the subsequent generation of activated intermediates, free radicals, and reactive oxygen species. Chemical structures likely to participate in such phototoxic mechanisms have tricyclic, heterocyclic, or porphyrin ring structures because, with light, they produce stable triplet reactive molecules leading to free radicals and reactive oxygen species. Recently, high efficiency light–emitting diode (LED) lighting, rich in the more phototoxic blue wavelengths of the spectrum, has come under scrutiny for potential contributions to retinal phototoxicity.

The phototoxic properties of chemicals are being exploited for photodynamic therapies where photoactive chemicals are delivered to pathological tissues such as cancerous tumors or inappropriate angiogenic vessels in age-related macular degeneration. Wavelength-specific light is introduced to the tissue causing the photoactive chemical to activate, thereby initiating a free-radical cascade that kills the pathological tissues.

Central Visual System Pharmacokinetics

The penetration of chemicals into visual areas of the central nervous system (CNS) is governed by the blood–brain barrier (Fig. 17–3). In some cases, toxic compounds may be actively

transported into the brain by mimicking the natural substrates of active transport systems. One area of the brain lacking a blood–brain barrier is the ON near the lamina cribrosa, which could cause this part of the central visual system to be vulnerable to exposures that do not affect much of the remainder of the brain.

EVALUATING CORNEAL, RETINAL, AND CENTRAL VISUAL FUNCTION

Evaluation of Corneal Irritancy and Toxicity

Topical injury to the eye, especially the cornea, can result from contact with household and workplace products and chemicals. The Draize test, with some additions and revisions, standardizes the process for evaluating the ocular irritation and safety of products and chemicals. Traditionally, albino rabbits were the subjects evaluated in the Draize test. The standard procedure involves instillation of 0.1 mL of a liquid or 100 mg of a solid into the conjunctival sac of one eye and then gently holding the eye closed for 1 second. The untreated eye serves as a control. Both eyes are evaluated at 1, 24, 48, and 72 hours after treatment. If there is evidence of damage in the treated eye at 72 hours, the examination time may be extended. The cornea, iris, and conjunctiva are evaluated and scored according to a weighted scale. The cornea is scored for both the degree of opacity and the area of involvement, with each measure having a potential range from 0 (none) to 4 (most severe). The iris receives a single score (0 to 2) for irritation, including degree of swelling, congestion, and degree of reaction to light. The conjunctiva is scored for redness (0 to 3), chemosis (swelling, 0 to 4), and discharge (0 to 3). The individual scores are then multiplied by a weighting factor: five for the cornea, two for the iris, and five for the conjunctiva. The results are summed for a maximum total score of 110. In this scale, the cornea accounts for 73% of the total possible points.

The Draize test has been criticized due to its high interlaboratory variability, subjective nature of scoring, poor predictive value for human irritants, and most significantly for causing undue pain and distress to the tested animals. Alternative testing procedures to assess the potential for chemicals to cause ocular corrosivity and irritation are available and accepted by U.S. Regulatory Agencies, the Organization for Economic Co-operation and Development (OECD), and by the European Union.

Ophthalmological Evaluations and the Pupillary Light Reflex

The many ophthalmological procedures for evaluating the health of the eye range from routine clinical screening evaluations to sophisticated imaging techniques for targeted purposes. Examination of the adnexa includes evaluating the eyelids, lacrimal apparatus, and palpebral (covering the eyelid) and bulbar (covering the eye) conjunctiva. The anterior structures, or anterior segment, include the cornea, iris, lens, and anterior chamber. The posterior structures include the retinal pigmented epithelium (RPE), retinal vasculature, choroid, optic nerve, and sclera.

An ophthalmological examination may also involve a standard or automated (i.e., infrared pupillography) examination of the pupillary light reflex (PLR). The direct PLR involves shining

a bright light into the eye and observing the reflexive pupil constriction in the same eye. The consensual PLR is observed in the eye not stimulated. Both the direct and consensual PLRs are dependent on function of a reflex arc involving cells in the retina, which travel through the optic nerve, optic chiasm, and optic tract to project to neurons in the pretectal area. The absence of a PLR is indicative of damage somewhere in the reflex pathway, and differential impairment of the direct or consensual reflexes can indicate the location of the lesion. The presence of a PLR, however, is not synonymous with normal visual function. PLRs can be maintained even with significant damage to the retina or optic nerve. In addition, lesions in visual areas outside of the reflex pathway, such as in the visual cortex, may also leave the reflex function intact.

Electrophysiological Techniques

Many electrophysiological or neurophysiological procedures available for non-invasively and objectively testing visual function in a toxicological context involve stimulating the eyes with visual stimuli and electrically recording potentials generated by visually responsive neurons. Commonly used procedures are the electroretinograms (ERGs: flash, pattern or PERG, multifocal or mfERG) and visual-evoked potentials (VEPs). These procedures have numerous variations of stimulation and recording protocols depending on the specific aspects of the visual system being evaluated.

ERGs are typically elicited with a brief flash of light and recorded from an electrode placed in contact with the cornea. A typical ERG waveform includes a negative a-wave that reflects the activation of photoreceptors, and a following positive b-wave that reflects the activity of retinal ON-bipolar cells. A standard set of ERG procedures includes the recording of (1) a response reflective of only rod photoreceptor function in the dark-adapted eye, (2) the maximal response in the dark-adapted eye, (3) a response developed by cone photoreceptors, (4) oscillatory potentials, and (5) the response to rapidly flickered light.

Visual evoked potentials (VEPs) are recorded from electrodes overlying the visual (striate) cortex. Consequently, VEPs reflect the activity of the retinogeniculostriate pathway and the activity of cells in the visual cortex. Pattern-elicited VEPs (PEPs), which are widely used in human clinical evaluations, have diagnostic value.

Behavioral and Psychophysical Techniques

Behavioral and psychophysical testing procedures typically vary the parameters of the visual stimulus and then determine whether the subject can discriminate or perceive the stimulus. Contrast sensitivity refers to the ability to resolve small differences in luminance contrast, such as the difference between subtle shades of gray. Contrast sensitivity should be measured for a series of visual patterns that differ in pattern size, or where the luminance changes across the pattern in a sinusoidal manner. Contrast sensitivity functions are dependent primarily on the neural as opposed to the optical properties of the visual system.

Some visual parameters that have been investigated include (1) the absolute luminance threshold, (2) visual acuity, (3) color and spectral discriminations, (4) critical flicker fusion frequency, and (5) the peak spatial and temporal contrast sensitivity functions at different luminance levels. Thresholds for detecting luminance, contrast, flicker, and color are primarily dependent on retinal and central mechanisms of neural function, although optical impairments

(e.g., cataracts) interfere with these functions. The assessment of visual acuity and contrast sensitivity has been recommended for field studies of humans potentially exposed to neurotoxic substances.

Color Vision Testing

Color vision deficits are either inherited or acquired. Most acquired color vision deficits, such as those caused by drug and chemical exposure, begin with a reduced ability to perform blue–yellow discriminations. With increased or prolonged low-level exposure, the color confusion can progress to the red–green axis as well. Because of the rarity of inherited tritanopia, it is generally assumed that blue–yellow deficits, when observed, are acquired deficits. Bilateral lesions in the area V4 of the visual cortex can also lead to color blindness (prosopagnosia).

Commonly used procedures in human toxicological evaluations include the Ishihara color plates and chip arrangement tests, such as the Farnsworth-Munson 100 Hue (FM-100) test and the simplified 15-chip tests using either the saturated hues of the Farnsworth D-15 or the desaturated hues of the Lanthony Desaturated Panel D-15. The Ishihara plates involve a series of colored spots arranged in patterns that take advantage of perceived difference in shades resulting from congenital protan or deutan anomalies. Normal observers perceive different sets of embedded numbers than those with color vision deficits. The Farnsworth-Munson procedure involves arrangement of 85 chips in order of progressively changing color. The relative chromatic value of successive chips induces those with color perception deficits to abnormally arrange the chips. The pattern is indicative of the nature of the color perception anomaly. The FM-100 is considered more diagnostically reliable but takes considerably longer to administer than the similar but more efficient Farnsworth and Lanthony tests. The desaturated hues of the Lanthony D-15 are designed to better identify subtle acquired color vision deficits.

Visual Toxicity Screening Procedures

Comprehensive visual toxicity studies should include ophthalmological, histological, and pathological evaluation of ocular tissues as well as assessments of simple and complex visual function. Many behavioral and observational evaluations of neurotoxicity involve presentation of sensory stimuli to human or animal subjects followed by the observation or measurement of a behavioral or motor response. In many cases, the inferences drawn from such measures are stated in terms of the cognitive abilities of the test subject, such as whether learning or memory have been compromised as a function of exposure to the test compound. If the subject was unable to clearly and precisely perceive the test stimuli, which are often complex patterns or contain color, task performance may be affected independently of any effect on cognition. Controlling for visual deficits may alter the interpretation of performance or cognitive tasks.

TARGET SITES AND MECHANISMS OF ACTION: CORNEA

First, as the anterior covering of the eye, the cornea must provide a clear refractive surface. The

air-to-fluid/tissue interface at the cornea is the principal refractive surface of the eye. The curvature of the cornea must be correct for the visual image to be focused at the retina. Second, the cornea provides tensile strength to maintain the appropriate shape of the globe. Third, the cornea protects the eye from external factors, including potentially toxic chemicals.

The cornea is transparent to wavelengths of light ranging between 310 nm (UV) and 2500 nm (IR) in wavelengths. Exposure to UV light below this range can damage the cornea. It is most sensitive to wavelengths of approximately 270 nm. Excessive UV exposure leads to photokeratitis and corneal pathology, the classic example being welder's arc burns.

The corneal epithelial cells can be damaged by topical or systemic exposure to drugs and chemicals (Table 17–1). Direct chemical exposure to the eye requires emergency medical attention. Acid and alkali chemicals that contact the cornea can be extremely destructive. Products at pH extremes ≤ 2.5 or ≥11.5 are considered dangerous ocular irritants that can cause severe ocular damage and permanent loss of vision. Damage that extends to the corneal endothelium is associated with poor repair and recovery. The most important therapy is immediate and adequate irrigation with large amounts of water or saline, whichever is most readily available. The extent of damage to the eye and the ability to achieve a full recovery depend on the chemical's physicochemical properties, the concentration and duration of exposure, and the speed and magnitude of the initial irrigation.

Several drugs cause keratopathies. A common finding is vortex dystrophy caused by cationic amphiphilic chemicals that easily cross cell membranes and produce intracellular phospholipid accumulation. Other drugs and chemicals cause corneal deposits. There are precious few studies on environmental or occupational corneal injury. An analysis of approximately 600 agricultural and industrial chemicals reported that over half of the materials tested caused no (18% to 31%) or minimal (42% to 51%) irritation. Depending on the chemical category, 9% to 17% of compounds were graded as slightly irritant, whereas 1% to 6% were graded as strong or extreme irritants.

Acids

Strong acids with a pH≤2.5 can be highly injurious and include hydrofluoric acid, sulfurous acid, sulfuric acid, chromic acid, hydrochloric acid, nitric acid, and acetic acid. Injuries may be mild if contact is with weak acids or with dilute solutions of strong acids. Compounds with a pH between 2.5 and 7.0 produce pain or stinging; but with only brief contact, they will cause no lasting damage. Following mild burns, the corneal epithelium may become turbid as the corneal stroma swells. Mild burns are typically followed by rapid regeneration of the corneal epithelium and full recovery. In more severe burns, the epithelium of the cornea and conjunctiva become opaque and necrotic and may disintegrate over the course of a few days.

Acid chemical burns of the cornea occur through hydrogen ion-induced denaturing and coagulation of proteins. As epithelial cell proteins coagulate, glycosaminoglycans precipitate and stromal collagen fibers shrink, causing the cornea to become cloudy. The protein coagulation and shrinkage of the collagen is protective in that it forms a barrier reducing further penetration of the acid. The collagen shrinkage, however, contracts the eye and can lead to a dangerous acute increase in intraocular pressure. Both the hydrogen ion and anionic portions of the acid molecules contribute to protein coagulation and precipitation. The tissue proteins also tend to act as buffers.

Bases or Alkalies

Compounds with a basic pH include ammonia or ammonium hydroxide, sodium hydroxide (lye), potassium hydroxide (caustic potash), calcium hydroxide (lime), and magnesium hydroxide. One of the reasons that caustic chemicals are so dangerous is their ability to rapidly penetrate the ocular tissues. This is particularly true for ammonia, which has been measured in the aqueous humor just seconds after application to the cornea. As with acid burns, the concentration of the solution and the duration of contact with the eye are important determinants of the eventual clinical outcome. Rapid and extensive irrigation after exposure is the immediate therapy of choice.

Depending on the extent of injury, direct damage from exposure is observed in the cornea, adnexa, and possibly in the iris, ciliary body, and lens. Hydroxide ions cause rapid necrosis of the corneal epithelium. Strong alkali substances attack membrane lipids, causing necrosis and enhancing penetration of the substance to deeper tissue layers. The cations also react with the carboxyl groups of glycosaminoglycans and collagen, leading to hydration of the collagen matrix and corneal swelling. The cornea may appear clouded or become opaque immediately after exposure as a result of stromal edema and/or proteoglycans precipitation. The denaturing of collagen and loss of protective covering of the glycosaminoglycans is thought to make the collagen fibrils more susceptible to subsequent enzymatic degradation. Intraocular pressure may increase as a result of initial hydration of the collagen fibrils and later through the blockage of aqueous humor outflow. Conversely, if the alkali burn extends to the ciliary body, the intraocular pressure may decrease due to reduced formation of aqueous humor. The acute phase of damage is typically followed by a second phase of corneal repair. The repair process may involve corneal neovascularization along with regeneration of the corneal epithelium. Approximately 2 to 3 weeks after alkali burns, damaging ulceration of the corneal stroma often occurs. The formation of these lesions is related to the inflammatory infiltration of polymorphonuclear leukocytes and fibroblasts and the release of degratory proteolytic enzymes. Clinically, anti-inflammatory therapy limits ulcerative damage. Stromal ulceration usually stops when the corneal epithelium is restored.

Organic Solvents

When organic solvents splash into the eye, the result is typically a painful immediate reaction. Exposure of the eye to solvents should be treated rapidly with abundant water irrigation. Highly lipophilic solvents can damage the corneal epithelium and produce swelling of the corneal stroma. Generally, the corneal epithelium will be repaired over the course of a few days and there will be no residual damage. Exposure to solvent vapors may produce small transparent vacuoles in the corneal epithelium or irritation and tears.

Surfactants

Surfactants in soaps, shampoos, detergents, cosmetics, and similar consumer products may be irritating or injurious to the eye. The hydrophilic portion of these compounds may be anionic, cationic, or neutral. In general, the cationic substances tend to be stronger irritants and more injurious than the other types, and anionic compounds more so than neutral ones. Because these compounds are by design soluble in both aqueous and lipid media, they readily penetrate the

sandwiched aqueous and lipid barriers of the cornea.

TARGET SITES AND MECHANISMS OF ACTION: RETINA

The adult mammalian retina is a highly differentiated tissue containing eight distinct layers plus the RPE, 11 major types of neurons, and a Müller glial cell (Fig. 17–1). The eight layers of the neural retina, which originate from the cells of the inner layer of the embryonic optic cup, are the nerve fiber layer (NFL), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), rod and cone photoreceptor inner segment layer (RIS; CIS), and the rod and cone photoreceptor outer segment layer (ROS; COS). The retinal pigment epithelium (RPE), which originates from cells of the outer layer of the embryonic optic cup, is a single layer of cuboidal epithelial cells that lies on Bruch's membrane adjacent to the vascular choroid. Between the RPE and photoreceptor outer segments lies the subretinal space. The 11 major types of neurons are the rod and cone photoreceptors, (depolarizing) ON-rod and ON-cone bipolar cells, (hyperpolarizing) OFF-cone bipolar cells, horizontal cells, numerous subtypes of amacrine cells, an interplexiform cell, and different types of retinal ganglions cells (RGCs): ON-RGCs, OFF-RGCs, and ipRGCs. The Müller glial cell (MGC) is the only glial cell in the retina. The somas of the MGCs are in the INL. The end feet of the MGCs in the proximal or inner retina along with a basal lamina form the internal limiting membrane of the retina. In the distal retina, the MGC end feet join with the photoreceptors and zonula adherens to form the external limiting membrane, which is located between the ONL and RIS/CIS.

The mammalian retina is highly vulnerable to toxicant-induced structural and/or functional damage due to (1) the highly fenestrated choriocapillaris that supplies the distal or outer retina as well as a portion of the inner retina; (2) the very high rate of oxidative mitochondrial metabolism, especially in photoreceptors; (3) high daily turnover of rod and cone outer segments; (4) high susceptibility of the rod and cones to degeneration due to inherited retinal dystrophies as well as associated syndromes and metabolic disorders; (5) presence of specialized ribbon synapses and synaptic contact sites; (6) presence of numerous neurotransmitter and neuromodulatory systems, including extensive glutamatergic, GABAergic, and glycinergic systems; (7) presence of numerous and highly specialized gap junctions used in the information signaling process; (8) presence of melanin in the choroid, RPE, uvea, and iris; (9) a very high choroidal blood flow rate, as high as 10 times that of the gray matter of the brain; and (10) the additive or synergistic toxic action of certain chemicals with light. The retina is the only part of the central nervous system also exposed to light.

Each retinal layer can undergo specific as well as general toxic effects. These alterations and deficits include visual field deficits, scotopic vision deficits such as night blindness and increases in the threshold for dark adaptation, cone-mediated (photopic) deficits such as decreased color perception, decreased visual acuity, macular and general retina edema, retinal hemorrhages and vasoconstriction, and pigmentary changes. The list of drugs and other chemicals that cause retinal alterations is extensive (Table 17–1).

Retinotoxicity of Systemically Administered Therapeutic Drugs

Cancer Chemotherapeutics—Ocular toxicity is a common side effect of cancer chemotherapy resulting in blurred vision, diplopia, decreased color vision and visual acuity, optic/retrobulbar neuritis, transient cortical blindness, and demyelination of the optic nerves. If not detected early, the ocular complications are often irreversible even after chemotherapy is discontinued.

Chloroquine and Hydroxychloroquine—Chloroquine and hydroxychloroquine can cause irreversible loss of retinal function. Chloroquine, its major metabolite desethylchloroquine, and hydroxychloroquine have high affinity for melanin, and these drugs accumulate in the choroid and retinal pigment epithelium (RPE), ciliary body, and iris during and following drug administration. Prolonged exposure of the retina to these drugs, especially chloroquine, may produce the pathognomonic “bull’s-eye retina” visualized as a dark, central pigmented area involving the macula, surrounded by a pale ring of depigmentation, which is surrounded by another ring of pigmentation. In humans and monkeys, long-term chloroquine administration results in sequential degeneration of the retinal ganglion cells (RGCs), photoreceptors, and RPE and the eventual migration of RPE pigment into the outer nuclear and plexiform layers, ONL and OPL, respectively. Hydroxychloroquine has fewer side effects and less ocular toxicity.

Digoxin and Digitoxin—The cardiac glycosides digoxin and digitoxin induce decreased vision, flickering areas of vision loss (scotomas), and altered color vision. The photoreceptors are a primary target site of the cardiac glycosides, and cone photoreceptors are more susceptible to the effects of cardiac glycosides than rod photoreceptors. Digoxin-binding studies show that the retina has the highest number of Na^+,K^+-ATPase sites of any ocular tissue.

Indomethacin—Chronic administration of 50 to 200 mg/day of indomethacin for 1 to 2 years has been reported to produce corneal opacities, discrete pigment scattering of the retinal pigment epithelium (RPE) perifoveally, paramacular depigmentation, decreases in visual acuity, altered visual fields, increases in the threshold for dark adaptation, blue–yellow color deficits, and decreases in ERG and electro-oculogram (EOG) amplitudes. Decreases in ERG a- and b-wave amplitudes, with larger changes observed under scotopic dark-adapted than light-adapted conditions, have been reported. Upon cessation of drug treatment, the ERG waveforms and color vision changes return to near normal, although the pigmentary changes are irreversible.

Erectile Dysfunction (ED) Drugs—Sildenafil citrate (Viagra) inhibits cGMP phosphodiesterase (PDE) 5 in the penis and PDE6 in rod and cone photoreceptors. Tadalafil and vardenafil are much more selective for PDE5 than PDE6 and thus have fewer retinal side effects. Following sildenafil usage, transient visual symptoms such as a blue tinge to vision, increased brightness of lights and blurry vision as well as alterations in scotopic and photopic ERGs have been reported. Sildenafil also has been associated with the occurrence of nonarteritic anterior ischemic optic neuropathy (NAION) in at-risk patients (i.e., those with small cup-to-disc ratios and/or arteriosclerotic risk profiles) within minutes to hours after the ingestion of the drug.

Tamoxifen—Chronic high-dose therapy (180 to 240 mg/day for approximately 2 years) caused widespread axonal degeneration in the macular and perimacular area. Clinical symptoms include a permanent decrease in visual acuity and abnormal visual fields, as the axonal degeneration is irreversible. Chronic low-dose tamoxifen (20 mg/day) can result in an increased incidence

(≤10%) of corneal lesion or keratopathy with minimal alteration in visual function. Following cessation of low-dose tamoxifen therapy, most of the keratopathy and retinal alterations, except the corneal opacities and retinopathy, were reversible.

Vigabatrin—Vigabatrin is an irreversible inhibitor of GABA-transaminase that has risk of retinopathy characterized by irreversible bilateral, concentric peripheral visual constriction, and decreased retinal nerve fiber layer (NFL) thickness. The drug is recommended for epileptic children and adults only when there are no alternative choices.

Retinotoxicity of Known Neurotoxicants

Inorganic Lead—Inorganic lead poisoning (mean blood lead [BPb] ≥80 μg/dL) in humans produces amblyopia, blindness, optic neuritis or atrophy, peripheral and central scotomas, paralysis of eye muscles, and decreased visual function. Moderate- to high-level lead exposure produces scotopic and temporal visual system deficits in occupationally exposed factory workers and developmentally lead-exposed monkeys and rodents. Gestational lead exposure increases retinal progenitor cell proliferation, and rod photoreceptor and bipolar cell neurogenesis, consistent with the supernormal ERGs, as well as delayed rod and bipolar cell differentiation.

Studies in Occupationally Exposed Lead Workers—Clinical and electrophysiological studies in lead-exposed factory workers observed retrobulbar optic neuritis and optic nerve atrophy following chronic moderate-level or acute high-level lead exposure. Most of these cases presented with fundus lesions, peripheral or paracentral scotomas, whereas severe cases also had a central scotoma. Generally, the scotomas were not observed until approximately 5 years of continuous lead exposure.

In Vivo and In Vitro Animal Studies with Lead— Lead exposure to adult animals and postnatally developing animals produces retinal, optic nerve, and visual cortical damage and functional deficits. Retinas from rhesus monkeys and mice exposed prenatally and/or postnatally to lead had decreased tyrosine hydroxylase immunoreactivity (TH-IR) in the large dopaminergic amacrine cells and a complete loss of TH-IR in a small subset of amacrine cells. Elevated levels of rod photoreceptor Ca^{2+} and/or Pb^{2+} play a key role in the process of apoptotic rod cell death in patients with retinitis pigmentosa and cancer-associated retinopathy, mice with retinal degeneration (*rd*), rats injected with antirecoverin monoclonal antibodies, rats with hypoxic-ischemic injury, or light-induced damage.

In vivo and in vitro data suggest that Pb^{2+} produces a dose (concentration)-dependent inhibition of rod cGMP PDE, which elevates rod cGMP and the rod Ca^{2+} concentration. Detailed kinetic analysis revealed that Pb^{2+} competitively inhibits rod cGMP PDE. Once inside the rod, both Ca^{2+} and Pb^{2+} enter the mitochondria via the Ca^{2+} uniporter and induce mitochondrial depolarization, swelling, and cytochrome *c* release. Following cytochrome *c* release, caspase-9 and caspase-3 are sequentially and selectively activated. These results suggest that Ca^{2+} and Pb^{2+} bind to the internal divalent metal binding site of the mitochondrial permeability transition pore and subsequently open it, which initiates the cytochrome *c*-caspase cascade of apoptosis in rods.

Methanol—Formic acid is the toxic metabolite that mediates the metabolic acidosis as well as the retinal and optic nerve toxicity observed in humans, monkeys, and rats with a decreased

capacity for folate metabolism. Human and nonhuman primates are highly sensitive to methanol-induced neurotoxicity due to their limited capacity to oxidize formic acid. The toxicity occurs in several stages. First there is a mild CNS depression, followed by an asymptomatic 12- to 24-hour latent period, then by a syndrome consisting of formic acidemia, uncompensated metabolic acidosis, ocular and visual toxicity, coma, and possibly death.

Acute methanol poisoning in humans, monkeys, and experimental rats resulted in profound and permanent structural alterations in the retina and optic nerve and visual impairments ranging from blurred vision to decreased visual acuity and light sensitivity to blindness. Ophthalmological studies of exposed humans and monkeys reveal varying degrees of edema of the papillomacular bundle and optic nerve head. Intraretinal metabolism of methanol is necessary for the formate-mediated alterations in the ERG. Formate also appears to directly and adversely affect the photoreceptors. The mechanism of methanol poisoning may involve disruption in oxidative energy metabolism, as formate is a mitochondrial poison that inhibits oxidative phosphorylation of photoreceptors, MGCs, and optic nerve.

Organic Solvents—*n-Hexane, Perchloroethylene, Styrene, Toluene, Trichloro-ethylene, Xylene, and Mixtures*—Loss of color vision (acquired dyschromatopsia) and contrast sensitivity have been reported in workers exposed to organic solvents such as alcohols, *n*-hexane, toluene, trichloroethylene, styrene, xylene, and solvent mixtures. The color vision losses were characterized initially as an increase in blue–yellow confusion errors, although more severe red–green deficits were reported with extended duration or higher concentrations of exposure. Generally, acquired blue–yellow losses may result from lens opacification or outer retinal alterations, whereas red–green losses are traditionally associated with retrobulbar or central visual pathway alterations. In addition, many of the occupationally exposed workers also exhibited lower contrast sensitivity at intermediate spatial frequencies, which likely reflects alterations in neural function. More detailed, well-designed, and well-executed studies are needed to determine the (1) specific solvents that cause alterations in color vision, (2) vulnerability of spatial and temporal contrast sensitivity, (3) dose (concentration)–response relations between exposures and effects, (4) possible sex differences, (5) potential reversibility of deficits if exposure is terminated, and (6) pathophysiological and toxicological basis for these changes.

Perchloroethylene (Tetrachloroethylene; PERC)—Long-term low-level occupational and environmental PERC exposure produces adverse retinal and central visual system effects. Dry cleaners exposed to low-level PERC for 8 to 10 years had blue–yellow deficits. The observed color vision and contrast sensitivity alterations were due to retinal changes (i.e., photoreceptors and parvocellular or P-type retinal ganglion cells).

Styrene—Workers exposed to mean atmospheric concentrations of styrene ranging from 20 to 70 ppm exhibit concentration-dependent alterations in color vision. The threshold for color visual impairments is ≤4 ppm styrene, which is well below the threshold limit value-time-weighted average (TLV–TWA) value for any country: range 20 to 50 ppm.

Toluene—Neurotoxic consequences of occupational toluene exposure are impaired color vision and reduced visual contrast sensitivity. Acute toluene exposure impairs oculomotor function and reduces pattern VEP amplitudes in proportion to estimated concentration of toluene in the brain. Moreover, toluene has a relatively low irritancy and a high euphoric potential making it a

favored selection among inhalant drug abusers. Abuse of toluene is associated with poor visual acuity, altered or unrecordable pattern VEPs, optic neuropathy, abnormal MRI signals in the visual cortex, decreased perfusion of the thalamus and cerebral cortex, and visual hallucinations.

Organophosphates—The evidence for organophosphate-induced retinal toxicity is strongest for fenthion (dimethyl 3-methyl-4-methylthiophenyl phosphorothionate). Currently, the mechanisms of ocular toxicity and sites of action are unknown.

TARGET SITES AND MECHANISMS OF ACTION: MITOCHONDRIAL OPTIC NEUROPATHIES (MONS)

The optic nerve consists primarily of retinal ganglion cell (RGC) axons carrying visual information from the retina to several distinct anatomic destinations in the CNS. Optic nerve axons have a high rate of oxygen consumption and the unmyelinated prelaminar portion of the optic nerve has a higher number of mitochondria and sodium-gated ion channels than the myelinated postlaminar region. Mitochondrial Optic Neuropathies (MON) include the genetic MONs (e.g., Leber's hereditary optic neuropathy and autosomal dominant optic atrophy), nutritional MONs (e.g., decreased folic acid and B12), and drug-induced MONs. The clinical features of MONs include relatively symmetric visual impairment, loss of high spatial frequency contrast sensitivity (decreased visual acuity), early and profound loss of color vision (often red), central visual field deficits with spared peripheral vision, (delayed) temporal pallor of the optic discs, and normal pupillary light reflex.

Acrylamide

Exposure to acrylamide produces a distal axonopathy in large-diameter axons of the peripheral nerves and spinal cord. Visual effects of acrylamide exposure occur at dose levels sufficient to cause substantial peripheral neuropathy. Although the large-diameter and long axons are most vulnerable to acrylamide in peripheral nerves and spinal cord, the middle diameter axons of the P_{β}-type retinal ganglion cells (RGCs) in the optic tract that project to the parvocellular layers of the LGN of New- and Old-World primates degenerate after prolonged treatment with acrylamide.

Carbon Disulfide

Workers chronically exposed to CS_2 experience loss of visual function accompanied by observable lesions in the retinal vasculature. The visual function and structural deficits reported in viscose rayon workers include central scotoma, depressed visual sensitivity in the peripheral visual field, optic atrophy, pupillary disturbances, blurred vision, and disorders of color perception. Although the mechanism of action of CS_2 is unknown, altered mitochondrial and energy metabolism underlie the pathophysiology.

Cuban Epidemic of Optic Neuropathy

During 1992 and 1993, over 50,000 people in Cuba suffered from optic neuropathy, sensory and autonomic peripheral neuropathy, high-frequency neural hearing loss, and myelopathy. The affected individuals were characterized as having bilateral low visual acuity, impaired color perception, impaired visual contrast sensitivity, central scotoma, optic disc pallor, and, in particular, loss of nerve fibers from the papillomacular bundle. Exposure to toxicants could not be documented in most of the people identified in the epidemic, suggesting nutritional deficit as the principal cause. Many of the clinical and behavioral effects observed in these affected individuals were consistent with MON.

Ethambutol

The dextro isomer of ethambutol produces dose-related alterations in the visual system, such as blue–yellow and red–green dyschromatopsias, decreased contrast sensitivity, reduced visual acuity, and visual field loss. The earliest visual symptoms appear to be a decrease in contrast sensitivity and color vision, although impaired red–green color vision is the most frequently observed and reported complaint. The symptoms are primarily associated with one of two forms of optic neuropathy. The most common form involves the central optic nerve fibers or papillomacular bundle (PMB) and typically results in a central or paracentral scotoma in the visual field and is associated with impaired red–green color vision and decreased visual acuity, whereas the second form involves the peripheral optic nerve fibers and typically results in a peripheral scotoma and visual field loss.

TARGET SITES AND MECHANISMS OF ACTION: THE CENTRAL VISUAL SYSTEM

Many areas of the cerebral cortex are involved in the perception of visual information. The primary visual cortex receives the primary projections of visual information from the lateral geniculate nucleus (LGN) and from the superior colliculus. Neurons from the LGN project to the visual cortex, maintaining a topographic representation of the receptive field origin in the retina.

Lead

Lead exposure during adulthood or perinatal development produces structural, biochemical, and functional deficits in the visual cortex of humans, nonhuman primates, and rats. Quantitative morphometric studies in monkeys exposed to either high levels of lead from birth or infancy to 6 years of age revealed a decrease in visual cortex areas V1 and V2, cell volume density, and the number of initial arborizations among pyramidal neurons. These alterations could contribute to the decreases in contrast sensitivity, flash and pattern-reversal evoked potentials, and visual function in lead-exposed laboratory animals or humans.

Methyl Mercury

Visual deficits are a prominent feature of methyl mercury intoxication in adult humans. Methyl mercury poisoned individuals experienced a striking and progressive constriction of the visual field (peripheral scotoma). The narrowing of the visual field gives the impression of looking through a long tunnel, hence the term “tunnel vision”. The damage was most severe in the regions of primary visual cortex that subserved the peripheral visual field, with relative sparing of the cortical areas representing the central vision. This regional distribution of damage corresponded with the progressive loss of peripheral vision while central vision was relatively preserved. Methyl mercury-poisoned individuals also experienced poor night vision (i.e., scotopic vision deficits), also attributable to peripheral visual field losses.

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QUESTIONS

1. In which of the following locations would one NOT find melanin?
   - **a.** iris.
   - **b.** ciliary body.
   - **c.** retinal pigment epithelium (RPE).
   - **d.** uveal tract.
   - **e.** sclera.

2. Systemic exposure to drugs and chemicals is most likely to target which of the following retinal sites?
   - **a.** RPE and ganglion cell layer.
   - **b.** optic nerve and inner plexiform layer.
   - **c.** RPE and photoreceptors.
   - **d.** photoreceptors and ganglion cell layer.
   - **e.** inner plexiform layer and RPE.

3. Which of the following structures is NOT part of the ocular fundus?
   - **a.** retina.
   - **b.** lens.
   - **c.** choriod.
   - **d.** sclera.
   - **e.** optic nerve.

4. Drugs and chemicals in systemic blood have better access to which of the following sites

because of the presence of loose endothelial junctions at that location?

a. retinal choroid.
b. inner retina.
c. optic nerve.
d. iris.
e. ciliary body.

5. All of the following statements regarding ocular irritancy and toxicity are true EXCEPT:
   a. The Draize test involves instillation of a potentially toxic liquid or solid into the eye.
   b. The effect of the irritant in the Draize test is scored on a weighted scale for the cornea, iris, and conjunctiva.
   c. The Draize test usually uses one eye for testing and the other as a control.
   d. The Draize test has strong predictive value in humans.
   e. The cornea is evaluated for opacity and area of involvement in the Draize test.

6. Which of the following statements regarding color vision deficits is FALSE?
   a. Inheritance of a blue–yellow color deficit is common.
   b. Bilateral deficits in the visual cortex can lead to color blindness.
   c. Disorders of the outer retina produce blue–yellow deficits.
   d. Drug and chemical exposure most commonly results in blue–yellow color deficits.
   e. Disorders of the optic nerve produce red–green deficits.

7. A substance with which of the following pH values would be most damaging to the cornea?
   a. 1.0.
   b. 3.0.
   c. 7.0.
   d. 10.0.
   e. 12.0.

8. Which of the following statements concerning the lens is FALSE?
   a. UV radiation exposure is a common environmental risk factor for developing cataracts.
   b. Cataracts are opacities of the lens that can occur at any age.
   c. The lens continues to grow throughout one's life.
   d. Naphthalene and organic solvents both can cause cataracts.
   e. Topical treatment with corticosteroids can cause cataracts.

9. Which of the following is NOT a reason why the retina is highly vulnerable to toxicant-induced damage?
   a. presence of numerous neurotransmitter systems.
   b. presence of melanin in the RPE.
   c. high choroidal blood flow rate.
   d. high rate of oxidative mitochondrial metabolism.
   e. lack of gap junctions.

10. A deficiency in which of the following vitamins can result in degeneration of optic nerve fibers?

a. vitamin A.
b. vitamin B_3.
c. vitamin C.
d. vitamin B_{12}.
e. vitamin E.

CHAPTER 18

Toxic Responses of the Heart and Vascular System

Matthew J. Campen

KEY POINTS

- Typical chemical-induced disturbances in cardiac function consist of effects on heart rate (chronotropic), contractility (inotropic), conductivity (dromotropic), and/or excitability (bathmotropic).
- Any xenobiotic that disrupts ion movement or homeostasis may induce a cardiotoxic reaction composed principally of disturbances in heart rhythm.
- All toxicants absorbed into the circulatory system contact vascular cells before reaching other sites in the body.
- Common mechanisms of vascular toxicity include (1) alterations in membrane structure and function, (2) redox stress, (3) vessel-specific bioactivation of protoxicants, and (4) preferential accumulation of the active toxin in vascular cells.

INTRODUCTION

Cardiovascular toxicology is concerned with the adverse effects of extrinsic and intrinsic stresses on the heart and vascular system. Extrinsic stress involves exposure to therapeutic drugs, natural products, and environmental toxicants. Intrinsic stress refers to exposure to toxic metabolites derived from nontoxic compounds such as those found in food additives and supplements, plus secondary neurohormonal disturbances such as overproduction of inflammatory cytokines derived from pressure overload of the heart and counter-regulatory responses to hypertension or uremic toxins that arise from renal failure. The manifestations of toxicological response of the heart include cardiac arrhythmia, hypertrophy, and overt heart failure. The responses of the vascular system include changes in blood pressure and lesions in blood vessels in the form of atherosclerosis, hemorrhage, and edema.

OVERVIEW OF THE HEART

Overview of Cardiac Structural and Physiological Features

Cardiac muscle is one of the excitable tissues of the body. It shares many bioelectrical properties with other excitable tissues, and also has unique features associated with cardiac structural and physiological specificities. Figure 18–1 illustrates the basic anatomy of the heart.

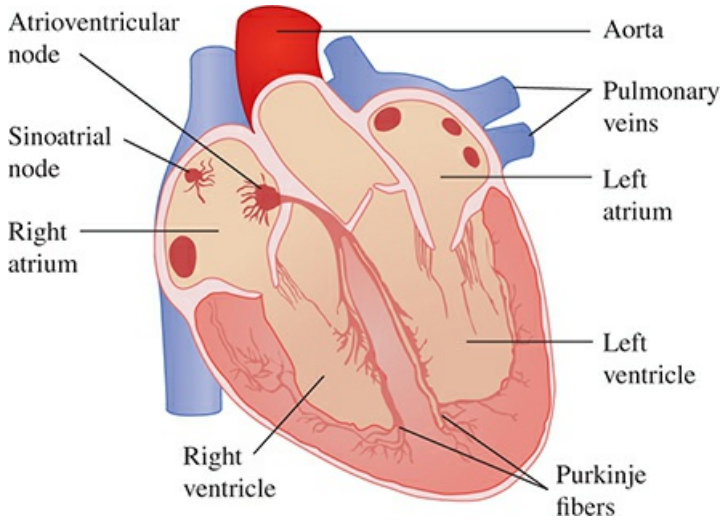

FIGURE 18–1 *Diagram illustrating the basic anatomy of the heart.*

Review of Cardiac Structure—Is the Cardiac myocytes are composed of several major structural features and organelles, as illustrated in Fig. 18–2. The contractile element, the myofibril, consists of smaller filaments (the thick and thin myofilaments). The thick filaments are special assemblies of the protein myosin, while thin filaments are made up primarily of the protein actin. Cardiac myosin is a hexamer composed of one pair of myosin heavy chains (MHCs) and two pairs of myosin light chains (MLCs). Two isoforms of MHC, α and β, are expressed in cardiac muscle. Similarly, two isoforms of actin are expressed in the heart (cardiac and skeletal α-actin).

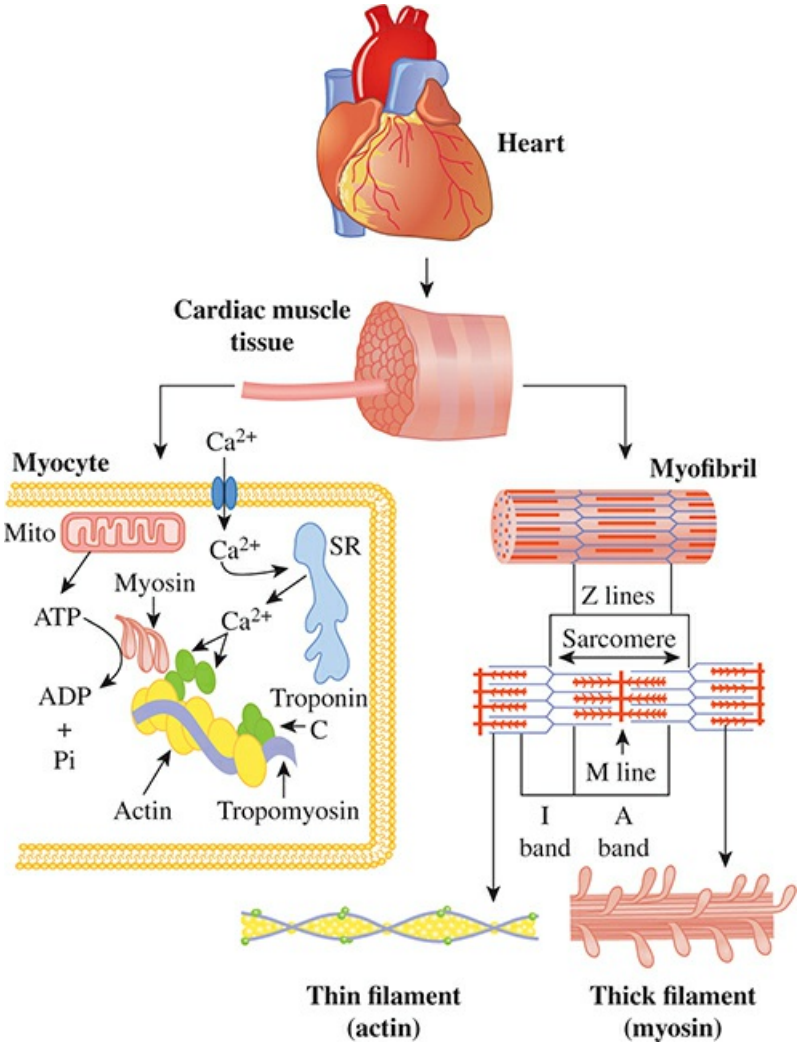

FIGURE 18–2 *Structural organization of cardiac muscle tissue.*

Under electron microscopy, these myocardial contractile proteins display alternating dark bands (A bands, predominantly composed of myosin) and light bands (I bands, predominantly composed of actin). Visible in the middle of the I band is a dense vertical Z line. The area between two Z lines is called a sarcomere, the fundamental unit of muscle contraction. Cardiac myocytes are joined end-to-end by dense structures known as intercalated disks having tight gap junctions that facilitate action potential propagation and intercellular communication.

Electrophysiology—In cardiac myocytes, calcium (Ca^{2+}), sodium (Na^{+}), and potassium (K^{+}) ions make a significant contribution to the bioelectricity of the heart. Each of the ions has specific channels and transporters (pumps) on the membrane of cardiac myocytes. Through the movement of these ions across the cell membrane, an action potential is generated and propagated from one cell to another, so that electric conductance is produced in the heart.

***Action Potential*—**The process of an action potential from depolarization to the completion of repolarization is divided into five phases in cardiac Purkinje fibers, as shown in Fig. 18–3. Phase 0 represents a rapid depolarization due to the inward current of Na^{+}, which is caused by the rapid

opening of voltage-gated sodium channels. Phase 1 is associated with an immediate rapid repolarization, during which the Na^+ inward current is inactivated and a transient K^+ outward current is activated, followed by an action potential plateau or phase 2, which is dominated by slowly decreasing inward Ca^{2+} current and a slow activation of an outward K^+ current. Phase 3 reflects a fast K^+ outward current and inactivation of the plateau Ca^{2+} inward current, and phase 4 is the diastolic interval for the resetting of the resting potential.

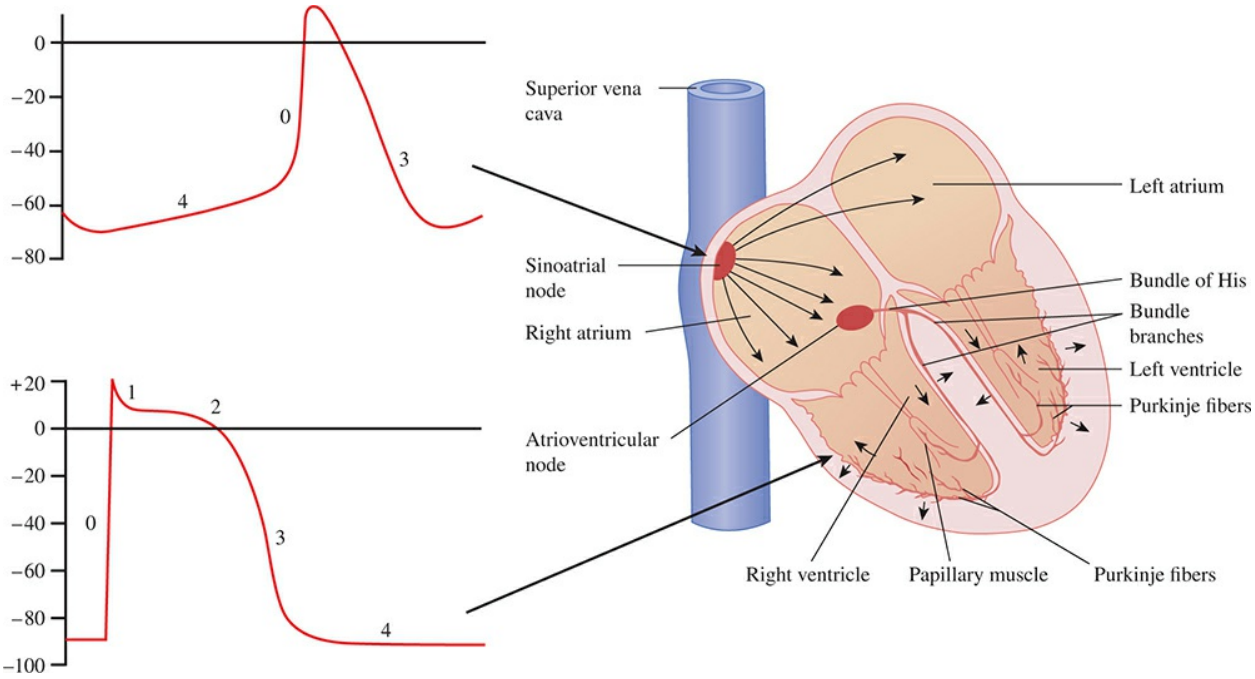

FIGURE 18–3 *Characteristic cardiac action potential recorded from sinoatrial node pacemaking cells (top) and Purkinje fibers (bottom) as indicated.* (Reprinted from Berne RM, Levy MN. *Physiology*. St. Louis, MO: Mosby; 1983.)

***Automaticity*—**The sinoatrial (SA) node pacemaking cells and Purkinje fibers in the ventricles repetitively generate and distribute each impulse through the heart in a highly coordinated manner to control the normal heartbeat. The sinus node pacemaker cells have only three distinct phases of action potential (Fig. 18–3): phase 0, rapid depolarization; phase 3, plateau and repolarization; and phase 4, slow depolarization, often referred to as *pacemaker potential*. The pacemaker potential brings the membrane potential to a level near the threshold for activation of the voltage-gated calcium channels and inward Ca^{2+} current, which triggers the phase 0 rapid depolarization. In pacemaker cells, phase 0 is mediated almost entirely by increased conductance of Ca^{2+} ions. Additionally, cells of the atrioventricular (AV) node exhibit a slower automaticity, such that under normal conditions the AV pace is entrained by the SA node rhythm. The AV node slows conduction through the heart to allow proper filling of ventricles prior to contraction.

Contractility—Myocyte contraction occurs when an action potential causes the release of Ca^{2+} from the SR as well as the entry of extracellular Ca^{2+} into the cell and is called *excitation–contraction coupling* (Fig. 18–4). The increase in Ca^{2+} concentrations in the cell allows Ca^{2+} to bind to troponin and tropomyosin, leading to interaction between the actin and myosin filaments through the crossbridges (myosin heads). Adenosine triphosphate (ATP) is hydrolyzed by

ATPase present in the crossbridges to release energy for the movement of the crossbridges in a ratchet-like fashion. This action increases the overlap of actin and myosin filaments, resulting in shortening of the sarcomeres and contraction of the myocardium. Synchronized contraction of atrial and ventricular myocytes leads to pumping of blood during the systolic phase of the cardiac cycle. After each contraction, the heart must then relax to permit filling from the atria, during diastole.

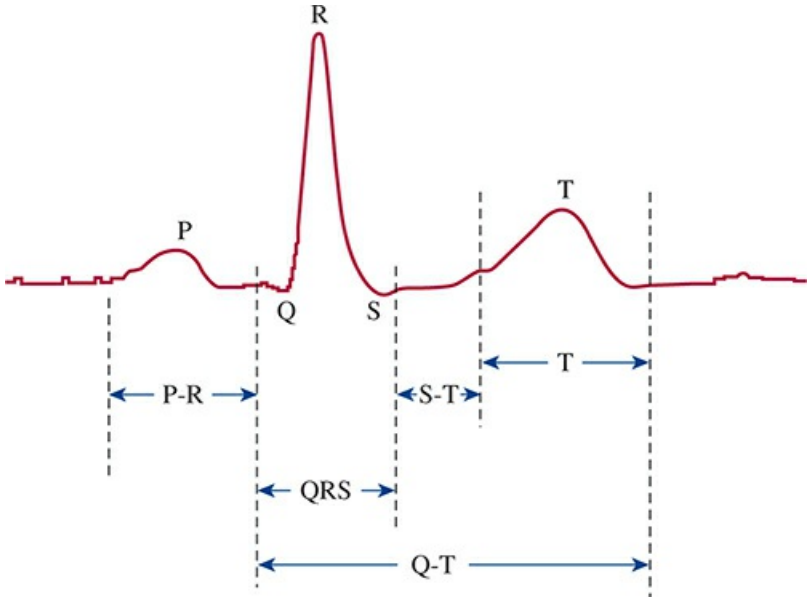

FIGURE 18–4 *A typical electrocardiogram (ECG) with the illustration of important deflections and intervals.*

Electrotonic Cell-to-Cell Coupling—Electrotonic cell-to-cell coupling via gap junctions permits major ionic fluxes between adjacent cardiomyocytes allowing electrical synchronization of myocardial cells for contraction. Each single gap junction is composed of 12 connexin 43 (Cx43) units, assembled in two hexametric connexons (hemichannels) that are contributed, one each, by two participating cells. The connexins are also important for cell-to-cell signaling and volume regulation. The distribution of gap junctions at the ends of myocytes facilitates a linear directionality of electrical conduction while discouraging transmission of abnormal “ectopic” electrical conductions traveling in the transverse direction to minimize risk of arrhythmic events.

Electrocardiograph—The pattern of the scalar electrocardiograph consists of P, QRS, and T waves, as shown in Fig. 18–5. The PR interval is a measure of the time from onset of atrial activation to the onset of ventricular activation. The QRS complex represents the conduction pathways through the ventricles. The ST segment is the interval during which the entire ventricular myocardium is depolarized. The QT interval is sometimes referred to as the period of “electrical systole” of the ventricles and mirrors the action potential duration of the mass of ventricular myocytes. QT interval prolongation is recognized as a major life-threatening factor of drug cardiac toxicity, which is brought about by a reduction of outward currents and/or enhanced inward currents during phase 2 and 3 of the action potential.

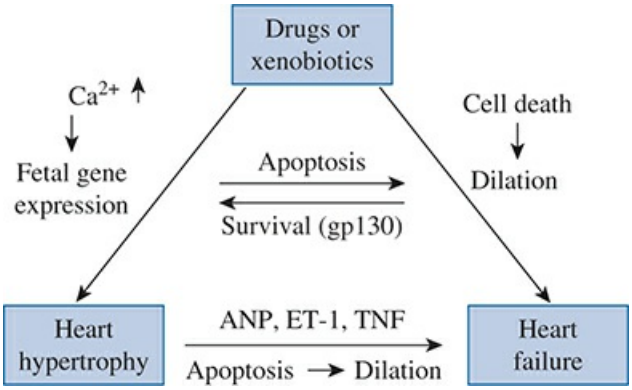

FIGURE 18–5 *Triangle analytical model of cardiac responses to drugs and xenobiotics.* Drugs or xenobiotics can directly cause both heart failure and heart hypertrophy. Under severe acute toxic insults, myocardial cell death becomes the predominant response leading to cardiac dilation and heart failure. In most cases, myocardial survival mechanisms can be activated so that myocardial apoptosis is inhibited. Surviving cardiomyocytes often undergo hypertrophy. If the toxic insult continues, counter-regulatory mechanisms against hypertrophy such as activation of cytokine-medicated pathways eventually lead to myocardial cell death through apoptosis or necrosis, dilated cardiomyopathy, and heart failure.

Neurohormonal Regulation—Although the heartbeat is governed by the automaticity of the sinus node P cells, neurohormonal regulation of cardiac electrophysiology and contraction controls cardiac function under normal and abnormal conditions. Toxicants often exert their effects on the cardiac system through interference with the neurohormonal regulation, thus this regulatory system is of significant relevance to cardiac toxicology.

Overview of Cardiac Energy Metabolism and Biochemistry

ATP and the Heart—The primary ATP-utilizing reactions are catalyzed by actomyosin ATPase in the myofibril, the Ca^{2+}-ATPase in the SR, and the Na^{+},K^{+}-ATPase in the sarcolemma. ATP is also needed for molecular synthesis and degradation in the heart as in other organ systems. ATP synthesis by oxidative phosphorylation in the mitochondria is usually sufficient to support the normal needs of the heart, even when the work output of the heart increases three- to fivefold. Under conditions of ischemia or impaired blood flow when oxygen is limited (e.g., myocardial infarction), ATP is rapidly consumed in the heart and reduced to adenosine, which can act as a vasodilator and have other protective effects.

Phosphocreatine and the Heart—A unique feature in cardiac energy metabolism is the use of energy reserve systems, such as phosphocreatine (PCr), to maintain a high phosphorylation potential to drive ATPase reactions under highly demanding conditions. The enzyme creatine kinase (CK) transfers the phosphoryl group between ATP and PCr at a rate about 10 times faster than the rate of ATP synthesis by oxidative phosphorylation. When ATP demand exceeds ATP supply, the use of PCr is a major pathway to maintain a constant supply of ATP.

Metabolic Pathways—Continuous synthesis of ATP via mitochondrial oxidative phosphorylation uses the oxidation of fatty acids (FA), providing about 65% of the total energy

demand, and oxidation of glucose provides about 30% of the total energy demand. In hypertrophic and failing hearts, FA oxidation is decreased and glucose utilization is increased. In response to toxic exposure, mitochondrial damage leads to impaired oxidative phosphorylation and reliance on glucose utilization.

Calcium, Calmodulin, and Calcineurin—Direct disturbance of calcium concentrations or channel function can have profound proarrhythmic consequences. Continually elevated levels of cytosolic calcium can also promote cardiomyocyte hypertrophy. In response to myocardial stress by environmental toxic exposures, calcium concentrations are increased in the myocardial cells. Notably, calcium levels become augmented in systole with increased myocyte force generation in response to increased vascular resistance. In later stages of heart failure, diastolic dysfunction is characterized by elevated resting levels of calcium.

A sustained increase in intracellular Ca^{2+} concentrations activates calcineurin, a ubiquitously expressed serine/threonine phosphatase that exists as a 59-kDa calmodulin (CaM)-binding catalytic A subunit and a 19-kDa Ca^{2+}-binding regulatory B subunit. Activation of calcineurin is mediated by Ca^{2+}binding to the regulatory subunit and CaM to the catalytic subunit. Calcineurin in turn dephosphorylates the transcription factor, nuclear factor of activated T cells (NFAT), which then translocates to the nucleus and transcribes a battery of pro-growth elements.

Certain G protein–coupled receptors (e.g., for endothelin and angiotensin) and other growth factor receptors can activate phospholipase C, leading to the formation of diacylglycerol and inositol triphosphate (IP_3). IP3 activates calmodulin and calmodulin kinase IIδ. Ras, mitogen-activated protein kinase (MAPK), and protein kinase C (PKC) signaling pathways are associated with increases in intracellular Ca^{2+} concentrations.

AMP-Activated Protein Kinase—Activation of AMPK occurs with changes in high-energy phosphate metabolism in hypertrophic and failing hearts. The increase in AMP/ATP ratio occurs when the PCr/ATP ratio decreases due to a decrease in PCr, with or without a concomitant decrease in ATP. The decrease in PCr/ATP ratio is an index of decreased energy reserve and correlates with the severity of heart failure and is of prognostic value. Activation of AMPK leads to translocation of the insulin-dependent glucose transporter (GLUT4) from intracellular stores to the sarcolemma. AMPK-dependent phosphorylation of 6-phosphofructo-2-kinase stimulates glycolysis. Thus, AMPK activation shifts the cardiac metabolism to reliance on glucose metabolism.

Mitogen-Activated Protein Kinases—MAPKs play a major role in cardiac response to toxic insults. The MAPKs consist of a series of successively acting kinases and three major branches are involved in the classic MAPK signaling pathway. These branches are divided based on their terminal effector kinases: the extracellular signal–regulated protein kinases (ERK), the *c*-jun NH_2-terminal kinases (JNK), and p38 MAPKs. The p38 MAPKs are important signaling molecules that mediate environmental stress responses in various cell types. In cardiac cells, p38 MAPKs are associated with the onset of apoptosis in ischemia reperfusion.

Protein Kinase C—PKC is a ubiquitously expressed serine/threonine kinase, which is activated predominantly by G_q/G_{11}-coupled receptors. The cPKCs are activated by Ca^{2+} and diacylglycerol (DAG), as well as phorbol esters. Disturbances in PKC signaling pathways lead to cardiac hypertrophy and heart failure.

Transcription Factors

***Activator Protein-1*—**AP-1 is a transcription factor composed of *Jun* and *Fos* gene family members. AP-1-binds TRE (12-*O*-tetradecanoyl phorbol 13-acetate response element) to initiate transcription of the target genes. Elevated levels of *c*-Jun are seen in cardiomyocytes with ischemia–reperfusion. Overstretching of myocardium induces Fas-dependent signaling pathways that can lead to myocardial cell apoptosis. AP-1 is implicated in transcriptional regulation of several genes associated with a hypertrophic response.

***Myocyte-Enhancer Factor 2*—**MEF2 binds to A-/T-rich DNA sequences within the promoter regions of a number of cardiac genes, including muscle CK gene, β-MHC, MLC1/3, MLC2v, skeletal α-actin, SR Ca^{2+}-ATPase, cardiac troponin T, C, and I, desmin, and dystrophin). The activation of MEF2 involves phosphorylation of the transcription factor by p38 MAPK or ERK5-MAPK. The ERK5-MEF2 pathway has been observed in the generation of cardiac hypertrophy. An important function of MEF2 is the convergence in the binary downstream pathway of Ca^{2+} signaling. Increased intracellular Ca^{2+} binds to and activates Ca^{2+}-binding proteins, which regulate calcineurin and Ca^{2+}/CaM-dependent protein kinase (CaMKs) to induce cardiac hypertrophy. CaMKs stimulate MEF2 through phosphorylation of the transcriptional suppressor, histone deacetylases (HDACs). Nuclear CaMK is considered a HDAC kinase whose activity is enhanced by calcineurin.

***Nuclear Factor of Activated T Cells 3*—**NFAT3 is a transcription factor that binds to the consensus DNA sequence GGAAAAT as monomers or dimers through a Rel homology domain. NFAT3 plays a major role in cardiac hypertrophy. Hypertrophic stimuli, such as angiotensin II and phenylephrine, cause an increase in intracellular Ca^{2+} levels, which results in activation of calcineurin, which dephosphorylates NFAT3 enabling NFAT3 to translocate to the nucleus to interact with GATA4.

***GATA*—**GATA factors are a family of six nuclear transcriptional regulatory proteins. GATA-4/5/6 regulates cardiogenesis. GATA-4 influences regulation of hypertrophic response in myocardial cells. Interactions between AP-1 and GATA-4, and between NFAT3 and GATA-4 are essential in myocardial hypertrophic responses.

CARDIAC TOXIC RESPONSES

Basic Concepts and Definitions

The ultimate functional effect of cardiac toxic manifestations is decreased cardiac output and peripheral tissue hypoperfusion. The critical cellular event leading to toxicological cardiomyopathy is myocardial cell death, which leads to extracellular matrix (ECM) remodeling (fibrotic scarring) in the place of lost cells, along with functional changes of surviving cells in response to the reduced contractile function.

A triangle model of cardiac toxicity in Fig. 18–5 considers the complexity of the interaction between environmental stresses and the heart, and the balance between myocardial protection and deleterious dose and time effects. First, chemical exposures can lead to heart failure without cardiac hypertrophy. Second, a chemical can activate both protective and destructive responses

in the myocardium and the balance of such responses, varying among individuals, determines the outcome. Third, long-term toxicological responses often result in maladaptive hypertrophy, which can ultimately lead to heart failure and/or predispose the heart for malignant ventricular arrhythmia, leading to sudden cardiac death.

Acute Cardiac Toxicity—A single exposure to a high dose of cardiotoxic chemicals is often manifested by cardiac arrhythmia or a profound depression of pump function. However, myocardial apoptosis is also involved in acute cardiac toxicity. As shown in Fig. 18–5, toxicant exposure can directly lead to heart failure. Acute exposures to certain toxins are capable of leading to chronic cardiac impacts from many different mechanisms. An important consideration is the reversibility of effects.

Chronic Cardiac Toxicity—Chronic cardiac toxicity from long-term exposure to chemicals is often manifested by cardiac hypertrophy and the transition to heart failure. About 25% of human patients with cardiomyopathy have idiopathic cardiomyopathy, some of which is due to chemical exposure.

Cardiac Arrhythmia—Arrhythmias may arise from chronic remodeling of the heart, which alters the normal electrical pathways, or acutely from the effect of toxicants on normal cellular electrophysiology. Additionally, acute induction of arrhythmias may occur due to sudden loss of blood flow and reduced oxygen availability, such as may be induced by coronary artery vasospasm. A cardiac arrhythmiais often manifested as tachycardia (fast heart rate) or bradycardia (slow heart rate). Classes of tachycardia include sinus tachycardia, atrial tachycardia, ventricular tachycardia, and Torsades de Pointes, a life-threatening ventricular tachycardia. Subclasses such as atrial fibrillation, atrial flutter, and accelerated idioventricular rhythm can be identified from standard electrocardiographic traces and provide etiologic information about the underlying abnormality.

Cardiac Hypertrophy—Concentric hypertrophy is often observed during pressure overload and is characterized by new contractile-protein units assembled in parallel, resulting in a relative increase in the width of individual cardiac myocytes. Eccentric or dilated hypertrophy is characterized by the assembly of new contractile-protein units in series resulting in a relatively greater increase in the length than in the width of individual myocytes. Toxicological cardiomyopathy is often manifested as eccentric hypertrophy. Cardiac hypertrophy can be divided into three stages: developing hypertrophy, during which period the cardiac workload exceeds cardiac output; compensatory hypertrophy, in which the workload/mass ratio is normalized and normal cardiac output is maintained; decompensatory hypertrophy, in which ventricular dilation develops and cardiac output progressively declines, and overt heart failure occurs. Monocrotaline is a unique toxin that is known for its ability to damage the pulmonary vasculature and cause right heart failure.

Heart Failure—Heart failure is defined as the inability of the heart to maintain cardiac output that is sufficient to meet the metabolic and oxygen demands of peripheral tissues. Changes in systolic and diastolic function may reflect specific alterations in ventricular function and abnormalities in various subcellular processes. Early stages of heart failure are characterized by physiological compensatory changes to offset compromised systemic perfusion. In addition to cardiac hypertrophy, the body also increases fluid retention through activation of the renin-

angiotensin system and increases overall sympathetic tone. When such pathways can no longer increase cardiac output and perfusion owing to severe cardiac deterioration, decompensation occurs relatively rapidly.

Myocardial Degeneration and Regeneration

Myocardial degeneration is the ultimate response of the heart to toxic exposure, which can be measured by both morphological and functional degenerative phenotypes. However, myocardial degeneration should not necessarily be considered an irreversible toxic response. Evidence now indicates myocardial regeneration and recovery from cardiomyopathy are possible, although a very slow process.

Myocardial Degenerative Responses—Myocardial cell death, fibrosis, and contractile dysfunction are considered degenerative responses that can result in cardiac arrhythmia, hypertrophy, and heart failure. If acute cardiac toxicity does not affect the capacity of myocardial regeneration, the degenerative phenotype may be reversible. Both acute and chronic toxic stresses can lead to irreversible degeneration should cardiac repair and regeneration mechanisms be overwhelmed. Cell death is the most common phenotype of myocardial degeneration. Both apoptosis and necrosis occur. Myocardial cell death is accompanied by hypertrophy of the remaining cardiac myocytes in the hypertrophic heart.

During myocardial remodeling after cell death, there is an increase in the size of cardiac myocytes and cardiac fibrosis. Myocardial fibrosis results from excess accumulation and modification of extracellular matrix (ECM) components. ECM fibers are noncontractile and offer resistance to both contraction and relaxation of the heart. Collagens, predominately types I and III, are the major fibrous proteins in ECM and their synthesis may increase in response to toxic insults. Matrix metalloproteinase (MMPs degrade the ECM) activities are altered during the processes of fibrogenesis and fibrinolysis. The MMPs are inhibited by specific endogenous tissue inhibitor of metalloproteinases (TIMPs). During heart remodeling the concentration of TIMPs increases.

Toxic Effect on Myocardial Regeneration—Cardiac progenitor cells may be responsible for cardiac repair because these cells can make myocytes and vascular structures. Also called cardiac stem cells. They are self-renewing, clonogenic, and multipotent. The effect of chemicals on the cardiac progenitor cells is unknown.

Myocardial vascularization is required for myocardial regeneration. Many toxic insults affect angiogenesis in the myocardium. During regeneration, coronary arterioles and capillary structures bridge the scar tissue and supply nutrients for survival of regenerated cardiomyocytes. The orderly organization of myocytes within the myocardium and a well-defined relationship between the myocytes and the capillary network is altered under cardiac toxic conditions; either toxicological hypertrophy or diminished capillary formation can lead to hypoperfusion of myocytes in the myocardium.

Reversible and Irreversible Toxic Responses—A key factor determining the reversibility of cardiac toxicity is the degree of scarification or ECM accumulation. Toxicity leading to substantial fibrosis and/or apoptosis of cardiomyocytes would be difficult to reverse, while cardiac effects from toxicants that act as ligands acutely affecting ion channel function or

autonomic nervous system tone are more readily reversible.

Myocardial Cell Death and Signaling Pathways

Apoptosis and Necrosis—Mild injuries can be repaired and mild disturbances of homeostasis can be balanced physiologically. However, severe injuries will lead to cell death in the modes of apoptosis and necrosis. If the cell survives the insults, structural and functional adaptations will take place.

The loss of cardiac myocytes is a fundamental component of myocardial injury, which initiates and aggravates cardiomyopathy. Myocytes that undergo apoptosis are lost and may not be replaced under toxicological conditions. Although myocardial regeneration is possible, xenobiotics often cause degeneration through apoptosis and inhibition of regeneration. The contribution of necrosis to cardiomyopathy induced by environmental toxicants and pollutants is particularly important. Apoptosis and necrosis can occur simultaneously in tissues and cultured cells. The intensity and duration of insults may determine the outcome. Triggering events can be common for both types of cell death. A downstream controller may direct cells toward a programmed execution of apoptosis. If apoptosis is aborted before this control point and the initiating stimulus is severe, cell death may occur by necrosis. Specific oligonucleotide probes that recognize different aspects of DNA damage, can, in combination with confocal microscopy, identify apoptotic and necrotic cell death in the heart after different pathogenic challenges.

Single-Strand DNA Breaks—A monoclonal mouse anti-ssDNA antibody that specifically reacts with ssDNA, but does not recognize dsDNA, can be combined with a terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) assay to distinguish repairable ssDNA breaks from apoptotic DNA damage in the heart.

Apoptotic DNA Damage—Apoptotic DNA damage produces end products that are fragments of double-strand DNA cleavage with three overhangs, which can be specifically identified by *Tag* polymerase-generated probe. This probe, in combination with fluorescence labeling of different cellular components, allows quantitative detection of apoptotic cells, with the possibility of identifying the apoptotic cell's origin (myocytes stained with α-sarcomeric actin, endothelial cells stained with factor VIII, and fibroblasts stained with vimentin in the heart).

Necrotic DNA Damage—Necrotic DNA damage is characterized by double-strand DNA cleavage with blunt ends. Endonucleases produce double-strand DNA cleavage with three overhangs. Exonucleases remove terminal nucleotides, leading to a blunt end of the damaged DNA. A probe generated by *pfu* polymerase can specifically recognize these blunt-end DNAs.

Proportion of Apoptotic and Necrotic Cell Death in the Heart—The proportion of apoptotic and necrotic cell death in the heart can be estimated by the combination of the above procedures. First, a conventional TUNEL procedure can be used to identify the total TUNEL-positive cells. Second, the procedure to define double-strand DNA breaks with blunt ends can be used to quantify the proportion of necrotic cells in the total TUNEL-positive population. Finally, the combination of the procedure to identify double-strand DNA breaks with 3′ overhangs and the specific antibody to identify total ssDNA breaks can distinguish the proportion of apoptotic cells from those with ssDNA breaks only.

Distinguishing Apoptotic Myocytes from Nonmyocytes in the Myocardium—Apoptotic myocytes are dually stained by TUNEL and α-sarcomeric actin, and apoptotic nonmyocytes are stained only by TUNEL. The gold standard for identification of apoptotic cells is morphological examination by electron microscopy. The immuno-gold TUNEL and electron microscopic procedure defines the cell type and morphological characteristics of apoptotic cells.

Telomeres and Telomerase—Telomeres are base pair repeats of TTAGGG at the 3′ chromosomal ends that provide genomic stability in the face of oxidative stress. The number of repeats, normally hundreds to thousands, can be reduced with aging, cellular division, and oxidative stress. When telomeres are truncated to a critical threshold, cells can enter a senescent phenotype that may progress to apoptosis. Telomere attrition appears most significant in extreme age (more than 80 years) and may contribute to cardiac failure late in life.

Mitochondrial Control of Cell Death—The role of mitochondria in myocardial response to toxicants as well as therapeutic drugs has long been a focus of investigation. Many toxicants cause modification of mitochondrial permeability transition (MPT).

***Mitochondrial Permeability Transition*—**MPT occurs under toxic insults and behaves like a membrane pore that allows diffusion of solutes less than 1500 Da in size. Although MPT can occur as a temporary event, it can rapidly become irreversible, with the resulting loss of mitochondrial homeostasis and high-amplitude mitochondrial swelling. Because the inner membrane has a larger surface area than the outer membrane, mitochondrial swelling can cause rupture of the outer membrane, releasing intermembrane proteins like cytochrome *c* into the cytosol. Mitochondrial cytochrome *c* release is triggered by overexpression of Bax under oxidative stress conditions, and is thought to be independent of MPT. The release of cytochrome *c* from mitochondria into the cytosol is a critical initiation step in myocardial apoptosis. Cytochrome *c* aggregates with apoptotic protease activating factor-1 (apaf-1, another factor released from mitochondria under oxidative stress), procaspase-9, and dATP, and subsequently activates caspase-9, which activates caspase-3.

Defective Mitochondrial Oxidative Phosphorylation—The early phase of defects in oxidative phosphorylation increases mitochondrial outer membrane permeability, leading to cytochrome *c* release, thus resulting in cytochrome *c*–mediated caspase-9 activation and thereby caspase-3 activation, leading to apoptosis. Defective oxidative phosphorylation also leads to depletion of cellular ATP levels, resulting in necrosis.

Abnormal Mitochondrial Biosynthesis—Both nuclear and mitochondrial DNA encode mitochondrial proteins. Nuclear DNA damage can lead to mutated products and abnormal mitochondrial biosynthesis. Mitochondrial DNA is subjected to far more oxidative injury than nuclear DNA due to the lack of histones and high exposure to reactive oxygen species (ROS) generated by the electron transport chain. Mitochondrial DNA repair mechanisms are limited compared to nuclear DNA repair.

Generation of Reactive Oxygen Species—Changes in mitochondrial membrane potential are critically involved in ROS generation. Two important potassium channels play important roles in mitochondrial membrane permeability. The first is the mitochondrial ATP-sensitive potassium channel, and the second is the Ca^{2+}-activated potassium channel in the cardiac inner

mitochondrial membrane.

Death Receptors and Signaling Pathways—TNF-α and Fas ligand can induce apoptosis of cardiomyocytes through the death receptor–mediated signaling pathway, which can be divided into mitochondrion-dependent and mitochondrion-independent signaling pathways. Under chronic toxic insults, the relative importance of mitochondrial MPT pore opening, cytochrome *c* release, and electron transport defects needs to be critically examined in order to understand the process or mode of cell death. Apoptosis is an energy-dependent process and the switch in the decision between apoptosis and necrosis depends on ATP concentrations. Drug and chemical exposures to myocardial cells causing ischemia result in significant reduction and eventual depletion of adenine nucleotides. Loss of more than 70% of the ATP pool in myocardial cells as observed in myocardial infarction results in a switch from apoptosis to necrosis.

Mitochondrial Dynamics and Autophagy

Autophagy occurs in all eukaryotic cells under the stress of starvation, hypoxia, and toxic insults. Selective autophagy of mitochondria, or mitophagy, is triggered by MPT pore opening and loss of mitochondrial membrane potential. In cardiomyocytes and other terminally differentiated cells, mitophagy is a continuous process of mitochondrial turnover, but the rate of this turnover is influenced by stresses. Nonselective autophagy has been observed in response to nutrient starvation; the degradation of cytosolic components including mitochondria via autophagy provides amino acids and lipid substrates for intermediate metabolism.

Mitochondrial Dynamics—Mitochondria are involved in generation of ATP through oxidative phosphorylation, regulation of apoptosis, the synthesis and degradation of essential metabolites, heme and steroid synthesis, regulation of cell proliferation, maintenance of plasma membrane potential, and calcium signaling. Mitochondrial fusion and fission constitute a major response of cardiomyocytes to stresses.

***Mitochondrial Fusion*—**Mitochondrial fusion is a process of an elongated interconnected mitochondrial network formation through coordinated joining of both the outer mitochondrial membrane (OMM) and the inner mitochondrial membrane (IMM). OMM fusion is directed by GTPases — mitofusins 1 and 2 (MFN1 and MFN2). In mammalian cells, early during fusion two mitochondria approach each other in a tethered docking step. The carboxy-terminal heptad repeats of MFN1 form an intermolecular antiparallel coiled coil that draws the membranes close together and initiates lipid bilayer mixing, and the GTPase provides biomechanical energy for outer membrane fusion.

IMM fusion is governed by a dynamin-related large GTPase optic atrophy 1 (OPA1) protein that is tethered to the IMM and in the intermembrane space. A normal membrane potential is required for fusion of IMM. Loss of membrane potential promotes cleavage of OPA1, causing outer membrane fusion to proceed in the absence of inner membrane fusion.

***Mitochondrial Fission*—**Fragmented discrete mitochondrial morphogenesis is regulated by dynamin-related protein 1 (DRP1) in mammals. This soluble protein contains an N-terminal GTPase, a middle domain, and a C-terminal GTPase effector. During fission, DRP1 is recruited from the cytoplasm to mitochondria. Mammalian fission protein 1 (FIS1) in the OMM interacts

with DRP1. FIS1 overexpression promotes mitochondrial fragmentation and FIS1 depletion stimulates interconnected mitochondrial networks. Depletion of mitochondrial fission factor (MFF) in the OMM suppresses mitochondrial fission in mammalian cells.

***Regulation of Mitochondrial Dynamics*—**Oxidative and nitrosative stress often induces mitochondrial fission. Activation of calcineurin (CaN) and calmodulin-dependent protein kinase 1 (CaMK1) by Ca^{2+} affects the recruitment of DRP1 and mitochondrial translocation. Inhibition of mitochondrial Ca^{2+} uptake attenuates mitochondrial fission.Increased metabolic rates upregulate mitochondrial fusion and dense packing of cristae. Downregulation of OPA1 or MFN leads to fragmented mitochondria with reduced membrane potential and oxygen consumption.

***Toxicological Significance of Mitochondrial Dynamics*—**Mitochondrial fusion and fission are essential in maintaining metabolic homeostasis and the balance between energy production and consumption. Disruption of this physiological process under stress conditions or toxicological exposures leads to overwhelming of mitochondrial fusion or fission, with cell death and maladaptation occurring and leading to toxicological pathogenesis.

Autophagy—The major catabolic pathway responsible for the disposal of damaged organelles and protein aggregates is autophagy, a lysosome-dependent proteolytic pathway capable of processing cellular components. Damaged organelles and proteins are encircled in a double-membrane vesicle, so-called autophagosome, and are hydrolyzed to yield amino acids, fatty acids, and substrates for ATP generation that can be recycled to synthesize new proteins, high-energy phosphates, and renewed cellular components.

Autophagy can be subdivided into three processes.Macroautophagy is characterized by the sequestration of organelles and proteins within an autophagosome. Microautophagy refers to protrusion of the lysosomal membranes per se around a region of cytoplasm. Chaperone-mediated autophagy is restricted to those proteins with a consensus peptide sequence recognized by specific chaperone complexes.

Cardiac Hypertrophy and Heart Failure

Adaptive and Maladaptive Responses—Myocardial adaptation, or remodeling, refers to the process by which the ventricular myocardium changes in structure and function. During maturation, myocardial remodeling is a normal feature for adaptation to increased demands. However, in response to pathological stimuli, such as environmental toxicants, myocardial remodeling is adaptive in the short term, but is maladaptive in the long term, and often results in further myocardial dysfunction. The central feature of myocardial remodeling is an increase in myocardial mass associated with a change in the shape of the ventricle.

***Adaptive Response*—**Physiological hypertrophy is considered an adaptive response in which cardiac function changes to meet an increased demand of cardiac output. Examples of adaptive hypertrophy include the increase in cardiac mass after birth and in response to exercise. Collagen accumulation does not accompany adaptive hypertrophy. Functionally, the increased mass is associated with enhanced contractility and cardiac output.

***Maladaptive Response*—**A distinction between adaptive and maladaptive hypertrophy is

whether the hypertrophy is necessary for the compensatory function of the heart under physiological and pathological stress conditions. Many studies support the hypothesis that cardiac hypertrophy is neither required nor necessarily compensatory. Cardiac hypertrophy increases the risk for malignant arrhythmia and heart failure, and is viewed as a maladaptive response.

Hypertrophic Signaling Pathways—Extrinsic and intrinsic stresses activate signaling transduction pathways leading to fetal gene program activation, enhanced protein synthesis of adult cardiomyocytes, and the eventual hypertrophic phenotype. The signaling pathways include several components: G protein–coupled receptors; protein kinases including MAPK, PKC, and AMPK; calcium and calcineurin; phosphoinositide 3-kinase (PI3K)/glycogen synthase kinase 3β (GSK3β); and transcription factors. Activation of each of the components is sufficient to induce myocardial hypertrophic growth. Figure 18–6 briefly summarizes these pathways and their interactions.

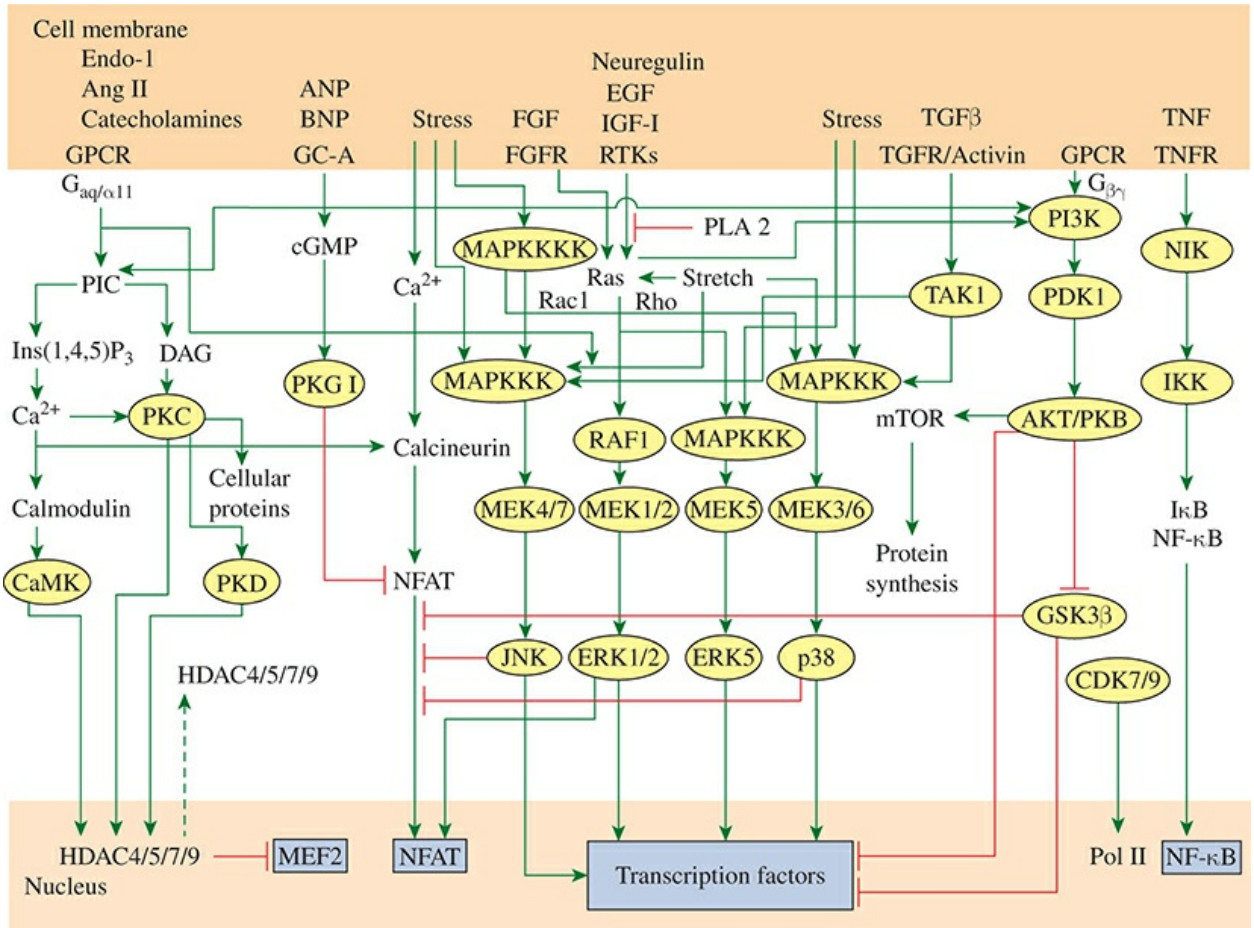

FIGURE 18–6 *Overview of signaling transduction pathways involved in cardiac hypertrophic growth and their cross-talk interactions.* The signaling at the sarcolemmal membrane is at the top. Intermediate transduction of signals by various kinases and phosphatases is in the middle. The nucleus is at the bottom. ANP, atrial natriuretic peptide; Ang II, angiotensin II; BNP, B-type natriuretic peptide; CaMK, calmodulin-dependent kinase; CDK, cyclin-dependent kinase; DAG, diacylglycerol; EGF, epidermal growth factor; Endo-1, endothelin-1; ERK, extracellular

signal-regulated kinase; FGF, fibroblast growth factor; FGFR, FGF receptor; GC-A, guanyl cyclase-A; GPCR, G protein–coupled receptors; GSK3β, glycogen synthase kinase-3β; HDAC, histone deacetylases; IκB, inhibitor of NF-κB; IGF-I, insulin-like growth factor-I; IKK, inhibitor of NF-κB kinase; Ins(1,4,5)P_3, inositol-1,4,5-trisphosphate; JNK, c-Jun N-terminal kinase; MAPKKK, mitogen-activated protein kinase kinasekinase; MAPKKKK, MAPKKK kinase; MEF, myocyte-enhancer factor; MEK, mitogen-activated protein kinase kinase; mTOR, mammalian target of rapamycin; NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor-κB; NIK, NF-κB-inducing kinase; PDK, phosphoinositide-dependent kinase; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PKC, protein kinase C; PKD, protein kinase D; PLA2, phospholipase A2; PLC, phospholipase C; Pol II, RNA polymerase II; RTK, receptor tyrosine kinase; TAK, TGF-β-activated kinase; TGF-β, transforming growth factor-beta; TGFR, TGF receptor; TNF-α, tumor necrosis factor-α; TNFR, TNF-α receptor. (Reprinted with permission from Heineke J, Molkentin JD. Regulation of cardiac hypertrophy by intracellular signalling pathways. *Nat Rev Mol Cell Biol*. 2006;7:589-600.)

***G Protein–Coupled Receptors*—**Myocardial adrenergic, angiotensin, and endothelin (ET-1) receptors belong to G protein–coupled receptors, which are coupled to three major classes of heterotrimeric GTP-binding proteins: $G_{\alpha s}$, $G_{\alpha q}$/$G_{\alpha 11}$, and $G_{\alpha i}$. Activation of $G_{\alpha q}$-coupled receptors induces myocyte hypertrophy in vitro. Cardiac overexpression of $G_{\alpha s}$, the downstream effector of β1-adenergic receptors, initially increases contractility, but eventually results in cardiac hypertrophy, fibrosis, and heart failure.

***Phosphoinositide 3-Kinase/Glycogen Synthase Kinase 3β Pathway*—**Activation of PI3K is found in both physiological and pathological hypertrophy. Overexpression of insulin-like growth factor IGF induces cardiac hypertrophy. IGF signals through PI3K to the serine/threonine kinase Akt or protein kinase B. Both PI3K and the Akt induce hypertrophic growth of adult hearts.

Transition from Cardiac Hypertrophy to Heart Failure—The critical cellular event of this transition is myocardial apoptosis triggered by inflammatory cytokines, such as TNF-α, and neurohormonal factors, such as atrial natriuretic peptide (ANP), which lead to dilated cardiomyopathy and deterioration of cardiac function. Toxicological exposures may cause dilated cardiomyopathy or heart failure without an intermediate hypertrophic stage. Myocardial cell death also plays an essential role in direct cardiac dilation pathogenesis. The most obvious myocardial dysfunction that occurs in the early responses to toxicants is cardiac arrhythmia.

From cardiac hypertrophy to heart failure, activation of compensatory mechanisms, including the sympathetic nervous system, the renin–angiotensin system, and increases in cytokines, occurs. Extensive biochemical, physiological, and molecular changes result in myocardial remodeling, ultimately leading to heart failure.

Arrhythmogenesis: QT Prolongation and Sudden Cardiac Death

Compromise of electrical conduction can lead to sudden death in an otherwise healthy individual. Pathological scenarios of remodeling or scarification can induce arrhythmias. Many drugs and other chemicals can adversely affect the monophasic action potential of cardiomyocytes, pacemaker cells, and Purkinje fibers to induce major arrhythmias.

Cardiomyocyte Monophasic Action Potential (MAP)—In pacemaking cells, the increased slope of phase 4 is due to a "leaky" pacemaker channel I_f, which causes a gradual increase in cellular potential until the voltage-gated calcium channels activate. Pacemaker cells initiate phase 0 depolarization through these calcium channels, rather than sodium. Pacemaker cells also lack a prolonged phase 2/3 plateau, as they are not contractile cells. Most cardiomyocytes have some degree of automaticity, but due to the reduced role of the I_f current, the time between automatic beats is much slower than pacemaker cells. Thus, the more rapidly firing pacemaking cells *entrain* the rest of the cardiomyocytes as the depolarization spreads through gap junctions.

Cellular Abnormalities: Afterdepolarizations—The normal monophasic action potential is dependent on specific timing of numerous ion channels in terms of three general biophysical states: Resting, Open, and Inactive. Ions can pass through the channel only during the Open state. Channels in the Inactive state cannot be opened until they reset to the Resting state; during the Inactive state, channels are said to be refractory, as they cannot be triggered to open. An afterdepolarization occurs when a given channel opens abnormally and leads to depolarization of the cell prior to the cell being fully recovered from the previous beat. In such scenarios, the cell may lose polarity, stimulate adjacent cells abnormally, or have a significant delay in recovery. Cells becoming independent from the normal conduction pattern become "ectopic" pacemaking cells that can significantly disrupt cardiac electrophysiological function.

Organ-level Abnormalities: Reentry and Capture—Interference with the normal spread of depolarizations leads to self-propagating waveforms, often leading to tachyarrhythmias or ventricular fibrillation. Capture often arises due to regions of myocardium that poorly conducts, causing the normal wavefront to travel around this region and possibly looping backwards as a result, a phenomenon called "reentry." Toxicants may contribute to such life-threatening electrocardiographic disturbances (1) by chronically causing abnormal growth that alters the normal pattern of wavefront propagation; (2) by acutely disrupting the MAP, leading to afterdepolarizations and causing ectopic conductors or areas of non-conducting tissue; and (3) through effects of increasing oxygen demand or limiting oxygen delivery (for instance, via coronary artery vasospasm).

QT Prolongation—Many drugs cause QT prolongation and Torsades de Pointes, a life-threatening ventricular arrhythmia. Clinically, long QT syndrome is defined when the QT interval is longer than 460 milliseconds. However, Torsades de Pointes occurs with an average increase in QT interval by approximately 200 milliseconds (a normal QT interval is about 300 milliseconds).

Molecular Basis of QT Prolongation—A reduction in net outward current and/or an increase in inward current are potential contributors to QT prolongation on the electrocardiogram. Three important channels play a critical role in the plateau phase (phase 2) of the cardiac action potential, sodium inward channels, and potassium outward channels (I_{Kr} and I_{Ks}).

Sodium channel dysfunction in congenital long QT syndrome is related to mutations in the *SCN5A* gene that encodes the α-subunits of sodium channels. Reopening the sodium channels during the plateau phase of action potential, even a small inward current, lengthens the duration of the cardiac action potential. Sodium channel inactivation immediately following depolarization (phase 1) is important for the transition to phase 2 of the action potential.

The I_{Kr} *potassium channels* critically affect the length of the plateau phase of the cardiac action potential. The human *ether-à-go-go*-related gene (*HERG*) expressed primarily in the heart encodes the α-subunit of the cardiac I_{Kr} potassium channel. The HERG α-subunits assemble with MiRP1 β-subunits to form cardiac I_{Kr} channels. During repolarization, the I_{Kr} channels open, increasing the magnitude of I_{Kr} current during the first half of phase 3 repolarization. Loss-of-function mutations in *HERG* make a critical contribution to the long QT syndrome due to the prolonged plateau phase of cardiac potential.

The I_{Ks} *potassium channel* is the other one of the two channels primarily responsible for the termination of the plateau phase of the action potential. The I_{Ks} potassium channel is assembled from KVLQT1 α-subunits and the min Kβ-subunits.

Torsade De Pointes and Sudden Cardiac Death—The trigger for arrhythmia in the long QT syndrome is thought to be a spontaneous early afterdepolarization that arises due to reactivation of the L-type calcium channels and/or activation of the sodium–calcium exchange current. Once triggered, a self-propagating waveform captures the rhythm of the heart creating a high-frequency waveform on the ECG referred to as Torsades de Pointes, French for "twisting of the points." Torsades de Pointes can spontaneously revert to normal rhythm, or become life-threatening and lead to sudden cardiac death.

Parameters Affecting QT Prolongation and Torsadogenesis—Electrotonic cell-to-cell coupling influences the dispersion of repolarization. If myocardial cells with intrinsically different durations of action potential are well coupled, electrotonic current flow attenuates the differences in action potential duration in individual cardiomyocytes. Therefore, torsadogenesis results from not only cardiomyocytes but also other types of cells and the interaction among these cells.

***Drugs and Environmental Toxicants*—**Drug-induced QT prolongation is a major acquired long QT syndrome. The so-called class III antiarrhythmics potassium channel blockers predictably elongate QT intervals and cause Torsades de Pointes in 5% to 7% of recipients. Environmental exposure to particulates in air is a risk factor for QT prolongation in elderly, children, and individuals with compromised hearts.

***Disturbances in Ion Homeostasis*—**Hypokalemia in combination with torsadogenic drugs is a recognized risk factor for QT prolongation and Torsades de Pointes. Also, sodium supplementation can diminish the long QT syndrome due to the gain-of-function mutations in sodium channels. Stress-induced Ca^{2+} overload in myocardial cells increases the likelihood of arrhythmia. Electrode imbalance exerts more effects on compromised hearts.

***Abnormal Gap Junction*—**Gap junction–mediated intercellular communication is essential in the propagation of electrical impulses in the heart. Under normal conditions, the gap junction electrotonic current flow attenuates the differences in action potential duration of myocardial cells. Toxic exposures that damage connexons can disrupt electrotonic cell-to-cell coupling.

***Myocardial Ischemic Injury*—**Acute myocardial ischemia can cause immediate arrhythmia due to disturbance in ionic homeostasis. Acute ischemia can induce myocardial infarction that can lead to the block of cardiac conductance. The areas separated by the scar tissue would be

uncoupled, making regional differences in action potential duration of myocardial cells apparent. The infarct heart thus is more susceptible to drug-induced QT prolongation and Torsades.

***Cardiac Hypertrophy*—**Normal distribution of Purkinje fibers in the myocardium is proportional to heart mass. Hypertrophic growth of cardiac myocytes would lead to unbalanced distribution of Purkinje fibers in the remodeling heart and interruptions in conduction of pacemaker potentials.

***Myocardial Fibrosis*—**Dilated cardiomyopathy in alcoholics often involves myocardial fibrosis, which simulates the effect of myocardial infarction on electrical conduction in the heart.

***Heart Failure*—**Heart failure presents a common, acquired form of the long QT syndrome. Selective downregulation of two potassium channels, I_{to1} and I_{K1}, is involved in action potential prolongation. I_{to1} is involved in phase 1 of the action potential and opposes the depolarization. The increase in depolarization may be adaptive in the short term because it provides more time for excitation–contraction coupling, mitigating the decrease in cardiac output. However, downregulation of potassium channels becomes maladaptive in the long term because it predisposes the individual to early afterdepolarization, inhomogeneous repolarization, and polymorphic ventricular tachycardia.

Biomarkers for Cardiovascular Toxicity

Myocardial structural changes and functional alterations can be indirectly observed by echocardiography and electrocardiogram in combination with stress testing. The data generated from these measurements can be considered in a broad sense as biomarkers. However, in clinical practice and experimental approach, biomarkers are molecules released from the myocardium under various injury conditions that are readily detectable from blood samples.

Validation of Biomarkers—Ideal cardiac toxic injury markers would have cardiac specificity, sensitivity, predictive value, robust, bridge preclinical to clinical, and noninvasive procedure/accessibility.

Availability of Biomarkers—Currently, validated biomarkers (Table 18–1) that are included in clinical diagnostic testing guidelines are all related to myocardial structural injury. For determination of pharmaceutical safety, a battery of complementary biomarkers is typically applied.

TABLE 18–1 Biomarkers for cardiovascular toxicity, injury, and inflammation.

Cardiac Markers	Background	USE
Creatine kinase	Elevation of specific isoform CK-MB in the serum is a specific marker of acute myocardial infarction	Routinely used clinical and preclinical myocardial injury marker
Myoglobin	Elevation of serum myoglobin is likely reflective of the extent of myocardial damage, although not specific to cardiac muscle	Readily available clinical and preclinical marker, although lack of specificity has led to reduced utilization
B-type natriuretic peptide	Cardiac neurohormone secreted by the ventricular myocardium in response to volume and pressure overload, and the release of BNP is a valuable indicator of heart failure	BNP is an important diagnostic marker as well as a drug used to alleviate congestive heart failure symptoms in cardiac decompensation. BNP has value for preclinical models of heart failure across species
Cardiac troponins	Cardiac troponin T (cTnT) and I (cTnI) are constituents of the myofilaments and expressed exclusively in cardiomyocytes. It is thus of absolute myocardial tissue specificity	"Gold Standard" for diagnosis of myocardial infarction. Value extends to preclinical safety and experimental models
Vascular Markers	**Background**	**USE**
Acute-phase proteins (e.g., CRP, serum amyloid A, and IL-6)	CRP is a marker of systemic and vascular inflammation, which appears to predict future cardiac events in asymptomatic individuals. SAA and IL6 are similarly reflective of systemic inflammation, but not used as extensively as CRP for clinical prediction	Clinically relevant to long-term disease, but may lack specificity/sensitivity for preclinical safety assessment
Soluble adhesion molecules	Soluble intracellular and vascular cell adhesion molecule (ICAM-1 and VCAM-1) and E- and P-selectins are cell surface receptors on endothelial cells that can be shed following injury or inflammatory activation of peptidases	Clinical and preclinical values, although not routinely used for cardiovascular diagnostic purposes
Endothelin-1	A potent vasoconstrictive peptide, appears elevated in response to a number of toxicants	Clearly involved in certain clinical pathologies, especially pulmonary hypertension. Mostly used in preclinical experimental settings
Thrombospondin-1	May be released from a variety of tissues and is a nonspecific biomarker for a number of diseases including vascular injury, but may also be a driver of inflammation and endothelial cell activation via the CD36 receptor	Principally preclinical and experimental research values
Vascular endothelial growth factor (VEGF)	A peptide that stimulates angiogenesis, may reflect vascular injury or conditions necessitating vascular growth or healing	Used clinically most often for cancer-related outcomes, but often used for preclinical safety related to vascular injury
Cytokines (MCP-1, CINC/IL-8, KC/GRO)	Numerous cytokines are released into the circulation from atheromas and areas of vasculitis. Specific cytokines may be more pertinent than others depending on the injury or pathology of interest	Used widely clinically and experimentally, but not specific enough for diagnostic or prognostic value

Biomarker Applications and Limitations—All the biomarkers described in Table 18–1 have been used as indices of myocardial or vascular injury in clinical practice and experimental studies. The major concern of most of the biomarkers is their specificity. Cardiac troponin T (cTnT), which has absolute myocardial tissue specificity and high sensitivity, is the biomarker of choice for assessing myocardial damage in humans. Its preclinical value for monitoring drug cardiac toxicity and in drug development needs to be evaluated.

CARDIAC TOXIC CHEMICALS

Many substances can cause cardiac toxic responses directly or indirectly (Table 18–2).

TABLE 18–2 Cardiotoxicity of key pharmaceutical agents.

Agents	Cardiotoxic Manifestations	Proposed Mechanisms of Cardiotoxicity
Antiarrhythmic drugs		
Class I (disopyramide, encainide, flecainide, lidocaine, mexiletine, moricizine, phenytoin, procainamide, propafenone, quinidine, tocainide)	↓ Conduction velocity Proarrhythmogenic	Na^+ channel blockade
Class II (acebutolol, esmolol, propranolol, sotalol)	Bradycardia, heart block	β-Adrenergic receptor blockade
Class III (amiodarone, bretylium, dofetilide, ibutilide, quinidine, sotalol)	↑ Action potential duration QTc interval prolongation Proarrhythmogenic	K^+ channel blockade
Class IV (diltiazem, verapamil)	↓ AV conduction Negative inotropic effect Negative chronotropic effect Bradycardia	Ca^{2+} channel blockade
Inotropic drugs and related agents		
Cardiac glycosides (digoxin, digitoxin)	Action potential duration AV conduction Parasympathomimetic (low doses) Sympathomimetic (high doses)	Inhibition of Na^+,K^+-ATPase, $\downarrow[Ca^{2+}]_i$
Ca^{2+}-sensitizing agents (adibendan, levosimendan, pimobendan)	↓ Diastolic function? Proarrhythmogenic	↓ Ca^{2+} sensitivity Inhibition of phosphodiesterase
Other Ca^{2+}-sensitizing agents (allopurinol, oxypurinol)	?	Inhibition of xanthine oxidase
Catecholamines (dobutamine, epinephrine, isoproterenol, norepinephrine)	Tachycardia Cardiac myocyte death	β_1-Adrenergic receptor activation Coronary vasoconstriction Mitochondrial dysfunction $\downarrow[Ca^{2+}]_i$ Oxidative stress Apoptosis
Bronchodilators (albuterol, bitolterol, fenoterol, formeterol, metaproterenol, pirbuterol, procaterol, salmeterol, terbutaline)	Tachycardia	Nonselective activation of β_1-adrenergic receptors
Nasal decongestants (ephedrine, ephedrine alkaloids, ma huang, phenylephrine, phenylpropanolamine, pseudoephedrine)	Tachycardia	Nonselective activation of α_1-adrenergic receptors
Appetite suppressants (amphetamines, fenfluramine, phentermine)	Tachycardia Pulmonary hypertension Valvular disease	↓ Serotonin? Na^+ channel blockade?

Antineoplastic drugs		
Anthracyclines (daunorubicin, doxorubicin, epirubicin)	Cardiomyopathy Heart failure	Altered $[Ca^{2+}]_i$ homeostasis Oxidative stress Mitochondrial injury Apoptosis
5-Fluorouracil	Proarrhythmogenic	Coronary vasospasm?
Cyclophosphamide	Cardiac myocyte death	4-Hydroxycyclophosphamide (metabolite) Altered ion homeostasis
Antibacterial drugs		
Aminoglycosides (amikacin, gentamicin, kanamycin, netilmicin, streptomycin, tobramycin)	Negative inotropic effect	↓ $[Ca^{2+}]_i$
Macrolides (azithromycin, clarithromycin, dirithromycin, erythromycin)	↓ Action potential duration QTc interval prolongation Proarrhythmogenic	K^+ channel blockade
Fluoroquinolones (grepafloxacin, moxifloxacin, sparfloxacin)	↓ Action potential duration QTc interval prolongation Proarrhythmogenic	K^+ channel blockade
Tetracycline	Negative inotropic effect	↓ $[Ca^{2+}]_i$
Chloramphenicol	Negative inotropic effect	↓ $[Ca^{2+}]_i$
Antifungal drugs		
Amphotericin B	Negative inotropic effect	Ca^{2+} channel blockade? Na^+ channel blockade? ↓ Membrane permeability?
Flucytosine	Proarrhythmogenic Cardiac arrest	5-fluorouracil metabolite Coronary vasospasm?
Antiviral drugs		
Nucleoside analog reverse transcriptase inhibitors (stavudine, zalcitabine, zidovudine)	Cardiomyopathy	Mitochondrial injury Inhibition of mitochondrial DNA polymerase Inhibition of mitochondrial DNA synthesis Inhibition of mitochondrial ATP synthesis
Centrally acting drugs		
Tricyclic antidepressants (amitriptyline, desipramine, doxepin, imipramine, protriptyline)	ST segment elevation QTc interval prolongation Proarrhythmogenic Cardiac arrest	Altered ion homeostasis Ca^{2+} channel blockade Na^+ channel blockade K^+ channel blockade
Selective serotonin reuptake inhibitors (fluoxetine)	Bradycardia Atrial fibrillation	Ca^{2+} channel blockade Na^+ channel blockade

Phenothiazine antipsychotic drugs (chlorpromazine, thioridazine)	Anticholinergic effects	Ca^{2+} channel blockade?
	Negative inotropic effect	
	QTc interval prolongation	
	PR interval prolongation	
Other antipsychotic drugs (clozapine)	Blunting of T waves	
	ST segment depression	
General inhalational anesthetics (enflurane, desflurane, halothane, isoflurane, methoxyflurane, sevoflurane)	Negative inotropic effect	Ca^{2+} channel blockade
	Decreased cardiac output	Altered Ca^{2+} homeostasis
	Proarrhythmogenic	β-Adrenergic receptor sensitization
Other general anesthetics (propofol)	Negative inotropic effect	Ca^{2+} channel blockade
		Altered Ca^{2+} homeostasis
		β-Adrenergic receptor sensitization
Local anesthetics		
Cocaine	Sympathomimetic effects	Na^{+} channel blockade
	Ischemia/myocardial	Coronary vasospasm, infarction
	Proarrhythmogenic	Altered Ca^{2+} homeostasis
	Cardiac arrest	Mitochondrial injury
	Cardiac myocyte death	Oxidative stress
		Apoptosis
Other local anesthetics (bupivacaine, etidocaine, lidocaine, procainamide)	Decreased excitability	Na^{+} channel blockade
	↓ Conduction velocity	
	Proarrhythmogenic	
Antihistamines (astemizole, terfenadine)	↓ Action potential duration	K^{+} channel blockade
	QTc interval prolongation	
	Proarrhythmogenic	
Immunosuppressants (rapamycin, tacrolimus)	Cardiomyopathy	Altered Ca^{2+} homeostasis
	Heart failure	
Miscellaneous drugs		
Cisapride	↓ Action potential duration	K^{+} channel blockade
	QTc interval prolongation	
	Proarrhythmogenic	
Methylxanthines (theophylline)	↓ Cardiac output	Altered Ca^{2+} homeostasis
	Tachycardia	Inhibition of phosphodiesterase
	Proarrhythmogenic	
Sildenafil	?	Inhibition of phosphodiesterase
Radiocontrast agents (diatrizoatemeglumine, iohexol)	Proarrhythmogenic	Apoptosis?
	Cardiac arrest	

Alcohol and Alcoholic Cardiomyopathy

Alcohol is considered the causal chemical in up to 40% of all patients with nonischemic, dilated

cardiomyopathy, which is characterized by an increase in myocardial mass, dilation of the ventricles, wall thinning, ventricular dysfunction, and heart failure. Asymptomatic ACM patients with changes in cardiac structure and function had a history of consuming more than 90 g/day of alcohol for more than 5 years, with symptoms beginning after longer exposures. Impaired liver function of alcoholics may generate quantities of acetaldehyde that can inhibit protein synthesis and Ca^{2+} sequestration by the SR, alter mitochondrial respiration, and disturb actin and myosin interaction. Reactive oxidative metabolites from ethanol biotransformation may be a major contributing factor for ACM.

Pharmaceutical Chemicals

Select pharmaceutical chemicals causing cardiac toxicity are listed with a proposed mechanism in Table 18–2. Additional drugs are listed below.

***Tyrosine Kinase Inhibitors*—**Hematologic malignancies have proven susceptible to small molecule tyrosine kinase inhibitors, including imatinib and sunitinib. However, these agents induce hypertension and ventricular systolic dysfunction, potentially by interfering with intracellular autophagy and accumulation in lysosomes. Dasatinib and nilotinib are reported to elicit rare occurrence of pulmonary hypertension and QT prolongation, respectively. Nilotinib does appear to promote a rare worsening of peripheral artery disease.

Anti-inflammatory Agents—Nonsteroidal anti-inflammatory drugs (NSAIDs) rofecoxib, celecoxib, and valdecoxib, which are selective inhibitors of COX-2, have an increased risk of cardiovascular events such as heart attack and stroke. The cardiovascular events induced by COX-2 inhibitors are presumably related to thrombotic events and other sequelae related to downregulation of prostacyclin production.

Natural Products

Natural products include naturally occurring catecholamines, hormones, and cytokines (Table 18–3). Many of these products have been shown to cause cardiac toxic responses, especially in higher exposure concentrations.

TABLE 18–3 Cardiotoxicity of naturally occurring substances.

Agents	Cardiotoxic Manifestations	Proposed Mechanisms of Cardiotoxicity
Estrogens		
Natural estrogens (17β-estradiol, estrone, estriol)	QTc interval prolongation?	Gender differences in K^+ channel expression?
Synthetic estrogens (diethylstilbestrol, equilin, ethinyl estradiol, mestranol, quinestrol)	Cardioprotection?	Antiapoptotic effects?
Nonsteroidal estrogens (bisphenol A, diethylstilbestrol, DDT, genistein)		Antioxidant activity?
		↑ Na^+,K^+-ATPase activity?
		Ca^{2+} channel blockade?
		Other mechanisms?
Progestins (desogestrel, hydroxyprogesterone, medroxyprogesterone, norethindrone, norethynodrel, norgestimate, norgestrel, progesterone)	Enhanced toxicity of cocaine?	Mechanisms?
Androgens		
Natural androgens (androstenedione, dehydroepiandrosterone, dihydrotestosterone, testosterone)	Myocardial infarction	Mitochondrial injury?
	Cardiac hypertrophy	Altered Ca^{2+} homeostasis?
Synthetic androgens (boldenone, danazol, fluoxymesterone, methandrostenolone, methenolone, methyltestosterone, nandrolone, oxandrolone, oxymetholone, stanozolol)		Other mechanisms?
Glucocorticoids		
Natural glucocorticoids (corticosterone, cortisone, hydrocortisone)	Cardiac hypertrophy	Increased collagen expression
Synthetic glucocorticoids (e.g., dexamethasone, methylprednisolone, prednisolone, prednisone)	Cardiac fibrosis	Other mechanisms?
Mineralocorticoids (aldosterone)	Cardiac fibrosis	Increased collagen expression
	Heart failure	Other mechanisms?
Thyroid hormones (thyroxine, triiodothyronine)	Tachycardia	Altered Ca^{2+} homeostasis
	Positive inotropic effect	
	Increased cardiac output	
	Cardiac hypertrophy	
	Proarrhythmogenic	
Cytokines		
Interleukin-1β	Negative inotropic effect	↑ Nitric oxide synthase expression
	Cardiac myocyte death	Apoptosis
Interleukin-2	Negative inotropic effect	↑ Nitric oxide synthase expression
Interleukin-6	Negative inotropic effect	↑ Nitric oxide synthase expression
Interferon-γ	Cardomyopathy	↑ Nitric oxide synthase expression
Tumor necrosis factor-α	Proarrhythmogenic	Altered ion homeostasis
	Negative inotropic effect	↑ Nitric oxide synthase expression
	Cardiac myocyte death	↑ Sphingosine production
		↓ Ca^{2+} transients
		Apoptosis

Environmental Pollutants and Industrial Chemicals

Airborne Particulate Matter—A clear relationship between ambient concentration of particulate matter and cardiovascular morbidity and mortality has been documented over the past 30 years. Particulate inhalation can lead to arrhythmias, electrocardiographic abnormalities, and negative impacts on cardiac function. Spillover of reactants from the lung can have inflammatory actions on cardiovascular cells, direct translocation of particulate matter or soluble particulate constituents to the cardiac tissue, or activation of pulmonary nerves that in turn modulate autonomic tone.

Solvents—Solvents may affect cardiac functions such as contraction and energy production by directly dispersing into plasma membranes, by affecting the neurohormonal regulation of cardiac function, by disrupting sympathetic and parasympathetic control of the heart, and releasing circulating hormones such as catecholamines, vasopressin, and serotonin.

Alcohols and Aldehydes—Oxidation of alcohols yields aldehydes, which have sympathomimetic activity as a result of releasing catecholamines. Acute cardiodepressant effects of alcohols and aldehydes may be related to inhibition of intracellular Ca^{2+} transport and/or oxidative stress. Acute cardiotoxicity from environmental exposure includes a negative dromotropic effect (reduced conductivity) and a decreased threshold for ventricular fibrillation.

Halogenated Alkanes—The highly lipophilic halogenated alkanes cross the blood–brain barrier readily producing CNS-depression and anesthesia, as well as depress heart rate, contractility, and conduction. In addition, some chemicals like the fluorocarbons sensitize the heart to the arrhythmogenic effects of β-adrenergic receptor agonists such as endogenous epinephrine.

Metals and Metalloids—Cadmium, lead, and cobalt exhibit negative inotropic and dromotropic effects and can also produce structural changes in the heart. The cardiotoxic effects of heavy metals are attributed to their ability to form complexes with intracellular macromolecules and to antagonize intracellular Ca^{2+}. Manganese, nickel, and lanthanum block Ca^{2+} channels at high concentrations (e.g., millimolar range). The arrhythmogenic effect of barium chloride has been utilized to screen antiarrhythmic agents. Arsenic has a high affinity for sulfhydryl proteins and can induce ventricular tachycardia, myocardial cell death through apoptosis, and decreased systolic and diastolic function of the heart.

Organophosphates—Acute poisoning with organophosphate pesticides such as malathion, chlorpyrifos, and dichlorvos, causes respiratory failure, bradycardia, and hypotension. Electrocardiographic abnormalities arising following poisoning include QT prolongation and slowing of conductance (AV nodal and bundle branch blockade).

OVERVIEW OF VASCULAR SYSTEM

Vascular Physiology and Structural Features

The vascular system consists of blood vessels of varying size and different cellular composition. Blood vessels can be divided into arterial, venous, and capillary systems. In addition, the lymphatic system only carries extracellular fluid, or lymph. The vascular system provides oxygen, hormones, cytokines, and nutrients to and removes carbon dioxide and metabolic by-

products from organ systems (Fig. 18–7). Endothelins produced by vascular endothelial cells alter cardiac rhythm and affect myocardial contractility. Many regulatory systems control the physiological function of the vascular system, so that the changes in the vascular system reflect either local action of chemicals or disruption of the regulatory systems, or both.

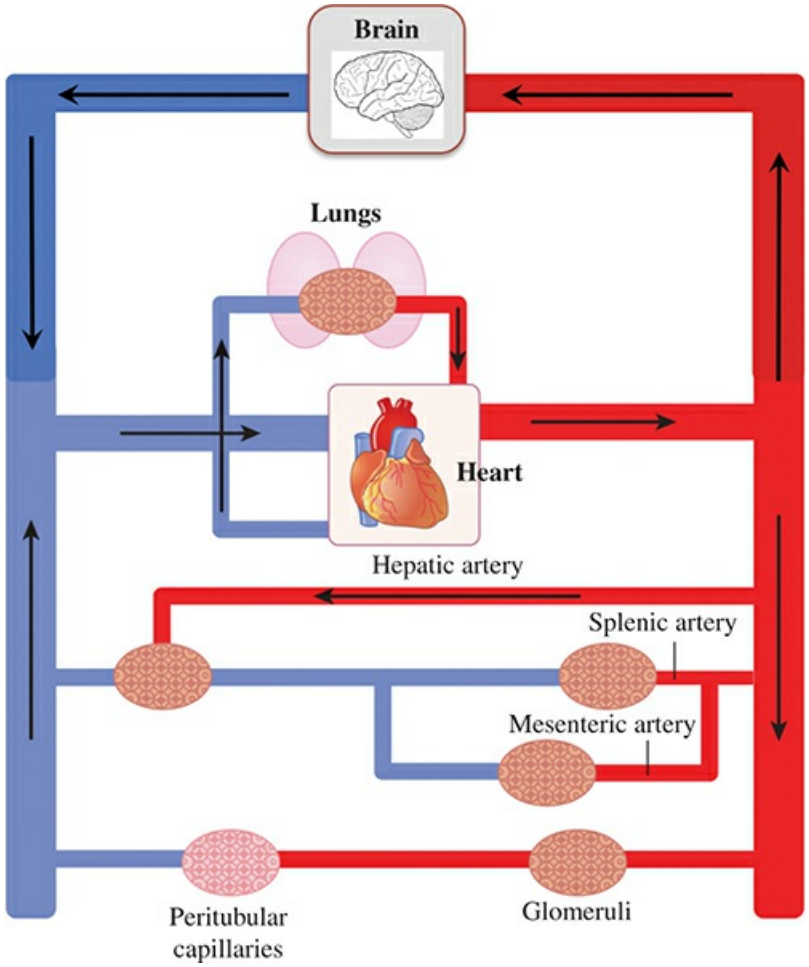

FIGURE 18–7 *Schematic diagram of vascular supply to selected organs.* The capillary beds are represented by a meshwork connecting the arteries (right) with the veins (left); inclusion of several organs indicates the importance of the vascular system in toxicology.

Endothelial Cells—All vessels of the circulatory system are lined with a single layer of endothelial cells that modulate blood flow, transport of circulating factors to end-target organs, and removal of waste products. Endothelial cells produce nitric oxide (NO) and prostacyclin, both of which also mediate vasodilation to increase regional blood flow. NO is a soluble gas derived from the metabolism of arginine to citrulline and NO by the enzyme endothelial NOS (eNOS). eNOS is a dimeric protein that appears to be readily downregulated (either by regulatory phosphorylation or uncoupled electrochemically) by oxidative stress and inflammation. In parallel, healthy endothelial function can be easily perturbed by a wide range of oxidative and inflammatory insults. Endothelial cells also produce other vasodilators, such as hydrogen sulfide, epoxyeicosatrienoic acids (EETs), and hydrogen peroxide. Endothelial cells can also create vasoconstrictors, including endothelin-1 and thromboxane A2.

The endothelium is a central player in inflammatory responses and chronic vascular pathologies. Endothelial cells respond to toxicants, injury, or certain pathogens by becoming activated and expressing cell surface adhesion molecules, such as intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). These adhesion molecules work in concert with chemokines to recruit leukocytes to regions of injury. During active inflammation, gaps between endothelial cells enlarge to facilitate extravasation of immune cells into the vessel wall. Over time, inflammatory cells build up in the vessel wall, contributing to the enlargement of atheromatous lesions.

Vascular Smooth Muscle Cells—Arteries and veins are surrounded by vascular smooth muscle. The thickness of the smooth muscle layer is greater in arteries than veins, lower in arterioles and venules, and absent in capillaries, except as a sphincter mechanism at the opening of capillaries to control the passage of erythrocytes. Smooth muscle controls the gauge, or diameter, of the vessels by constriction and dilation.

Arterial System and Physiological Function—The arterial system is composed of the aorta, major arteries, and small arterioles. The aorta and major arteries are thick-walled structures with vascular smooth muscle, elastic, and connective tissues (Fig. 18–8). Blood flow within the arterial system is initiated by contraction of the heart and begins at the ascending aorta. Blood is distributed to the organ systems of the body through the major arteries that branch from the aorta. All these arteries further branch to give rise to smaller arteries and become arterioles that connect to capillaries for the delivery of oxygen and nutrients to target tissues.

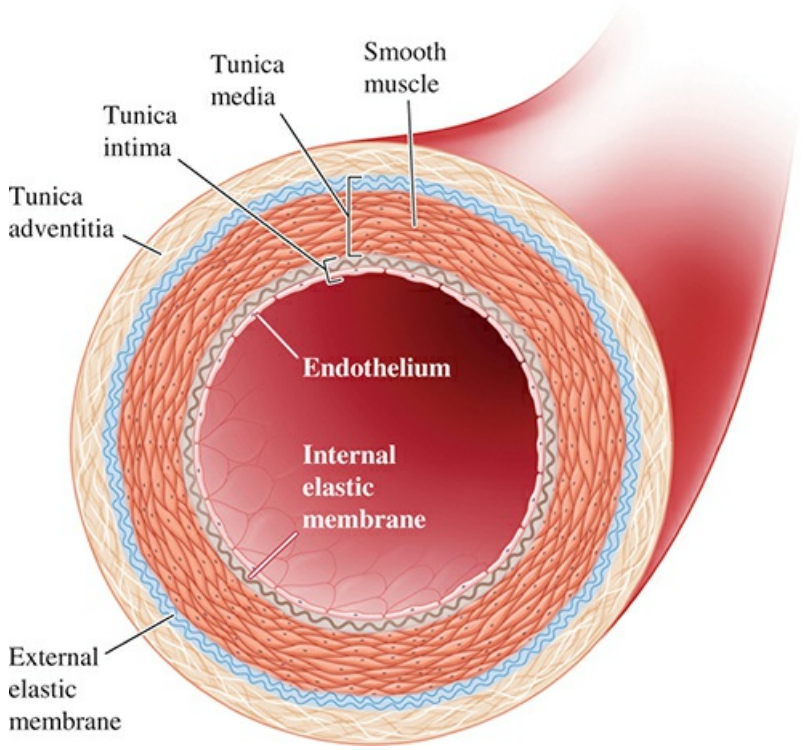

FIGURE 18–8 *Cross-sectional representation of the vascular wall of large and medium-size blood vessels.* The tunica intima, composed of endothelial cells facing the vessel lumen, rests on a thin basal lamina. The tunica media consists mainly of vascular smooth muscle cells

interwoven with collagen and elastin. The tunica adventia is a layer of fibroblasts, collagen, elastin, and glycosaminoglycans.

***Aorta and Large Arteries*—**Chemicals can affect directly the arterial systems leading to alterations in blood flow or indirectly act through the regulatory system to change the blood flow.

***Arterioles and Vascular Resistance*—**The vascular smooth muscle cells are critical for the regulation of vascular resistance, which alters vessel diameter. The arterioles are primarily responsible for regulation of peripheral vascular resistance and blood flow. Chemicals affecting the structure and function of the vascular smooth muscle cells can change the physiological regulation of vascular resistance.

Capillaries and Microcirculation—Capillaries serve as the communication site between blood and tissues and constitute the major part of the microcirculation where nutrients, water, gases, hormones, cytokines, and waste products are exchanged between blood and tissues. Capillaries are only one cell-layer thick. The passage of molecules across the capillary wall can occur both between and through the endothelial cells. The endothelial cells are targets of xenobiotics.

Venous System and Physiological Function—The venous system is composed of venules, veins, and vena cava. The blood flow in the venous system starts from the thin-walled venules that collect the blood from the target tissues of the body and return blood to the heart. The large veins contain vascular smooth muscle cells that can increase the blood return to the heart by constriction and increase capacitance by dilation. Xenobiotics can exert adverse effects on the vascular smooth muscle cells to compromise capacitance of the venous system.

Lymphatic System and Physiological Function—The lymphatic system begins as blind-ended lymphatic end bulbs that drain into a meshwork of interconnected lymphatic vessels. This low-pressure system collects excess tissue water and plasma proteins and maintains a negative interstitial pressure. Toxic insults to the lymphatic system can lead to elevated interstitial pressures and subsequent tissue edema.

Unique Vascular Beds

***Coronary*—**Coronary arteries branch from the aorta immediately past the mitral valve and supply oxygen and nutrients to the heart. The coronary blood flow can increase about fourfold to supply additional oxygen needed by the heart muscle under demanding conditions, such as during exercise. Heart tissue extracts most of the oxygen from blood during resting conditions, and the coronary blood flow increases with the workload demands on the heart. The increase in blood flow is the major mechanism of the heart to increase energy production. Xenobiotics can affect coronary vasculature leading to reductions in cardiac blood flow and cardiac work.

***Cerebrovasculature and the Blood–Brain Barrier*—**The brain is uniquely protected by exceptionally tight endothelial cell gap junctions, with a secondary barrier of pericyte cells and astrocytes. Under normal conditions, the blood–brain barrier (BBB) is permeable to water, soluble gases, and small molecule mediators, but larger circulating proteins and molecules cannot penetrate to the neural compartment. Transporter proteins remove larger hydrophobic molecules and prevent their uptake into the brain. Toxicants can directly or indirectly impair the

BBB allowing chemical penetration into the CNS.

***Placenta*—**The placenta develops during and grows throughout pregnancy. The organ transfers nutrients to and removes waste products from the growing fetus. As such, it is predominantly an interface of two vascular systems (maternal and fetal) and is largely populated by endothelial cells (in addition to immune cells, fibroblasts, stem cells, etc.).

Regulatory Mechanisms of the Vascular System

Neurohormonal Regulation—Most arteries, arterioles, venules, and veins, with the exception of those of the external genitalia, receive sympathetic innervation only. Norepinephrine is the usual transmitter that binds to α_1-adrenergic receptors causing contraction of the vascular smooth muscle and thus the constriction of blood vessels. Activation of β_2-adrenergic receptors by circulating epinephrine leads to vascular smooth muscle relaxation and vasodilation. Coronary and skeletal muscle arteries are highly responsive to the epinephrine-induced vasodilation. In addition, the blood vessels of skeletal muscles receive sympathetic cholinergic innervation to stimulate vascular smooth muscle relaxation and vasodilation. The CNS regulates the activity of the autonomic nerves at several levels.

***The Renin–Angiotensin–Aldosterone System*—**Renin is released from the kidney in response to reduced arterial pressure and blood volume and catalyzes conversion of angiotensinogen to angiotensin I, which is further converted to angiotensin II, a powerful arteriolar vasoconstrictor that also causes the release of aldosterone. Aldosterone reduces renal sodium excretion, resulting in water retention and increased blood volume.

***ADH and ANP*—**ADH is a vasoconstrictor that is released under the conditions of hemorrhage, decreased atrial stretch receptor firing, and increased plasma osmolarity. ADH acts to increase water retention by the kidney, thus increasing blood volume. ANP and related B-type natriuretic peptide are released from cardiac muscle cells when they are stretched. ANP and BNP increase the excretion of sodium so that it decreases the blood volume.

Local Metabolic Regulation—Oxygen is a major regulator of microcirculation. Oxygen is not stored in the end organs and is replenished constantly from blood flow. Large changes in oxygen tension signal vascular muscles to relax or constrict. Endothelium-derived relaxing factor, or NO, works to increase in cGMP and the subsequent activation of intracellular signaling pathways to relax vascular smooth muscle cells. The endothelium-derived relaxing factor also suppresses platelet activation and reduces adhesion of leukocytes to endothelial cells.

VASCULAR SYSTEM TOXIC RESPONSES

Mechanisms of Vascular Toxicity

Responses of Vascular Endothelial Cells to Toxic Insults—NO rapidly reacts with superoxide to form peroxynitrite, which can form nitrotyrosine adducts. Substances mimicking agonists activate numerous types of receptors on the endothelial cells and trigger intracellular signaling

transduction, leading to activation of nuclear factor kappa-B (NF-κB) and MAPK activity. The downstream pathways triggered by NF-κB, MAPK, NO, and ROS then activate gene expression and regulate posttranslational modification of proteins leading to cytoprotective action against toxic insults, or the production of cytokines, chemokines, and adhesion molecules to protect the circulatory system and the affected organ systems.

Angiogenesis helps form new blood vessels that will deliver nutrients and oxygen to damaged tissue. Xenobiotics can both promote and suppress angiogenesis, and the primary target is the vascular endothelial cell. The mechanisms and molecular signaling pathways leading to apoptosis of vascular endothelial cells are basically the same as described for cardiomyocytes. Circulating bone marrow–derived endothelial progenitor cells accelerates re-endothelialization and limits atherosclerotic lesion formation.

Responses of Smooth Muscle Cells to Toxic Insults—Damage to vascular smooth muscle cells involves changes in vascular tone and atherosclerosis. Toxic substances influence calcium homeostasis in multiple ways impacting contractility. Targets of toxic agents include the calcium-binding proteins, the calcium homeostasis regulatory proteins, the calcium-activated proteins such as calcineurin, and the calcium storing and releasing process.

Proliferation and migration of medial smooth muscle cells are primarily responsible for the formation of sclerosis. These differentiated smooth muscle cells synthesize collagen and accumulate low-density lipoproteins along with a loss of filaments, as occurs in atherosclerosis.

Oxidative Stress and Vascular Injury—Many xenobiotics generate ROS during their biotransformation in endothelial and smooth muscle cells, producing oxidative injury. An increased ROS in endothelial cells is related to stimulation by angiotensin II, as well as other neurohormonal factors and cytokines. Xenobiotics can activate the enzyme system and lead to more extensive production of or inhibit the antioxidant system, resulting in accumulation of ROS in the cells.

Inflammatory Lesions—The initial injury to endothelial cells and the release of chemicals from the injured cells are responsible for the initiation of the inflammatory response, including recruitment of inflammatory cells to the injured site. Cytokines released from activated inflammatory cells propagate the inflammatory response leading to a lesion or vasculitis.

Toxic Responses of Blood Vessels

Hypertension and Hypotension—Hypertension results from excessive constriction of the arterial vasculature and/or increased resistance of the microcirculation system. However, the primary problem of sustained hypertension is an elevated vascular resistance in all organs. Once established, hypertension becomes a disease of the microvasculature. The vascular wall becomes hypertrophied. Additionally, all vascular smooth muscle cells become exceptionally responsive to norepinephrine. Toxic substances may directly or indirectly affect the sympathetic nervous system, alter the turnover of catecholamines in the circulation, or cause more complicated changes in the microcirculation, resulting in hypertension.

Baroreceptors, volume receptors, chemoreceptors, and pain receptors are all involved in the maintenance of adequate blood pressure. During chemical exposure, these mechanisms may be affected individually or jointly resulting in a disturbance in the integration of blood pressure

regulation.

Atherosclerosis—The most common vascular structural injury is atherosclerosis, causing mechanical occlusion of blood vessels such that blood flow is inadequate for the metabolic demands of the organs. Additionally, the intimal surface of atherosclerotic lesions has reduced capacity to generate antiplatelets and anticoagulant factors, providing a risk for adherent thrombi. Once stimulated, the vascular smooth muscle cells proliferate, differentiate into synthetic phenotype cells, and migrate to the lesion site. These synthetic cells increase production of type I and II collagen, dermatan sulfate, proteoglycan, and stromelysins. In addition, the smooth muscle cells produce cytokines including macrophage colony-stimulating factor, TNF-α, and monocyte chemoattractant protein-1. Recruitment of inflammatory cells to the lesion site perpetuates the process of atherosclerosis. Chemicals affecting oxidative metabolism of lipids in the circulatory system trigger the formation and development of the lipid core of atherosclerosis. Toxic effects on smooth muscle cells can also initiate the formation of atherosclerotic plaques, which may be different from the lipid core–mediated atherosclerosis. Macrophages and monocytes are also targets for xenobiotics and may be involved in the genesis of atherosclerosis.

Hemorrhage—A direct mechanical injury to blood vessels causes bleeding. Chemical-induced hemorrhages are seen when damage to capillaries or blood clotting cascade takes place.

Edema—The capillary exchange of fluid is bidirectional as capillaries and venules alter the balance of hydrostatic and colloid osmotic pressure. Filtration occurs most likely at the arteriolar end of capillaries, where filtration exceeds absorption. The absorption of water occurs in the venular end of the capillary and small venules. The capillary pressure is determined by the resistance of, and the blood pressure in, arterioles and venules. Xenobiotics can change the pressure gradient and cause more filtration than reabsorption of the extracellular liquid by the capillary system. The removal of excess fluid as well as plasma proteins that diffuse into the interstitial space occurs via the lymphatic system. Toxic insults to the lymphatic system can lead to elevated interstitial pressures and subsequent tissue edema.

VASCULAR SYSTEM TOXIC CHEMICALS

Blood vessels are the target organ of many chemicals. Also, vascular toxicity affects the organs in which the vessels are localized and is often accompanied with functional defects of the organ.

Pharmaceutical Chemicals

Sympathomimetic Amines—The sympathomimetic amines, including epinephrine, norepinephrine, dopamine, and isoproterenol, can damage the arterial vasculature by a variety of mechanisms. Large or repeated doses of norepinephrine produce toxic effects on the endothelium including degenerative changes, focal areas of unusual endothelial cytoarchitecture, and atherosclerotic lesions. Formation of arteriosclerotic lesions in certain forms of hypertension may be initiated and/or potentiated by high levels of circulating catecholamines.

Nicotine—At pharmacological concentrations, nicotine increases heart rate and blood pressure

as a result of stimulation of sympathetic ganglia and the adrenal medulla. Nicotine is a causative or aggravating factor in myocardial and cerebral infarction, gangrene, and aneurysm.

Cocaine—The central actions of cocaine are owing to increased circulating levels of catecholamines that cause a generalized state of vasoconstriction. Hypertension, coronary vasospasm, and cerebral strokes are common vascular complications. Cocaine enhances leukocyte migration across the cerebral vessel wall during inflammatory conditions.

Psychotropic Drugs—Postural hypotension is a common side effect of TCAs and anxiolytics. Along with drugs like cocaine, nicotine, and caffeine, psychotropic agents causing acute myocardial distress (angina, infarction, heart failure) may relate to a cluster of physiological responses that misbalance oxygen supply and demand to the heart.

Antineoplastic Drugs—The vasculotoxic responses elicited by antineoplastic drugs range from asymptomatic arterial lesions to thrombotic microangiopathy. Pulmonary venoocclusive disease has been reported after use of 5-fluorouracil, doxorubicin, and mitomycin. Cyclophosphamide causes cerebrovascular and viscerovascular lesions, resulting in hemorrhages.

Analgesics and Nonsteroidal Anti-inflammatory Drugs—Aspirin can produce endothelial damage. Regular use of phenacetin has been associated with an increased risk of hypertension and cardiovascular morbidity. NSAIDs may induce glomerular and vascular renal lesions.

Oral Contraceptives—Oral contraceptive steroids, especially high doses, can produce thromboembolic disorders and an increased risk of myocardial infarction, cerebral thrombosis, hemorrhage, venous thrombosis, and pulmonary embolism.

Natural Products

Bacterial Endotoxins—Bacterial endotoxins are potent toxicants that cause swelling of endothelial cells and adhesion of platelets to sinusoid walls. In the lung, endotoxins produce increased vascular permeability and pulmonary hypertension. The terminal phase of the effects of endotoxin on the systemic vasculature results in marked hypotension.

Homocysteine—Moderately elevated homocysteine levels are associated with atherosclerosis and venous thrombosis, cardiomyopathy and heart failure. Oxidative injury to vascular endothelial and/or smooth muscle cells may lead to deregulation of vascular smooth muscle growth, synthesis and deposition of matrix proteins, and adverse effects on anticoagulant systems.

Hydrazinobenzoic Acid—Hydrazinobenzoic acid present in the cultivated mushroom *Agaricusbisporus* causes smooth muscle cell tumors, vascular leiomyomas and leiomyosarcomas, in the aorta and large arteries of mice when administered over the life span of the animals. Smooth muscle cell lysis with vascular perforation apparently precedes malignant transformation.

T-2 Toxin—Trichothecene mycotoxins are naturally occurring cytotoxic metabolites of *Fusarium* species. These mycotoxins, including T-2 toxin, are major contaminants of foods and

animal feeds and may cause an initial decrease in heart rate and blood pressure, followed by tachycardia and hypertension and finally by bradycardia and hypotension. Acute T-2 toxin exposure causes extensive destruction of myocardial capillaries, while repeated dosing promotes thickening of large coronary arteries.

Vitamin D—Toxicity of vitamin D may result from its structural similarity to 25-hydroxycholesterol, a potent vascular toxin. Manifestations of vitamin D hypervitaminosis include medial degeneration, calcification of the coronary arteries, and smooth muscle cell proliferation in laboratory animals.

β-Amyloid—Accumulation of amyloid-β (Aβ) in the brain of Alzheimer's patients produces extensive vascular disruption, including endothelial and smooth muscle damage, adhesion, and migration of leukocytes across arteries and venules.

Uremic Toxins—Small molecule waste products known as uremic toxins build up in the circulation in patients with kidney disease. Among these metabolites, indoxyl sulfate, urea, cresyl sulfate, and acrolein have direct toxicity to vascular endothelial cells.

Environmental Pollutants and Industrial Chemicals

The environmental pollutants and industrial chemicals discussed in the cardiac toxicity section have toxic effects on the vascular system.

Carbon Monoxide—It is difficult to distinguish the direct effects of carbon monoxide from those of chemicals such as sulfur oxides, nitrogen oxides, aldehydes, and hydrocarbons on humans because most sources of carbon monoxide are complex mixtures of chemicals. Short-term exposure to carbon monoxide is associated with direct damage to vascular endothelial and smooth muscle cells. Injury to endothelial cells increases intimal permeability and allows the interaction of blood constituents with underlying components of the vascular wall. Carboxyhemoglobin decreases the oxygen-carrying capacity of blood, eventually leading to functional anemia. In addition, carbon monoxide interacts with myoglobin and cytochrome *c* oxidase and elicits a direct vasodilatory response of the coronary circulation.

Particulate Matter—Elevated levels of ambient particulate matter air pollution are associated with endothelial dysfunction, promotion of atherosclerotic lesions, and increased cardiovascular and respiratory morbidity and mortality. Vascular lesions lead to release or secretion of cytokines and chemokines, worsening cardiac complications.

1,3-Butadiene—1,3-Butadiene increases the incidence of cardiac hemangiosarcomas. Toxic effects of 1,3-butadiene depend on metabolic activation by cytochrome P450 to toxic epoxides. Outcomes of exposure may be influenced by GSH-mediated detoxification of oxidative metabolites.

Metals and Metalloids—The vascular toxicity of food- and water-borne elements (selenium, chromium, copper, zinc, cadmium, lead, and mercury) as well as airborne elements (vanadium and lead) involves reactions of metals with sulfhydryl, carboxyl, or phosphate groups. Metals such as cobalt, magnesium, manganese, nickel, cadmium, and lead also interact with and block

calcium channels. Intracellular calcium-binding proteins, such as CaM, are targets of heavy metals, including cadmium, mercury, and lead. Cadmium increases sodium retention, induces vasoconstriction, increases cardiac output, produces hyperreninemia and hypertensive effects. Lead is associated with essential hypertension in a large percentage of patients. Lead activates the renin–angiotensin–aldosterone system. Mercury produces vasoconstriction of preglomerular vessels and disrupts the integrity of the blood–brain barrier. Mercury added to platelet-rich plasma causes a marked increase in platelet thromboxane B_2 production and platelet responsiveness to arachidonic acid. Finally, arsenic poisoning causes vasodilation and capillary dilation resulting in extravasation, transudation of plasma, and decreased intravascular volume.

Aromatic Hydrocarbons—Aromatic hydrocarbons, including polycyclic aromatic hydrocarbons and polychlorinated dibenzo-*p*-dioxins, have been identified as vascular toxicants that can initiate and/or promote the atherogenic process in experimental animals. The atherogenic effect is associated with cytochrome P450–mediated conversion of the parent compound to toxic metabolic intermediates, but aromatic hydrocarbons can also initiate the atherogenic process.

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QUESTIONS

1. In which of the following locations would one NOT find spontaneous depolarization?
   - **a.** SA node.
   - **b.** myocardium.
   - **c.** AV node.
   - **d.** bundle of His.
   - **e.** Purkinje fibers.

2. Which of the following scenarios would increase contractility of the myocardium?
   - **a.** increased activity of the Na^+/K^+-ATPase.
   - **b.** increased activity of sacroplasmic reticulum Ca^{2+} ATPase.
   - **c.** decreased activity of sacroplasmic reticulum Ca^{2+} ATPase.
   - **d.** decreased intracellular calcium levels.
   - **e.** increased intracellular K^+ levels.

3. All of the following statements regarding abnormal cardiac function are true EXCEPT:
   - **a.** Ventricular arrhythmias are generally more severe than atrial arrhythmias.
   - **b.** Ventricular hypertrophy is a common cause of ventricular arrhythmias.
   - **c.** Coronary artery atherosclerosis is a major cause of ischemic heart disease.
   - **d.** Right-sided heart failure results in pulmonary edema.
   - **e.** Tachycardia is classified as a rapid resting heart rate (>100 beats/min).

4. Ion balance is very important in maintaining a normal cardiac rhyth**m.** Which of the

following statements is TRUE?

a. Blockade of K^+ channels decreases the duration of the action potential.
b. Blockade of Ca^{2+} channels has a positive inotropic effect.
c. Inhibition of Na^+ channels increases conduction velocity.
d. Blockage of the Na^+/K^+-ATPase increases contractility.
e. Calcium is transported into the cell via a Ca^{2+}-ATPase.

5. Which of the following is most likely NOT a cause of myocardial reperfusion injury?
a. cellular pH fluctuations.
b. damage to the sarcolemma.
c. generation of toxic oxygen radicals.
d. Ca^{2+} overload.
e. inhibition of the electron transport chain.

6. Which of the following statements regarding the cardiotoxic manifestations of ethanol consumption is FALSE?
a. Acute ethanol toxicity causes decreased conductivity.
b. Chronic alcohol consumption is associated with arrhythmias.
c. Acute ethanol toxicity causes an increased threshold for ventricular fibrillation.
d. Chronic ethanol toxicity can result in cardiomyopathy.
e. Acetaldehyde is a mediator of cardiotoxicity.

7. Cardiac glycosides:
a. increase the activity of the Na^+/K^+-ATPase.
b. make the resting membrane potential more negative.
c. can have sympathomimetic and parasympathomimetic effects.
d. decrease ventricular contractility.
e. increase AV conduction.

8. Which of the following is NOT a common cardiotoxic manifestation of cocaine abuse?
a. parasympathomimetic effects.
b. myocardial infarction.
c. cardiac myocyte death.
d. ventricular fibrillation.
e. ischemia.

9. Using high doses of anabolic-androgenic steroids is NOT likely associated with which of the following?
a. an increase in LDL.
b. cardiac hypertrophy.
c. myocardial infarction.
d. increased nitric oxide synthase expression.
e. a decrease in HDL.

10. Which of the following is NOT a common mechanism of vascular toxicity?
    a. membrane disruption.
    b. oxidative stress.
    c. bioactivation of protoxicants.
    d. reduction and accumulation of LDL in endothelium.
    e. accumulation of toxin in vascular cells.

CHAPTER 19

Toxic Responses of the Skin

Donald V. Belsito

KEY POINTS

- Skin participates directly in thermal, electrolyte, hormonal, metabolic, and immune regulation.
- Percutaneous absorption depends on the xenobiotic's hydrophobicity, which affects its ability to partition into epidermal lipid, and rate of diffusion through this barrier.
- Cells of the epidermis and pilosebaceous units express biotransformation enzymes.
- Irritant dermatitis is a nonimmune-related response caused by the direct action of a chemical on the skin.
- Allergic contact dermatitis represents a delayed (type IV) hypersensitivity reaction, whereby minute quantities of material elicit overt reactions.

SKIN AS A BARRIER

A large (10% of body mass; 1.5-2 m^2) and highly accessible organ, the skin protects the body against external insults, thus maintaining homeostasis. Physiologically, the skin participates directly in thermal, electrolyte, hormonal, metabolic, antimicrobial, and immune regulation. Rather than merely repelling noxious agents, the skin may react to them with various defensive mechanisms preventing widespread cutaneous and/or internal injuries. If an insult is severe or sufficiently intense to overwhelm the protective function of the skin, acute or chronic injury becomes manifest. The specific presentation depends upon a variety of intrinsic and extrinsic

factors, including body site, duration of exposure, other environmental conditions, and the physicochemical properties of the insult (Table 19–1).

TABLE 19–1 Factors Influencing Cutaneous Responses

Variable	Comment
Body site	
Palms/soles	Thick stratum corneum—good physical barrier
	Common site of contact with chemicals
	Occlusion with protective equipment
Intertriginous areas (axillae, groin, neck, finger webs, umbilicus, and genitalia)	Moist, occluded areas
	Chemical trapping
	Enhanced percutaneous absorption
Face	Exposed frequently
	Surface lipid interacts with hydrophobic substances
	Chemicals frequently transferred from hands
Eyelids	Poor barrier function—thin epidermis
	Sensitive to irritants/allergens
Postauricular area	Chemical trapping
	Occlusion
Scalp	Chemical trapping
	Hair follicles susceptible to metabolic damage
Predisposing cutaneous illnesses	
Atopic dermatitis	Increased sensitivity to irritants
	Impaired barrier function
Psoriasis	Impaired barrier function
Genetic factors	Predisposition to skin disorders
	Variation in sensitivity to irritants
	Susceptibility to contact sensitization
Temperature	Vasodilation—improved percutaneous absorption
	Increased sweating—trapping
Humidity	Increased sweating—trapping
Season	Variation in relative humidity
	Chapping and wind-related skin changes

Skin Histology

The skin consists of two major components, the outer epidermis and the underlying dermis, which are separated by a basement membrane (Fig. 19–1). The junction ordinarily is not flat, but undulating (rete ridges). In addition, epidermal appendages (hair follicles, sebaceous glands, and eccrine glands) span the epidermis and embed into the dermis. In thickness, the dermis comprises approximately 90% of the skin, and largely has a supportive function. A high content of collagen and elastin provide skin resilience and elasticity. Separating the dermis from underlying tissues is a layer of adipocytes, whose accumulation of lipids has a cushioning and thermoregulatory action. The blood supply to the epidermis originates in capillaries in the rete ridges at the dermoepidermal junction. Capillaries also supply the bulbs of the hair follicles and the secretory cells of the sebaceous and eccrine glands. The ducts from the eccrine glands carry a dilute salt solution to the surface of the skin, where evaporation provides thermoregulation. The ducts of the sebaceous gland provide a protective oil covering to the skin.

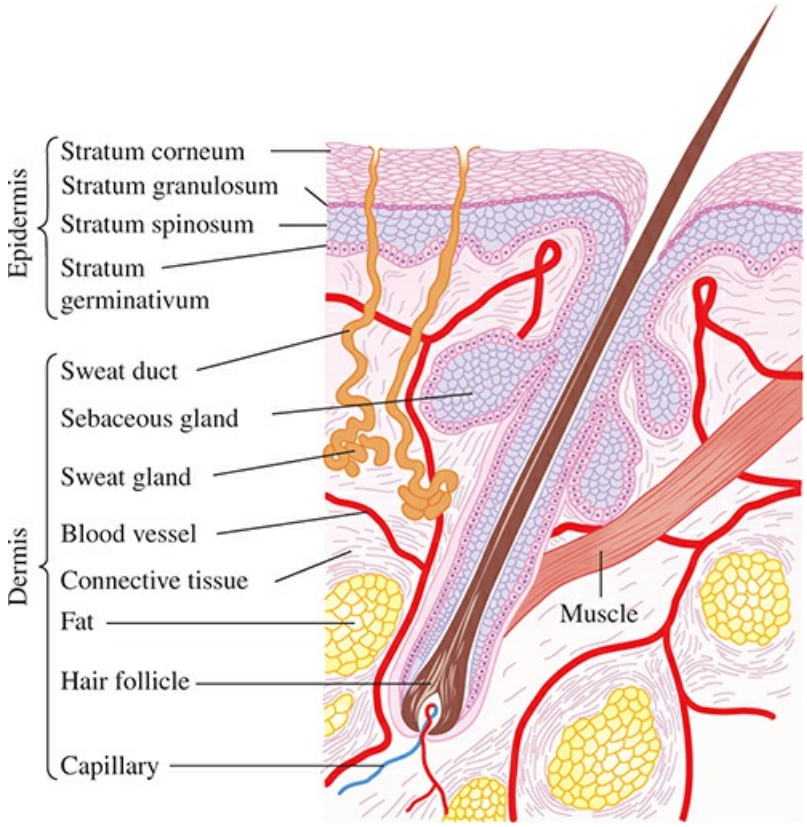

FIGURE 19–1 *Diagram of a cross-section of human skin.* The epidermis and pilosebaceous units are shown in pink and purple.

The interfollicular epidermis is stratified squamous epithelium consisting primarily of keratinocytes. These cells are tightly attached to each other by desmosomes and to the basement membrane by hemidesmosomes. Melanocytes, whose melanin granules give the skin its color, are interspersed among the basal cells and distributed in the papillae of hair follicles. Migrating through the epidermis are numerous Langerhans cells, which are dendritic, antigen presenting cells involved in the immune response of skin to foreign xenobiotics.

The cellular composition of the epidermis is dominated by the keratinocyte, which comprises the basal layer of the stratum germinativum. When a basal cell divides, one of the progeny detaches from the basal lamina and migrates upwards. As cells move toward the skin surface, they undergo programmed terminal differentiation, gradually expressing new protein markers, and accumulating keratin proteins. These keratins form insoluble intermediate filaments, which account for nearly 40% of the total cellular protein in the spinous layer of the epidermis. At the granular layer of the epidermis, keratinocytes undergo a striking morphological transformation, becoming flattened and increasing in volume nearly 40-fold. Lipid granules fuse with the plasma membrane at the granular layer/stratum corneum interface, filling intercellular spaces of the stratum corneum with lipid and replacing the aqueous intercellular environment of the underlying viable epidermis. These surface lipids prevent diffusion of water and ions out of the body and access of toxicants and bacteria into the skin. Meanwhile, the plasma membranes of the now moribund keratinocytes become permeable, lose their reducing environment, and undergo extensive disulfide bonding of the keratin proteins. Cell organelles are degraded, while a protein envelope is synthesized immediately beneath the plasma membrane. These membrane alterations are characterized by loss of phospholipids and addition of sphingolipids.

This programmed terminal differentiation and cell death, beginning as keratinocytes leave the basal layer, produces the outermost chemicals layer of the skin, the stratum corneum. No longer viable, the mature cells (called corneocytes) are approximately 80% keratin in content. They are gradually shed from the surface and replaced from beneath. Epidermal turnover typically takes 28 days: 2 weeks for basal cells to reach the stratum corneum, and another 2 weeks to be shed from the surface.

Percutaneous Absorption

The stratum corneum has been recognized as the primary barrier. Diseases (e.g., atopic dermatitis) or other conditions (e.g., abrasions/wounding) in which this barrier is compromised can permit greatly increased uptake of poorly permeable substances. The viable layer of epidermis provides a much less protective barrier than does the stratum corneum, because hydrophilic chemicals can readily diffuse into the intercellular water, while hydrophobic chemicals can partition into the cell membranes, and each can gain access to the blood supply in the rete ridges of the dermis.

The stratum corneum prevents water loss from underlying tissues by evaporation. Its hydrophobic characteristics result from the lipid content of its intercellular spaces. The lipids, a major component being sphingolipids, have a high content of long chain ceramides, removal of which seriously compromises barrier function as measured by transepidermal water loss. The stratum corneum is hydrated (approximately 20% water), the moisture residing in its protein content. However, the stratum corneum can take up considerable amounts of water upon prolonged immersion, thereby reducing the effectiveness of the barrier to hydrophilic chemicals. Indeed, occlusion of the skin with impermeable wraps, permitting the retention of perspiration underneath, is employed to enhance uptake of chemicals, particularly medications, applied to the skin’s surface. Penetration from air is generally too low to be of concern.

The rate of xenobiotic uptake through the skin facilitates estimating the consequences of environmental exposure. Dermal uptake is incorporated into pharmacokinetic modeling to estimate potential risks. The degree of uptake depends upon exposure conditions and is typically proportional to solute concentration (assuming it has been diluted), time, and the area of the

exposed skin surface. Intrinsic factors that contribute to the absorption rate of a given compound include its hydrophobicity, which affects its ability to partition into epidermal lipid, and its rate of diffusion through the corneal barrier. A measure of hydrophobicity is the commonly used octanol/water partitioning ratio (K_{ow}). However, partitioning of a chemical into the skin is greatly affected by its solubility in, or adhesion to, the medium in which it is contained (including soil). Similarly, hydrophobic compounds, once in the stratum corneum, may diffuse only very slowly into the less hydrophobic regions below. Also, hydrophobic agents of low molecular weight permeate the skin better than those of high-molecular-weight, or low-molecular-weight hydrophilic. For small molecules, hydrophobicity is the dominant factor in penetration.

Considerable empirical information has been collected on some compounds (pharmaceuticals, pesticides, and pollutants) to allow for in silico quantification of penetration based upon structural/activity relationships. Mathematical formulas to estimate skin penetration (P_{cw}) have been put forward using empirically derived constants (C_1, C_2, C_3):

$$\log P_{cw} = C_1 - C_2\,(\mathrm{MW}) + C_3 \log K_{ow}$$

Such relations describe steady-state conditions, in which a chemical exits the stratum corneum at the same rate as it enters. Because rates of transfer of various hydrophobic chemicals into the aqueous phase of the spinous layer are slow, the stratum corneum can provide a depot leading to continued penetration into the body for relatively long periods after external exposure has stopped.

Diffusion through the epidermis is considerably faster at some anatomical sites than others. A list in order of increasing permeability gives the following: foot/sole, palm, forehead, and abdomen. Scrotal skin has the highest permeability for most topical chemicals. Absorption through the epidermal appendages is usually neglected, despite the ability of chemicals to bypass the stratum corneum by this route, because the combined appendageal surface area is a relatively small fraction of the total available area for uptake. However, if loading of the stratum corneum is slow, penetration through the appendages can constitute an appreciable fraction of the total amount following short exposures.

Transdermal Drug Delivery—Eloquently designed patches are currently used to deliver an increasing number of different drugs (including clonidine, estradiol, fentanyl, testosterone, nitroglycerin, scopolamine, nicotine, etc.) for therapeutic purposes. The advantage of the transdermal approach over oral dosing includes (a) providing a steady infusion of drug over extended periods, thereby avoiding large variations in plasma concentrations; (b) preventing exposure to the acidic pH of the stomach, thus preventing biodegradation; and (c) avoiding biotransformation in the gastrointestinal tract from first pass removal by the liver. The contrast in plasma concentration kinetics between different methods of delivery is evident for chemicals that are rapidly metabolized, such as nitroglycerin, which has a half-life of minutes. In addition, encapsulation of drug into small vesicles of phospholipids/nonionic surfactants (micelles) and nanoparticle technologies can improve delivery through the epidermis and hair follicles. Microneedle systems have been used to deliver drugs (esp. insulin), vaccines, and gene therapy across the skin.

Measurements of Penetration—A pharmacokinetic approach to penetration has used experimental animals. To simplify determination of penetration kinetics, skin flaps have been

employed and the capillary blood flow monitored. Pig skin is useful. Penetration through rodent skin is usually faster than through human skin, providing an overestimate of penetration. Human skin grafts onto athymic mice minimize species differences, because human skin survives well on athymic mice and retains its normal barrier properties. In pharmaceutical studies, volunteers are dosed, plasma and/or urine concentrations are quantified at suitable intervals, and amounts excreted from the body are estimated. Key factors such as xenobiotic permeability, octanol/water partition coefficient (K_{ow}), the partition coefficient (Log P), acid dissociation constant (pK_a), and molecular weight are being used for in silico methods.

Biotransformation—The ability of the skin to metabolize chemicals that diffuse through it contributes to its barrier function. Phase I metabolism in skin usually is only a small fraction (approximately 3%) of that in the liver, but it is capable of affecting the outcome of exposure. A significant fraction of the pharmaceuticals used in clinical dermatology are cytochrome P450 inducers, inhibitors, or substrates. Additional phase I enzymes expressed in the skin include flavin-dependent monooxygenases, aldehyde dehydrogenases, carboxylesterases, and glutathione peroxidases. Phase II enzyme families expressed in skin include UDP glucuronosyltransferase, sulfotransferase, and glutathione *S*-transferase. Other enzymes include sulfatases, β-glucuronidase, *N*-acetyl transferases, esterases, and reductases. The intercellular region of the stratum corneum has catabolic activities, e.g., protease, lipase, glycosidase, and phosphatase.

CONTACT DERMATITIS

Overall, contact dermatitis falls into two major categories, irritant and allergic. Both involve inflammatory processes and can have indistinguishable clinical characteristics of erythema (redness), induration (thickening and firmness), scaling (flaking), and vesiculation (blistering) in areas of direct contact with the chemical. Allergic and chronic cumulative irritant contact dermatitis may coexist. Figure 19–2 shows examples of many types of contact dermatitis resulting from occupational exposures.

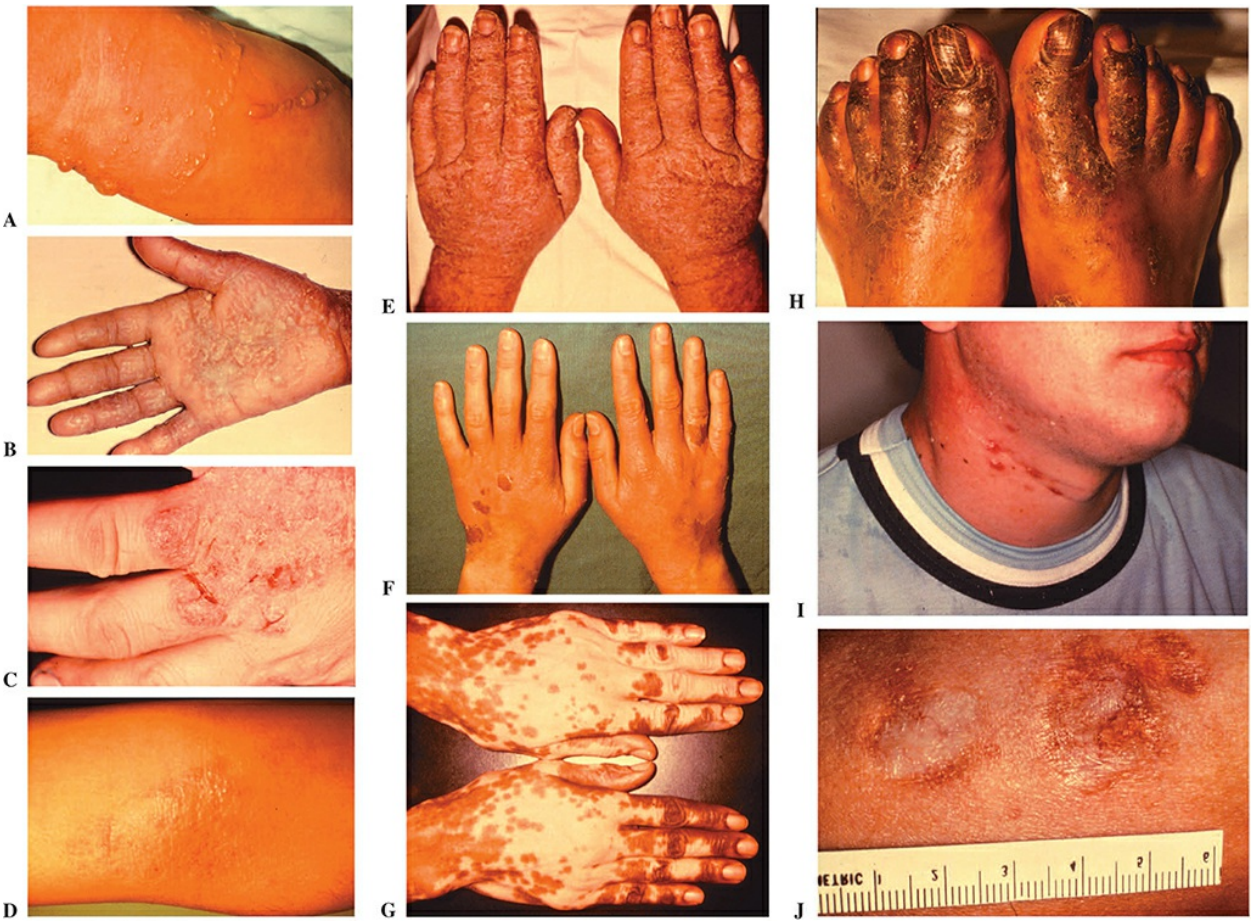

FIGURE 19–2 *Examples of occupational skin toxicity.* (**A**) Burn from ethylene oxide; (**B**) Burn from alkali exposure; (**C**) chronic cumulative irritant contact dermatitis from cutting oil; (**D**) atopic dermatitis; (**E**) allergic contact dermatitis to dichromate; (**F**) phototoxicity from lime juice; (**G**) leukoderma from rubber antioxidants; (**H**) post-inflammatory hyperpigmentation following allergic contact dermatitis due to mercaptobenzothiazole; (**I**) acne from cutting oil; and (**J**) beryllium granulomas. (Images from The National Institute for Occupational Safety and Health (NIOSH) website (https://www.cdc.gov/niosh/index.htm).niosh/ocderm1.html.)

Irritant Dermatitis

Accounting for nearly 80% of all contact dermatitis, irritant dermatitis arises from the direct action of agents on the skin. A chemical in this category is anticipated to give an adverse reaction to anyone if the concentration is high enough, the amount supplied sufficient, and the exposure conditions long enough. Irritant dermatitis can be broadly subcategorized as corrosive (chemical burns), acute, and chronic cumulative irritation.

Information on the irritancy of chemicals toward human skin has traditionally been obtained using laboratory animals (mice, rats, rabbits, and guinea pigs). The prototypical methodology has been Draize testing for irritation. After 24 hours of exposure, the skin is scored for erythema and eschar formation and for edema. For development of new cosmetics, particularly in the European Union, a great need exists for an in vitro system to determine the potential for irritant responses

because animal tests are prohibited for cosmetic products and for cosmetic ingredients.

Chemical Burns (Corrosion)—A chemical burn produces immediate coagulative necrosis resulting in considerable tissue damage with ulceration and sloughing. Also referred to as a severe second- or third-degree burn, it does not have a primary inflammatory component. Examples of such include burns from strong acids and alkalis, ethylene oxide, and other severe dermatotoxicants. Examples are shown in Figs. 19–2A and B. If the chemical is not quickly and completely removed, damage to the skin may continue and, with increased access to the circulation, systemic injury can occur. Table 19–2 lists some important corrosive agents found in the occupational setting.

TABLE 19–2 Selected Chemicals Causing Skin Burns

Chemical	Comment
Ammonia	Potent skin corrosive Contact with compressed gas can cause frostbite
Calcium oxide (CaO)	Severe chemical burns Extremely exothermic reaction—dissolving in water can cause heat burns
Chlorine	Liquid and concentrated vapors cause cell death and ulceration
Ethylene oxide	Solutions and vapors may burn Compressed gas can cause frostbite
Hydrogen chloride (HCl)	Severe burning with scar formation
Hydrogen fluoride (HF)	Severe, painful, slowly healing burns from high concentration Lower concentration causes delayed cutaneous injury Systemic absorption can lead to electrolyte abnormalities and death Calcium-containing topical medications and quaternary ammonium compounds are used to limit damage
Hydrogen peroxide	High concentration causes severe burns and blistering
Methyl bromide	Liquid exposure produces blistering and deep burns
Nitrogen oxides	Moist skin facilitates the formation of nitric acid causing severe yellow-colored burns
Phosphorus	White phosphorus continues to burn on skin in the presence of air
Phenol	Extremely corrosive even in low concentrations Systemic absorption through burn sites may result in cardiac arrhythmias, renal disease, and death
Sodium hydroxide	High concentration causes deep burns, readily denatures keratin
Toluene diisocyanate	Severe burns with contact Contact rarely may also result in respiratory and/or skin sensitization

Given its severity, in vitro models for corrosion were considered essential given the EU's stance on animal testing. Validated in vitro alternatives included in the EU regulations for skin corrosion testing are the TER (rat skin transcutaneous electrical resistance test; OECD TG 430) and tests on reconstructed human epidermis (EpiSkin™, EpiDerm™, SkinEthic™, and epiCS®).

Acute Irritation—Certain chemicals at sufficient concentration can produce an acute irritant response, sometimes called a first degree or mild second degree chemical burn. These acute irritants include acid, alkalis, and oxidizing or reducing chemicals that substantially disrupt the cornified layer producing cytotoxicity directly.

Chronic Cumulative Irritant Dermatitis—This most common irritation arises from repeated exposures to mild irritants such as soaps, detergents, solvents, and cutting oils. An example from cutting oil is shown in Fig. 19–2C. Currently, there is no in vitro test to predict chronic cumulative irritation. This typically has been done using human volunteers in what has been termed the Human Repeat Insult Patch Test.

In addition to chemical irritants, chronic friction and trauma can mechanically damage the stratum corneum, leading to further loss of its cohesiveness. As a result, the penetration of exogenous chemicals is enhanced at sites of barrier damage.

The skin at some anatomic sites is more easily irritated than at other sites, which can be demonstrated by measurement of transepidermal water loss. The eyelids have a thin epidermis and are quite sensitive. The back is more sensitive than the forearm. Individuals can vary greatly in their sensitivity to irritant dermatitis, especially the chronic cumulative variety.

Patients with atopic dermatitis (Fig. 19–2D) are especially prone to irritation. Atopic individuals are more sensitive to irritants, exhibit a propensity to produce specific IgE antibodies to allergens, and typically suffer from hay fever and allergic asthma. Atopic dermatitis has a strong genetic component. In many atopics, defects in the intermediate filament aggregating protein, filaggrin, among other defective genetic barrier genes, are detectable.

Allergic Contact Dermatitis

Allergic contact dermatitis (ACD) is a delayed (T-cell mediated) hypersensitivity reaction (Gell and Coombs Type IV immune response). To induce sensitization through the skin, chemical haptens generally penetrate the lipid barrier and attach themselves to carrier proteins. The complete antigens are processed by Langerhans cells (epidermal dendritic cells) and displayed on their surfaces with major histocompatibility complex II molecules. Following migration to the paracortical area of the draining lymph nodes, the Langerhans cells present the processed peptide to naive T-helper cells (Th_0) with specific receptors for the complexed allergen. This antigen presentation leads to the release of a variety of interferons, growth factors, and chemokines, which result in a proliferation of a specific clone of sensitized Th_1. This phase of the response is termed the induction, during which, over a 1- to 3-week period, memory T-cells are generated and enter the circulation.

Upon subsequent exposure to a specific antigen previously recognized by the immune system, a more vigorous and rapid response occurs due to homing of specific memory T cells to the skin, where they release cytokines and chemokines that attract other nonspecific inflammatory cells. This phase of the immune response is termed elicitation, which may develop within hours to

several days following antigenic exposure. The resultant epidermal spongiosis (intercellular edema) and perivascular dermal infiltrate of lymphohistiocytic cells is characteristic, but not pathognomonic, of ACD.

Thousands of chemicals have been reported to cause allergic contact dermatitis. Table 19–3 lists some common contact allergens. Because most chemicals are only weakly active or infrequently encountered, much effort has focused on finding the major allergens in the population by systematic patch testing of patients presenting with suspected allergic contact dermatitis.

TABLE 19–3 Common Contact Allergens

Source	Common Allergens	
	Antibiotics	**Therapeutics**
Topical medications	Bacitracin Neomycin Polymyxin Aminoglycosides Sulfonamides	Benzocaine Corticosteroids
	Preservatives	**Others**
Personal care products	Formaldehyde Formaldehyde releasers • Quaternium-15 • Imidazolidinyl urea • Diazolidinyl urea • DMDM hydantoin • 2-bromo-2-nitropropane-1,3-diol Methylchloroisothiazolone/Methylisothiazolinone (MCI/MI)	Ethylenediamine Lanolin *p*-Phenylenediamine Propylene glycol Fragrances
Plants and trees	Mixtures: • Balsam of Peru • Rosin (colophony)	Defined chemical structure: • Pentadecylcatechols (poison ivy/oak/sumac) • Sesquiterpene lactones • Tuliposide A
Antiseptics	Chlorhexidine Chloroxylenol Dichlorophene Glutaraldehyde	Hexachlorophene Thimerosal (Merthiolate) Mercurials Triphenylmethane dyes
Rubber products	Diphenylguanidine Hydroquinone Mercaptobenzothiazole *p*-Phenylenediamine derivatives	Resorcinol monobenzoate Benzothiazolesulfenamides Dithiocarbamates Thiurams
Leather	Formaldehyde Glutaraldehyde	Hexavalent chromium Cobalt
Glues and bonding agents	Bisphenol A/Epichlorohydrin Phenol formaldehyde resins Acrylic monomers Cyanoacrylates	Epoxy resins Toluene sulfonamide resins Urea formaldehyde resins
Metals	Chromium Cobalt	Gold Nickel

The chemicals chosen for patch testing vary with the geographic location to account for local exposures. Additionally, the choice may be directed to specific anatomic sites, such as the foot. Panels may also be adapted to detect emerging trends, as new products appear and others decline in use. An example of contact allergy to hexavalent chromium in cement is shown in Fig. 19–2E.

Sensitization to ingredients in topical preparations is a common problem and one that changes as formulations evolve. As with other consumer products, reduction in use of the most prevalent allergenic chemicals, and their replacement by less allergenic substitutes in topicals, is advocated. Caution in using less characterized chemicals as a replacement must be exercised, because their allergenicity may not become evident until they reach large populations of users.

Immunoregulation—The route of primary sensitization has a profound effect on the subsequent immunologic response. Aged individuals have been shown to have various defects in the induction and/or elicitation of ACD. The precise reason is unknown and many aspects of cell-mediated responses are likely involved. Competency of immune responses in children is also controversial. The effects of gender on the incidence of ACD seem related to the likelihood of exposure.

In experimental models and clinical practice, downregulation of ACD has been achieved with ultraviolet radiation (UVB or psoralen + UVA (PUVA), glucocorticoids, and calcineurin inhibitors. Each of these acts somewhat differently. UVB and PUVA significantly decrease the density of epidermal Langerhans cells (LCs), induce epidermal hyperplasia (making it more difficult for the antigen to reach any remaining epidermal antigen-presenting cells), upregulate IL-10, and induce CD4+/CD25+ regulatory T cells. Glucocorticoids also inhibit the type IV reaction at multiple points: epidermal LCs are decreased in number, the production and function of IL-1, IL-2, and IFN-γ are inhibited, and T cell proliferation is decreased. In contrast, cyclosporine A and other macrolactams act primarily to inhibit activation of cNF-AT via inhibition of calcineurin.

Diagnosis and Testing— Patch testing is the gold standard for determining what chemical is responsible for causing allergic contact dermatitis. On the washed back of patients who are not exhibiting dermatitis at the sites to be tested, nor using corticosteroids or other immunosuppressive treatments, patches are placed that contain a small amount of the putative allergen. After 2 days, during which time a reaction begins to develop, the patches are removed and the sites of exposure are scored for degree of response. These sites are additionally re-evaluated at any time from 3 to 7 days after the initial application. The relevance of any positive reaction to the patient's actual environment must be considered so that exposure to putative allergens in daily life can be minimized to appropriate chemicals. Interpretation of the results, and resulting modifications in lifestyle, should consider cross-sensitivity, where reactivity to a compound may occur because it shares functional groups that have provoked sensitization with another compound. Table 19–4 lists some common cross-reacting chemicals.

TABLE 19–4 Common Cross-Reacting Chemicals

Chemical	Cross Reactor(s)
Balsam of Peru	Pine resin, cinnamates, benzoates
Bisphenol A	Diethylstilbestrol, monobenzyl ether of hydroquinone
Canaga oil	Benzyl salicylate
Chlorocresol	Chloroxylenol
Diazolidinyl urea	Imidazolidinyl urea
Hydroquinone	Resorcinol
Alkyl hydroxybenzoates	Parabens, hydroquinone, monobenzyl ether of hydroquinone
p-Aminobenzoic acid	*p*-Aminosalicylic acid, sulfonamide
Para-phenylenediamine	*p*-Aminobenzoic acid, aminobenzoate anesthetics
Propyl hydroxybenzoate	Hydroquinone monobenzyl ether
Phenol	Resorcinol, cresols, hydroquinone

In animal testing, a chemical is injected intradermally with or without Freund's complete adjuvant. The reaction of the skin to subsequent challenge with a chemical can then be observed and graded. While this approach has been successful, the extrapolation of sensitivity measurements from laboratory animals to humans presented large uncertainties. Therefore, the local lymph node assay (LLNA), performed in mice, has become the predominant in vivo animal tool to measure the sensitization capacity of a specific chemical. The LLNA also has the capability to rank the potency of allergens from very strong to weak.

There are four key biological components in the "adverse outcome pathway" (AOP) for skin sensitization: (1) the molecular initiating event; (2) activation of keratinocytes as detected by gene expression associated with specific cell signaling pathways and inflammatory responses; (3) dendritic cell activation as determined by specific expression of cell surface markers; and (4) T-cell proliferation. Assessment of only one aspect of the AOP will not provide sufficient information to replace the use of animals for the assessment of chemical allergenicity. Non-testing methods (in silico data, analogue based "read across" information) and in vitro approaches that evaluate the key events in the AOP will be needed.

Four assays have been developed to address the key events in the sensitization AOP:(1) The Direct Peptide Reactivity Assay or "DPRA" measures the ability of a chemical to react with peptides, via histidine and cysteine residues. (2) The KeratinoSens™ assay assesses the sensitizing potential of a chemical using immortalized adherent human keratinocytes (HaCaT cell-line) transfected with a plasmid to quantify luciferase gene induction as a measure of activation of Keap1-NRF2-antioxidant/electrophile response element (ARE)-1. (3) Dendritic cell activation can be determined using either the MUSST (myeloid U937 skin sensitization test) or h-CLAT (human cell line activation test) methodologies. (4) Currently there is no validated methodology to assess the fourth component of the AOP, T-cell activation.

Currently, the Weight of Evidence or the best "two out of three" approach in assessing sensitization based upon the testing of the first three key events in the AOP has found the greatest success. Chemicals are identified as non-sensitizers if they are negative in both the DPRA and either the KeratinoSens™ assay or the MUSST/h-CLAT assays. However, these current approaches have drawbacks. Pre-haptens (abiotically transformed materials, e.g., oxidized in the environment) and pro-haptens (biotically transformed materials via metabolism in the skin) need to be detected. The peroxidase peptide reactivity assay "PPRA" looks at identification of pro-haptens via the use of peroxidase in the DPRA.

PHOTOSENSITIVITY

The ultraviolet and visible spectra of solar radiation that reach the earth's atmosphere extend from 290 to 700 nm. Wavelengths beyond this range are either filtered by the earth's atmosphere or are insufficiently energetic to cause cutaneous pathology. Adequate doses of artificially produced UVC (100 to 289 nm) or x-rays (less than 10 nm) can produce profound physical and toxicologic skin changes. The protective skin pigment, melanin, synthesized by the melanocytes, absorbs a broad range of radiation from ultraviolet B (290 to 320 nm) through the visible spectrum (400 to 700 nm). Other chromophores in the skin include amino acids, particularly tryptophan and tyrosine, and their breakdown products (e.g., urocanic acid), which absorb light in the UVB range. Biologically, the most significant chromophore in the skin is DNA.

Phototoxicity

Phototoxic reactions from exogenous chemicals may be produced either by systemic or topical exposures. In acute reactions, the skin can become red and blistered within minutes to hours following ultraviolet light exposure, typically UVA (320 to 400 nm). Occupational examples include coal tar, which, in combination with sunlight, can produce an immediate stinging sensation referred to as "tar smarts." Over time, significant hyperpigmentation of the skin occurs.

Phototoxic chemicals most commonly absorb in the UVA range, thereby assuming a higher energy, excited, triplet state; the subsequent transfer of electrons to other molecules or reduction to more highly reactive free radicals results in the observed tissue damage. Oxygen dependent photodynamic reactions commonly occur as these excited molecules return to their ground state and transfer their energy to oxygen, forming highly reactive singlet oxygen. These reactive products then damage cellular macromolecules, especially unsaturated membrane lipids, which results in cell death. The release of immune mediators from dying keratinocytes and the recruitment of inflammatory cells to the skin are histopathologic signs of phototoxicity. Chemicals most often associated with phototoxic reactions are listed in Table 19–5.

TABLE 19–5 Selected Phototoxic Chemicals

Furocoumarins
8-Methoxypsoralen
5-Methoxypsoralen
Trimethoxypsoralen
Polycyclic aromatic hydrocarbons
Anthracene
Fluoranthene
Acridine
Phenanthrene
Pyridine
Drugs
Amiodarone
Tetracyclines
Sulfonamides
Sulfonylureas
Nalidixic acid
Thiazides
Phenothiazines
Nonsteroidal anti-inflammatories
Dyes
Anthraquinones
Eosin
Acridine orange
Rose Bengal
Porphyrin derivatives
Hematoporphyrin

Psoralens (furocoumarins) are an example of chemicals producing phototoxicity without production of singlet oxygen. Psoralens intercalate within DNA to form covalent adducts, and cross-link when activated by UVA. The result is inhibition of DNA synthesis and repair causing

the cells to die. Psoralens can be found in various foods, including lime and celery. Phototoxicity from furocoumarin present in lime peel is shown in Fig. 19–2F.

Nails, an appendage of the skin, may also suffer phototoxic reactions to drugs and, less commonly, to topically applied chemicals. While various types of nail pathology may result, including changes in cell growth and pigmentation, detachment of the various layers of the nail plate from each other (onycholysis) is the commonly observed effect of phototoxicity. Agents such as coal tar and tetracyclines produce photodynamic onycholysis, via generation of oxygen radicals. In contrast, topical or systemic psoralens produce onycholysis by a non-photodynamic mechanism that does not require oxygen.

Photoallergy

Most photoallergens respond to light in the UVA range (Table 19–6). Except for the need for photoactivation of the allergen, photoallergy is a typical delayed type IV hypersensitivity response. The reaction may take hours to days to develop and can persist for several weeks following exposure if the agent is retained within the skin. Photoallergy may result from exposure to a concentration too low to produce a phototoxic irritant response, but sufficient to elicit photoallergy.

TABLE 19–6 Selected Photoallergens

Halogenated salicylanilides
Tetrachlorosalicylanilide
Dibromosalicylanilide
Trichlorocarbanilide
Bithionol
Whiteners
Stilbene
Fragrances
Musk ambrette
6-methyl coumarin
Drugs
Ketoprofen
Phenothiazines
Chlorpromazine
Promethazine
Fentichlor
Sunscreens
PABA esters
Benzophenones
Octocrylenes

The diagnosis of photoallergy is best confirmed by photopatch testing, which includes exposure of the allergens to light and a control of nonirradiated allergens. Because the offending agent may not be obvious from the patient's history due to the delay between exposure to the agent and sunlight, a standard panel of photoallergens, as well as the patient's own sunscreen(s) and personal care products containing potentially photoactivated materials, are tested.

In Vitro Determination of Phototoxicity and Photoallergenicity

One way to assess the ability of a material to cause a phototoxic or photoallergic reaction is its ultraviolet absorption spectrum. If absorption is below the molar extinction coefficient of less than 1000 L × mol^{-1}× cm^{-1}, then the material has little to no phototoxic or photosensitization hazard. If absorption occurs, then phototoxicity and photosensitization are both possible. Other tests include the reactive oxygen species (ROS) assay, the 3T3 Neutral Red Uptake in vitro

phototoxicity test, the photo-exposed “3D skin models,” and a fluorometric ROS assay.

The development of in vitro systems to assess photosensitization is critical, since testing for photoallergenicity in humans is considered unethical because of the potential for the induction of persistent light reactivity, in which the individual remains extremely sensitive to sunlight even in the absence of the initially inducing photoallergen.

As is the case for non-photoactivated allergens, there remains a concern that in vitro testing may miss pre-and pro-photoallergens. Two models of photoantigen formation, the photo-hapten model and the pro-hapten model, exist. In the induction phase of photoallergy, photoallergens can produce ROS and induce apoptosis in keratinocytes, but the use of either or both biomarkers would detect both photoallergy and phototoxicity and would not distinguish the two. Therefore, other investigators have focused on the in vitro formation of photo-antigens. In these studies, the photobinding properties of putative photoallergens to human serum albumin have been used.

Endogenous Photosensitivities

Endogenous photosensitivity results from both endogenous and exogenous factors. In some genetic diseases, such as xeroderma pigmentosum, the cell’s ability to repair ultraviolet light induced damage is significantly impaired. Systemic lupus erythematosus, an autoimmune connective tissue disease, features an abnormal sensitivity to UVB. In hereditary or chemically induced porphyria, enzyme abnormalities disrupt the biosynthetic pathways that produce heme, leading to accumulation of protoporphyrin precursors throughout the body, including the skin, which are activated by visible light (400 to 410 nm). One such porphyria, porphyria cutanea tarda, can be precipitated by alcohol, estrogens, or certain antibiotics in individuals with hereditary abnormalities in porphyrin synthesis. Pellagra (photodermatitis, diarrhea, dementia, and death), due to alteration in tryptophan metabolism, can be induced by isonicotinic acid and hydralazine; the treatment for pellagra is oral niacin (vitamin B3).

Other Responses to Electromagnetic Radiation

The most evident acute feature of ultraviolet radiation exposure is erythema (redness or sunburn). The minimal erythema dose (MED), the smallest dose of ultraviolet light that induces an erythematous response, varies greatly from person to person, largely dependent upon skin pigmentation. In the sunburn reaction, vasodilatation, responsible for the color change, is accompanied by the production of inflammatory mediators from injured keratinocytes. These mediators are responsible for the systemic symptoms that may be associated with sunburn: fever, chills, and malaise. UVB (290 to 320 nm) is the most effective wavelength for inducing erythema in human skin. UVA’s efficiency in generating erythema in humans is about 1000-fold less than that of UVB. Both UVA and UVB have been implicated in the development of melanoma and non-melanoma skin cancers. Because of its wavelengths and greater depth of penetration, UVA is more responsible for some of the long-term effects of sun exposure: wrinkling, thinning (atrophy), and easy bruisability.

Overt pigment darkening after ultraviolet exposure is accomplished by enhanced melanin production by melanocytes and/or photo-oxidation of melanin. Tanning (increased pigmentation occurring within 3 days of ultraviolet exposure) is to be contrasted with the immediate pigment darkening of photo-oxidation. The tanning response is most readily produced by exposure to

UVB and may be induced, along with erythema and DNA repair, by DNA damage. Tanning serves to augment the protective effects of melanin in the skin; however, this protective effect may be insufficient in preventing the damage it induces, especially in fair-skinned individuals.

Chronic exposure to radiation results in characteristic skin changes that accelerate or mimic aging. Lighter skin people suffer from chronic sun exposure with a greater frequency than more darkly pigmented individuals. Photo-exposed areas (head, neck, backs of hands, and the sternal area of the chest) are more readily damaged by light. Pigmentary changes, such as freckling and hypomelanotic areas (idiopathic guttate hypomelanosis), wrinkling, telangiectasias (fine dilated superficial blood vessels), pre-cancerous lesions (actinic keratoses and porokeratoses), and malignant skin lesions (basal and squamous cell carcinoma; malignant melanoma) are all consequences of chronic exposure to ultraviolet light. One significant pathophysiologic response of chronic exposure to light is a pronounced decrease in the Langerhans cells. Chronically sun exposed skin may have up to 50% fewer of these cells compared to photo-protected areas. This decrease may lessen immune surveillance of neoantigens on malignant cells and may allow for such transformations to proceed unchecked. For these reasons, a tan obtained either naturally or in a tanning salon is not to be recommended.

Ultraviolet radiation is critical for the conversion of 7-dehydrocholesterol to pre-vitamin D3, without which normal endogenous production of vitamin D would not take place. Blue light (420 to 490 nm) can be lifesaving in premature infants due to its ability to photoisomerize bilirubin in the skin. In addition, the toxic effects of ultraviolet light have been exploited for decades through artificial light sources for treatment of skin disorders such as psoriasis.

URTICARIA

The skin is capable of IgE-mediated reactions, resulting in allergic urticaria. For those allergens to which IgE antibodies have been elicited by previous or ongoing systemic or topical exposures, subsequent cutaneous and/or systemic contact can lead to development of hives through an immediate, Gell and Coombs type I, hypersensitivity reaction. Hives are raised wheals that usually itch or sting and appear reddish. The symptoms of allergic urticaria result from degranulation of cutaneous mast cells induced by binding of IgE to receptors on the cell surface, leading to release of histamine and other vasoactive substances. Food allergies and pharmaceuticals are major causes of acute allergic urticaria. Some agents (e.g., opiates) can bring about the direct release of histamine from mast cells without antibody mediation, while others (NSAIDs) do so through effects on arachidonic acid metabolism or other uncertain mechanisms. Contact urticaria can arise from cutaneous exposure to a plant, animal, or other protein sources (allergic), but can also have a nonallergic basis. Many substances that elicit contact urticaria are highlighted in Table 19–7.

TABLE 19–7 Substances Reported to Elicit Contact Urticaria

Chemically Defined	Chemically Undefined
Acetylsalicylic acid	**Animals:**
Acrylic monomer	Amniotic fluid
Alcohols (amyl, ethyl, benzyl, butyl, isopropyl, propyl)	Blood
	Brain
Aliphatic polyamide	Cockroaches
Alpha amylase	Hair
Aminophenazone	Dandruff
Aminothiazole	Intestine
Ammonium	Collagen
Ampicillin	Placenta
Bacitracin	Saliva
Balsam of Peru	Seminal fluid
Benzocaine	Serum
Benzoic acid	**Food:**
Benzophenone	Cheese/milk
Black rubber mix	Eggs
Butylhydroxytoluene (BHT)	Flour
Carba mix	Honey
Cellulose	Rice
Cephalosporins	**Fruits and vegetables:**
Chloramine	Beans
Chloramphenicol	Asparagus
Chlorhexidine	Carrot
Chlorocresol	Olives
Chlorpromazine	Cauliflower
Citric anhydride	Eggplant
Rosin (colophony)	Endives
Cornstarch	Pepper
Denatonium benzoate	Onion
Diethyltoluamide	Spinach
Epoxy resin	Pea
Formaldehyde and its resins	Parsley

Gentamicin	Yucca
Glucocorticoids	Watercress
Hydrolyzed wheat	Latex (cross reaction)
Iodohydroxyquinone	**Grains:**
Lidocaine	Barley
Lindane	Oats
Mechlorethamine	Rye
Menthol	Wheat
Mercaptobenzothiazole	**Meat:**
Methimazole	Beef
Mezlocillin	Chicken
Monoamylamine	Liver
Neomycin	Turkey
Nickel	Lamb
Oleyl amide	**Nuts/Seeds:**
Papain	Almond
Parabens	Peanut
para-aminodiphenylamine	Sesame
para-phenylenediamine	**Seafood:**
Penicillin	Fish
Pentamidine	**Spices:**
Phenylmercury propionate	Cilantro
Phosphorus sesquisulfide	Cumin
Platinum salts	Curry
Propylene glycol	Dill
Polysorbate	Garlic
Polyvinylpyrrolidone	Paprika
Potassium ferrocyanide	**Textiles:** silk, cotton
Promethazinek2 Propyphenazone Proteases Reactive paints	**Plants and wood:** birch, chrysanthemum, tulip, lichens, teak, mahogany
Rhodium Rifampicin Salicylic acid Sodium hypochlorite Sodium silicate	**Cosmetics:** hair gel, henna, hydrolyzed collagen, hydrolyzed wheat, nail polish, perfume, blush

Sodium sulfite	**Miscellaneous:** copy paper chemical, plastic, rubber, tinofix S
Sorbitan monolaurate	
Streptomycin	
Sulfur dioxide	
Terpinyl acetate	
Vanilla	
Virginiamycin	
Vinyl pyridine	
Xylanase	
Zinc diethyldithiocarbamate	

Data from Amaro C, Goossens A. Immunological occupational contact urticaria and contact dermatitis from proteins: A review. *Contact Dermatitis.* 2008;58:67–75; Amin S, Tanglertsampan C, Maibach HI. Contact urticatia syndrome: 1997. *Am J Contact Dermatitis.* 1997;8:15–19; Burdick AE, Mathias CGT. The contact urticaria syndrome. *Dermatol Clin.* 1985;3:71–84; Brancaccio RR, Alvarez MS. Contact allergy to foods. *Dermatol Ther.* 2004;17:302–313; Lahti A. Contact urticaria to plants. *Clin Dermatol.* 1986;4:127–136.; von Krogh G, Maibach HI. The contact urticaria syndrome – 1982. *Semin Dermatol.* 1982;1:59–66.

PIGMENTARY DISTURBANCES

Melanocytes help protect the skin from the harmful effects of ultraviolet light by producing melanin, which results from a series of oxidative reactions involving the tyrosine in the presence of the enzyme tyrosinase. As indicated in Table 19–8, several agents can interfere with normal pigmentation to yield either excessive or reduced amounts of melanin. Depigmentation can occur following exposure to phenols, catechols, quinones, and related compounds. Many chemicals inducing leukoderma (depigmentation) do so as a result of their metabolism by tyrosinase to ROS that are toxic to melanocytes. Depigmentation caused by exposure to monobenzyl ether of hydroquinone is shown in Fig. 19–2G.

TABLE 19–8 Selected Causes of Cutaneous Pigmentary Disturbances

I. **Hyperpigmentation**
- Ultraviolet light exposure
- Post-inflammatory changes (melanin and/or hemosiderin deposition)
- Hypoadrenalism
- Internal malignancy
- Chemical exposures
  - Coal tar volatiles
  - Anthracene
  - Picric acid
  - Mercury
  - Lead
  - Bismuth
  - Furocoumarins (psoralens)
  - Hydroquinone (paradoxical)
- Drugs
  - Chloroquine
  - Amiodarone
  - Bleomycin
  - Zidovudine (AZT)
  - Minocycline

II. **Hypopigmentation/Depigmentation/Leukoderma**
- Post-inflammatory pigmentary loss
- Chemical leukoderma/hypopigmentation
  - Hydroquinone
  - Monobenzyl, monoethyl, and monomethyl ethers of hydroquinone
  - *p*-(*t*-Butyl)phenol
  - Mercaptoamines
  - Phenolic germicides
  - *p*-(*t*-Butyl)catechols
  - Butylated hydroxytoluene
- Vitiligo (autoimmune)

Other causes of toxic leukoderma may occur via effects on melanocytic signaling pathways. The cancer chemotherapeutic drug, imatinib mesylate, causes pigment loss in the epidermis, likely through inhibition of c-kit tyrosine kinase pathway essential for melanocyte function. Paradoxically, imatinib mesylate may cause hyperpigmentation over the hard palate and repigmentation of gray hair.

The use of skin lightening creams containing hydroquinone, corticosteroids, or mercurials is common. These agents produce their effects by inhibiting tyrosinase activity or expression, but long-term hyperpigmentation, referred to as pseudo-ochronosis, may paradoxically occur.

Pigmentary alterations may also be seen in the nails. Characteristic pigmentation of nail beds has followed systemic exposure to phenolphthalein, arsenic, silver, anti-neoplastics, anti-malarials, and other pharmaceutical chemicals.

Hyperpigmentation, termed post-inflammatory, results from exposure to phototoxic agents including coal tars, furocoumarin found in certain foods such as limes (see Fig. 19–2F), and azo-aniline dyes present in the printing and textile industries. Furthermore, many inflammatory conditions of the skin may result in hyperpigmentation (Fig. 19–2H). Pseudo-hyperpigmentation may occur due to deposition of metals such as lead, bismuth, and arsenic within the skin. Finally,

many drugs and other xenobiotics may cause hyperpigmentation via their effect on synthesis/release of melanocortin peptides, tyrosinase activity, melanocyte signaling pathways, and other unknown mechanisms.

ACNE

Acne is a common affliction of the pilosebaceous units especially of the face, chest, and back. The condition arises from blockage of the duct leading from the sebaceous gland to the cutaneous surface, resulting retention of sebum and enlargement of the gland. In its most common form (acne vulgaris), hormones associated with puberty lead to high sebum production, hyperproliferation and cornification of the ductal cells, plugging of the orifice, and retention of corneocytes in the follicular lumen. Proliferation of resident bacteria and inflammation within the blocked pore/sebaceous gland results. Topically, long chain fatty acids induce an acne-like response in animal models and in some individuals exposed to cosmetics. Petroleum products (oils, coal tar) in the workplace can give rise to acneiform eruptions. Insoluble cutting oils used in the machine industry may have this effect, as illustrated in Fig. 19–2I.

Chloracne

The most disfiguring form of acne in humans, chloracne, is caused by exposures to halogenated aromatic hydrocarbons. Chloracnegenic chemicals, such as polychlorinated biphenyls (PCBs) and naphthalenes, polychlorinated dibenzofuran and dioxin contaminants, are formed during the synthesis of these agents. The recalcitrant nature of chloracne and its preventability mark it as an important occupational and environmental illness. Typically, blackheads (comedones) and cysts are present behind the ears and about the eyes, on the shoulders, back, and genitalia. As opposed to acne vulgaris, the nose is almost invariably spared. In addition to acne, increased hair in atypical locations (hypertrichosis), hyperpigmentation, brown discoloration of the nails, conjunctivitis, and eye discharge may be present. Chloracne exhibits progressive degeneration of the sebaceous units with transformation of the sebocytes to keratinized cells.

GRANULOMATOUS DISEASE

Foreign body reactions, isolating invading substances that cannot be readily removed, occur following the introduction of a variety of foreign agents into the skin through injection or subsequent to a laceration or abrasion. These reactions can take three forms: (1) nontoxic (the mere presence of an inert substance in the dermis); (2) toxic, in which the substance induces activations of macrophages; and (3) immunologic, in which T cells are activated. Toxic and immunologic granulomas can produce persistent lesions with abundant inflammatory cells resembling chronic infectious conditions (e.g., tuberculosis, leprosy, Leishmaniasis, etc.), and can present diagnostic challenges.

The cutaneous response to graphite intradermally injected into the skin via puncture with a “lead pencil” is a common example of the nontoxic granuloma. The resultant “carbon tattoo” may persist as a grayish dot visible on the skin surface for years. However, there is no

inflammatory reaction, and, no sequela following this intradermal injection. Silica or talc (magnesium silicate), paraffin, mineral oil, beryllium (see Fig. 19–2J), metallic mercury and zirconium compounds, and tattoo dyes (including cobalt, chromium, mercury, and others) can also induce immunologic granulomatous reactions.

SCLERODERMATOUS SKIN DISORDERS

Scleroderma encompasses a group of rare diseases that involve the hardening and tightening of the skin and connective tissues in other organs. Signs and symptoms vary, depending on which structures are affected. Scleroderma results from the overproduction and accumulation of collagen in body tissues. Symptoms may be triggered by exposure to silica, polyvinyl chloride, epoxy resins, *meta*-phenylenediamine, bleomycin, and denatured rapeseed oil.

TOXIC EPIDERMAL NECROLYSIS

Toxic epidermal necrolysis (TEN) is a rare life-threatening skin disease. This syndrome involves detachment of ≥30% of the epidermal surface from the dermis, commonly accompanied by severe erosions of the mucous membranes. Mucous membrane involvement can result in gastrointestinal hemorrhage, respiratory failure, ocular abnormalities, and genitourinary complications. Even with intensive treatment and prompt identification of causality, it may have a fatality rate of approximately 30%.

Stevens–Johnson syndrome is a milder, but still serious, condition that has been considered by some to fall within the spectrum of TEN; however, many dermatologists consider this a separate entity induced by drugs, viruses, and other often unknown precipitating factors. In Stevens–Johnson syndrome, up to 10% of skin surface area may be affected and there is a proportionally lower fatality rate.

TEN is commonly associated with drugs; however, the disorder has other potential etiologies, including infection, malignancy, and vaccinations. At onset, it may resemble an upper respiratory tract infection (fever, cough, sore throat, and malaise), but prompt diagnosis when the cutaneous lesions develop several days later improves survival. Ongoing exposure to drugs first encountered during the previous month should be stopped, and treatment like that for severe burn patients initiated: fluid and electrolyte replacement, protection from infection, and nutritional assistance. Nearly 200 drugs have been reported to cause this syndrome with the major contributors being the anticonvulsants, NSAIDs, sulfonamides, and allopurinol.

TEN is an immune-related, cytotoxic T cell reaction that causes apoptosis of keratinocytes. It mimics a hypersensitivity reaction, with its characteristic delayed reaction to an initial exposure and a more rapid response following repeated exposure. The proposed immunopathologic pathways leading to TEN include: Fas ligand activation on keratinocyte membranes leading to death receptor–mediated apoptosis; release of destructive proteins (perforin and granzyme B) from cytotoxic T lymphocytes generated following an interaction with cells expressing major histocompatability complex (MHC) class I; overproduction of T cell- and/or macrophage-derived cytokines, such as interferon-γ, tumor necrosis factor-α, and various interleukins; and drug-induced secretion of granulysin from cytotoxic T cells, natural killer cells, and natural killer T

cells.

HAIR

Hair, like the nail, is an “appendage” of the skin. It is a keratinous protein filament that grows from follicles found in the dermis. The highly structural and organized cortex, or middle layer of the hair, is the primary source of mechanical strength and water uptake. The cortex contains melanin, which gives the hair its color. Toxic effects on hair occur from systemic exposures affecting the anagen (growth) phase. The most commonly encountered toxic effect is hair loss (alopecia) and is referred to as anagen effluvium. The principal causes are systemic cancer chemotherapeutics and heavy metals (mercury, bismuth, and thallium).

Overstimulation of the anagen phase can result in excess hair (hypertrichosis). Acquired, toxic hypertrichosis may be seen with certain drugs (minoxidil and anabolic steroids), but has also been associated with various cancers and eating disorders. Hexachlorobenzene has also been reported to cause hypertrichosis, although this is usually seen in hexachlorophene-induced porphyria cutanea tardas.

SKIN CANCER

UV-Induced Skin Cancer

Most skin cancers in the United States are UV-induced and may be aggravated by genetic variations in the melanocortin receptor, which are associated with an increased skin cancer risk that is independent of hair type or skin color. The most common are the non-melanoma skin cancers, and less commonly malignant melanoma. Basal cell carcinoma (BCC) is the most common form of skin cancer, and squamous cell carcinoma is the second most common form. Actinic keratosis is the most common pre-cancer. Melanoma is the most lethal form of not only skin cancer but most other malignancies. The vast majority of melanoma is caused by ultraviolet radiation—both natural and artificial (tanning salons). Regular daily use of an SPF 15 or higher sunscreen reduces the risk of developing squamous cell carcinoma by about 40% and the risk of developing melanoma by 50%.

UVB induces pyrimidine dimers and 8-oxoguanine modifications, thereby eliciting mutations in critical genes. Damage to the p53 tumor suppressor gene is detectable in most UV-induced squamous cell carcinomas. Because the p53 protein arrests cell cycling until DNA damage is repaired, and may also induce apoptosis, its loss destabilizes the genome of initiated cells and gives them a growth advantage. UV light also has immunosuppressive effects that may help skin tumors survive. Skin cancer incidence is highest in the tropics in pale complexioned individuals, particularly at sites on the head and neck that receive the most intense exposure.

Chemically Induced Skin Cancers

Substances rich in polycyclic aromatic hydrocarbons (coal tar, creosote, etc.) have become recognized as skin carcinogens in both humans and animals. Polycyclic aromatic compounds are

relatively inert chemically, but they can accumulate in cellular membranes and thus perturb function if not removed. They are hydroxylated by several cytochrome P450 isoenzymes, primarily 1A1 and 1B1 in epidermal cells, and then conjugated for disposal from the body. Oxidative biotransformation can produce electrophilic epoxides that form DNA adducts. Phenols, produced by rearrangements of the epoxide, can be oxidized further to electrophilic quinones that generate ROS leading to DNA adducts, oxidized bases, and abasic sites. Occupations at risk of skin cancer from exposure to these compounds (e.g., roofing) often involve considerable sun exposure as well.

Skin Tumor Promotion

Promotion is reversible and requires frequent exposure to the promoter to enhance survival of the transformed cell. Many natural products are tumor promoters and often alter phosphorylation pathways. The active ingredient of croton oil, 12-*O*-tetradecanoylphorbol-13-acetate, transiently stimulates then chronically depletes protein kinase C in epidermis. Other phorbol esters have similar promoting effects.

Other groups of promoting agents include okadaic acid, a phosphatase inhibitor, calcium channel modulators, and free radical generators. Sensitivity to tumor promotion is an important factor in the relative sensitivity to skin carcinogenesis among different animal species. Hexavalent chromium (a human lung carcinogen) in drinking water generates ROS that can be effective skin cancer promoters.

Arsenic

Arsenic is an abundant element near the earth’s crust that is encountered routinely in small doses in the air, water, and food. High exposures can occur from medications, from pesticides, from dust from mining operations, from smelting fumes, or from leachate in waterways. Glass manufacturing and food dried using coal with high arsenic content can also contribute to exposures. The environmental burden is increased by disposal of large volumes of wood preserved with chromated copper arsenic, among other sources. Well water derived from geological formations with high arsenic content is now recognized as a major health concern.

An association exists between high arsenic exposure and altered skin pigmentation, including hyperkeratosis of the palms and soles, impaired circulation reflecting endothelial cell damage (“black foot” disease), and carcinoma (skin, lung, bladder, liver, and other organs). Furthermore, there appears to be synergism between the toxic effects of arsenic and smoking (lung and bladder), sun exposure (skin), and fertilizer use (liver).Arsenic is a very weak mutagen in bacterial and mammalian cell lines, but it does induce DNA deletions and chromosome aberrations. It appears to act as a co-mutagen by interfering with DNA repair.

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QUESTIONS

1. Which of the following statements is FALSE regarding skin histology?
   a. Blood supply to the epidermis originates in the epidermal–dermal junction.
   b. Melanin is made and stored by melanocytes.
   c. The stratum corneum is made up of nonviable cells.
   d. It takes approximately 2 weeks for cells to be sloughed off from the stratum corneum.
   e. Stem cells in the basal layer replenish the keratinocytes of the layers of epidermis.

2. Transdermal drug delivery does NOT:
   a. prevent drug exposure to low pH.
   b. avoid first-pass metabolism.
   c. provide steady infusion over an extended period of time.
   d. avoid large variation in drug plasma concentration.
   e. increase safety of drug delivery.

3. Irritant and contact dermatitis are marked by all of the following characteristics EXCEPT:
   a. softness.
   b. erythema.
   c. flaking.
   d. induration.
   e. blistering.

4. Nickel is a common cause of allergic contact dermatitis, which is which type of hypersensitivity reaction?
   a. type I.
   b. type II.
   c. type III.
   d. type IV.
   e. type V.

5. All of the following statements regarding phototoxicology are true EXCEPT:
   a. Melanin is primarily responsible for the absorption of UV-B radiation.
   b. UV-A is the most effective at causing sunburn in humans.
   c. IL-1 release is responsible for systemic symptoms associated with sunburn.
   d. Melanin darkening is a common response to UV exposure.
   e. UV radiation exposure causes thickening of the stratum corneum.

6. Photoallergies:
   a. represent a form of type III hypersensitivity reaction.
   b. can occur without exposure to UV radiation.

c. are hapten-mediated.
d. cannot be tested for as contact dermatitis allergies can.
e. often occur on first exposure.

7. Diffusion through the epidermis would occur most slowly across skin at which of the following locations?
a. palm.
b. forehead.
c. scrotum.
d. foot sole.
e. abdomen.

8. Which of the following statements regarding photosensitivity is FALSE?
a. Porphyrias cause light sensitivity because of the lack of heme synthesis.
b. Lupus patients are unable to repair damage caused by UV light.
c. Chronic phototoxic responses often result in hyperpigmentation.
d. Photoallergy represents a type IV hypersensitivity reaction.
e. UV radiation causes cycloadducts between pyrimidine bases.

9. Acne is caused by all of the following EXCEPT:
a. clogged sebaceous glands.
b. hormones.
c. viruses.
d. genetics.
e. environmental factors.

10. All of the following statements regarding urticaria are true EXCEPT:
a. Urticaria is a delayed-type hypersensitivity reaction.
b. Hives are mediated partly by histamine release from mast cells.
c. Latex is a common chemical cause of urticaria.
d. Select foods have been reported to elicit contact urticaria.
e. Urticaria is mediated by IgE antibodies.

CHAPTER 20

Toxic Responses of the Endocrine System

Patricia B. Hoyer and Jodi A. Flaws

KEY POINTS

- Endocrine glands are collections of specialized cells that synthesize, store, and release their secretions directly into the bloodstream.
- Each type of endocrine cell in the adenohypophysis is under the control of a specific releasing hormone from the hypothalamus.
- Toxicants can influence the synthesis, storage, and release of hypothalamic-releasing hormones, adenohypophyseal-releasing hormones, and the endocrine gland-specific hormones.

INTRODUCTION

Higher animals regulate their internal environment, independent of wide fluctuations in external factors, via an endocrine system that consists of (1) an endocrine gland that secretes hormone, (2) the hormone itself, and (3) a target tissue. A hormone produced by a ductless endocrine gland is secreted into the blood, which carries it to a target organ. The hormone-producing glands include the pituitary (hypophysis), thyroid and parathyroids, adrenals, gonads, and pancreas. In response to a stimulus, endocrine cells synthesize and secrete hormones into the blood. Hydrophilic hormones (peptides and proteins) are freely dissolved in plasma. Hydrophobic hormones (steroids, amino acid derivatives) bind specialized serum binding proteins or albumin. Hormones elicit biological responses by binding to a receptor in target tissue. Receptors can be located on the plasma membrane (peptides, proteins, and catecholamines) or in the cellular nucleus (steroids, thyroid, and vitamin D hormones). The interaction of the hormone with its receptor initiates a chain of intracellular events leading to its physiological response.

PITUITARY GLAND

The pituitary may be divided into four major subdivisions: the pars distalis (adenohypophysis or anterior pituitary), the pars intermedia between the adenohypophysis and neurohypophysis, the pars tuberalis, and the pars nervosa (neurohypophysis or posterior pituitary). There are two basic types of communication between the hypothalamus and pituitary (Fig. 20–1).

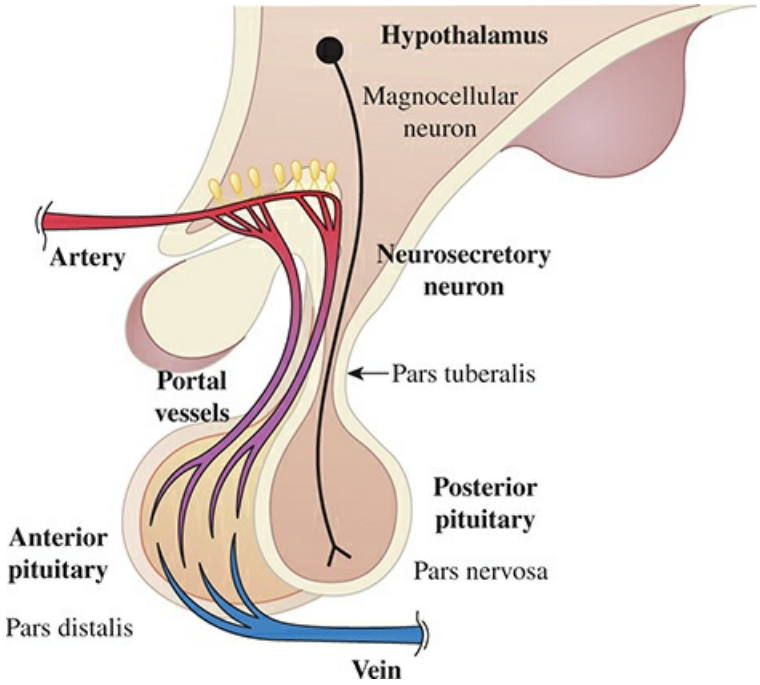

FIGURE 20–1 *Communication between the hypothalamus and the pituitary.* The anterior pituitary receives endocrine input from the hypothalamus in the form of peptide releasing hormones, which are transported in portal vessels to stimulate release of hormones in the anterior pituitary cells. The posterior pituitary is innervated by axons extending from magnocellular neurons with their cell bodies located in the hypothalamus. Stimulation of these cell bodies results in hormonal secretion in the posterior pituitary. (Adapted from Hedge GA, Colby HD, Goodman RL, eds. *Clinical Endocrine Physiology*. Philadelphia, PA: W.B. Saunders; 1987.)

Each type of endocrine cell in the adenohypophysis is under the control of a specific releasing hormone from the hypothalamus (Fig. 20–2). These releasing hormones are small peptides synthesized, secreted by the hypothalamus into capillaries, and conveyed by the hypophyseal portal system to specific trophic hormone-secreting cells in the adenohypophysis. Each hormone stimulates the rapid release of preformed secretory granules containing a specific trophic hormone. Specific releasing hormones have been identified for thyroid-stimulating hormone (TSH), follicle-stimulating hormone (FSH), luteinizing hormone (LH), adrenocorticotropic hormone (ACTH), and growth hormone (GH). Prolactin (PRL) secretion is stimulated by a number of factors including thyrotropin-releasing hormone (TRH). Dopamine serves as the major prolactin-inhibitory factor to suppress PRL secretion. Another hypothalamic release-inhibitory factor is somatostatin, which inhibits secretion of growth hormone.

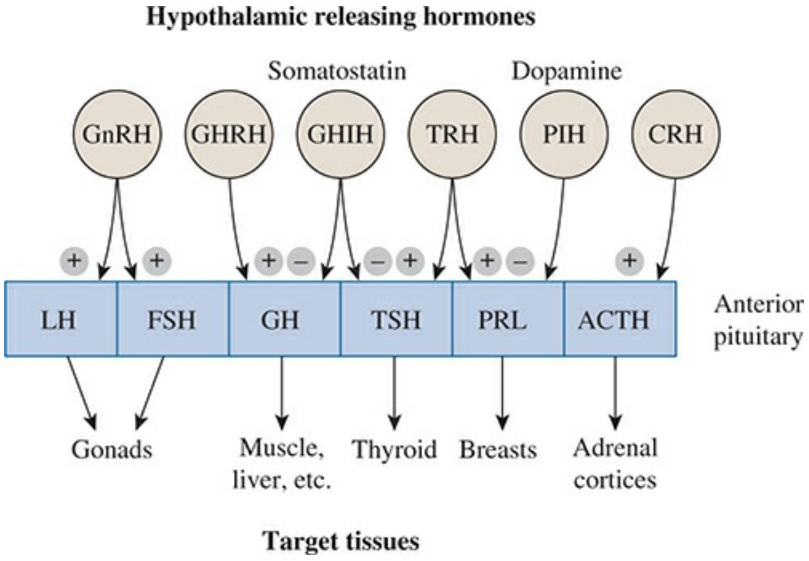

FIGURE 20–2 *Regulation of anterior pituitary hormone release (rectangles) by hypothalamic releasing hormones (circles).* Major target tissues for the anterior pituitary hormones are indicated. ACTH, adrenocorticotropic hormone; CRH, corticotropin releasing hormone; FSH, follicle-stimulating hormone; GH, growth hormone; GHIH, growth hormone inhibiting hormone (somatostatin); GHRH, growth hormone releasing hormone; GnRH, gonadotropin releasing hormone; LH, luteinizing hormone; PIH, prolactin inhibiting hormone (dopamine); TRH, thyrotropin releasing hormone; TSH, thyroid stimulating hormone. +, stimulatory; −, inhibitory. (Adapted from Hedge GA, Colby HD, Goodman RL, eds. *Clinical Endocrine Physiology*. Philadelphia, PA: W.B. Saunders; 1987.)

The pars nervosa (neurohypophysis; posterior pituitary) in the human secretes two important hormones. These are vasopressin, VP (or antidiuretic hormone [ADH]), and oxytocin. ADH (VP) has two major effects corresponding to its two names: (1) it enhances reabsorption of water by the distal tubule and collecting ducts of the kidney producing an antidiuretic effect, which decreases osmotic pressure of the blood, and (2) it causes contraction of vascular smooth muscle producing a generalized pressor effect. Oxytocin stimulates contraction of smooth muscles located in the uterine myometrium to regulate fetal parturition, and in breast alveoli to regulate milk letdown during lactation.

Pituitary Toxicity

Studies consistently show that heavy metals may target pituitary gland structure or function. Cadmium inhibits prolactin, LH, and FSH secretion. Cadmium exposure increases ACTH levels in rodents exposed during puberty and decreases ACTH levels in animals exposed during adulthood. Studies indicate that acute exposure to cadmium decreases circulating GH levels, while treatment for a longer period (14 days) increases circulating GH levels. Lead and mercury inhibit secretion of LH and FSH, whereas acute arsenic and manganese co-exposure decreases levels of FSH and LH in rats.

Environmental contaminants such as polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) inhibit release of LH, FSH, and TSH. The insecticide dimethoate causes pituitary tumors in male and female rats. Methoxychlor, dieldrin, and endosulfan

stimulate prolactin and LH. The phytoestrogen coumestrol reduces pulsatile LH secretion and suppresses the pituitary response to exogenous gonadotropin-releasing hormone (GnRH).

Flame retardants (tetrabromo- and tetrachloro-bisphenol A) increase proliferation of GH3 cells, a pituitary cell line. The plasticizers bisphenol A and 2-ethyl-phthalate stimulate proliferation of rat pituitary cells. 2-Mercaptobenzothiazole, which is used in rubber products, causes pituitary tumors in chronically exposed rats and mice.

ADRENAL GLANDS

The adrenals are small glands situated on the superior poles of each kidney. Each gland is divided into two morphologically and functionally distinct regions: the outer cortex and the interior medulla. The cortex synthesizes and secretes steroid hormones that function to regulate salt and fluid balance, glucose homeostasis, and a long-term response to stress. The medulla secretes the catecholamines, epinephrine, and norepinephrine.

ADRENAL CORTEX

The adrenal cortex can be divided at the cellular level into three separate zones. The zona glomerulosa produces the mineralocorticoid aldosterone. The glucocorticoid hormones, cortisol and corticosterone, are both secreted by the zona fasciculata and, to some extent, the zona reticularis. The zona reticularis also secretes the androgens, dehydroepiandrosterone, and androstenedione.

The adrenal cortex regulates many physiological functions such as the immune system, inflammation, water and electrolyte balance, and carbohydrate and protein metabolism involving such target organs as the liver, kidney, heart, bone, and nervous system. The adrenal cortex is predisposed to the toxic effects of xenobiotic chemicals because the adrenocortical cells contain large stores of lipids in which lipophilic toxicants can accumulate. Cell membranes contain high levels of unsaturated fatty acids that are susceptible to the generation of reactive free radicals via lipid peroxidation. Finally, adrenocortical cells express enzymes involved in steroidogenesis, including those of the cytochrome P450 (CYP) family. Toxic xenobiotic chemicals can be metabolized to reactive species causing direct toxicity by covalent interactions with cellular macromolecules, or through lipid peroxidation.

Steroidogenesis

Adrenal steroids are synthesized from cholesterol by specific enzyme-catalyzed reactions that involve a complex shuttling of steroid intermediates between the mitochondria and endoplasmic reticulum. The common biosynthetic pathway from cholesterol is the formation of pregnenolone, the basic precursor for the three major classes of adrenal steroids (Fig. 20–3).

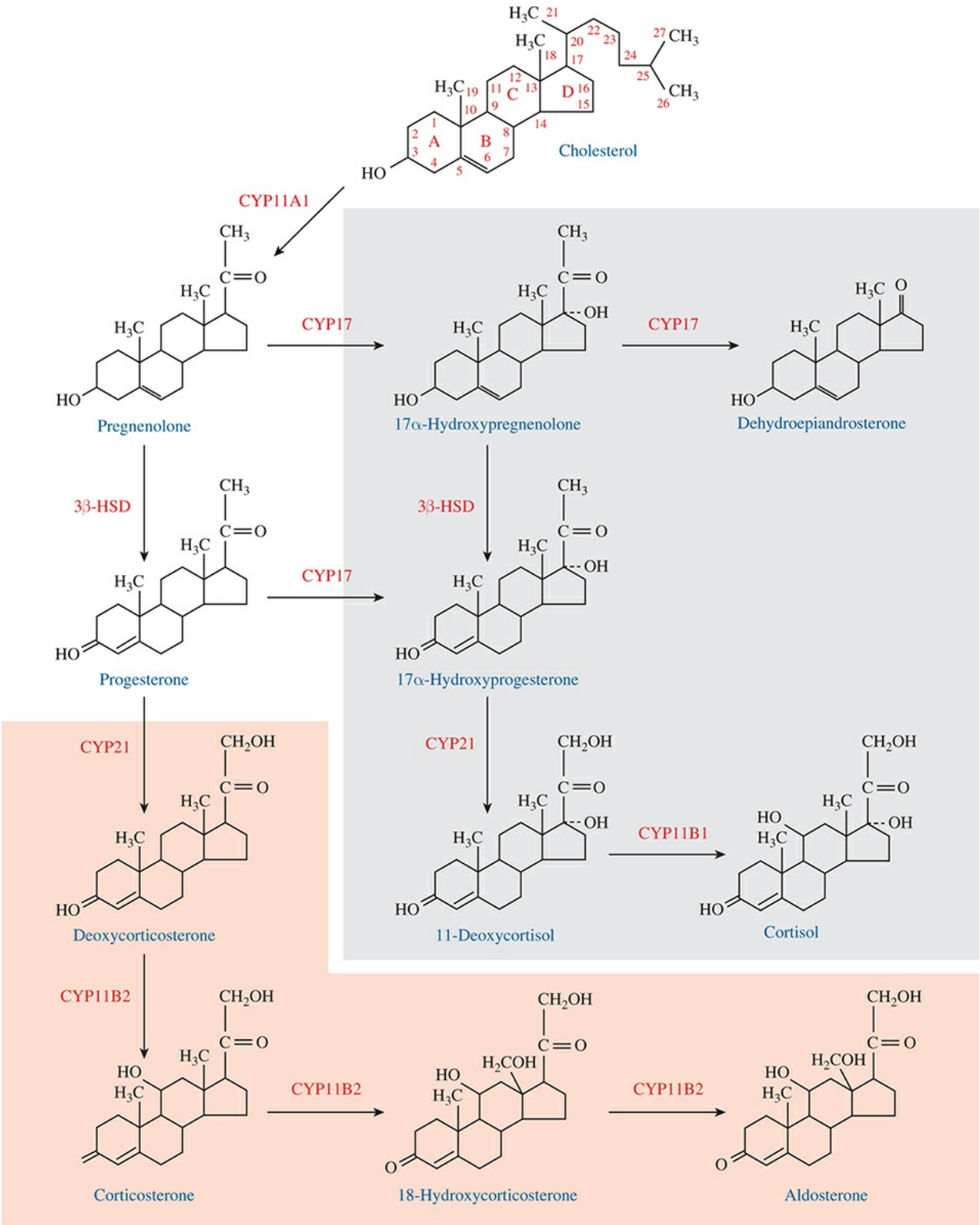

FIGURE 20–3 *Adrenocortical hormone steroidogenic pathway.* Synthesis of the steroids uses cholesterol as a substrate. A series of cytochrome P450 (CYP) enzymes participate in synthesis of aldosterone (zona glomerulosa) or cortisol and adrenal androgens (zonae fasciculata and reticularis). The zona glomerulosa does not express the enzyme 17α-OH

(CYP17A1), whereas the zonae fasciculata and reticularis do not express the enzyme 18OH (CYP11B2). (Reproduced with permission from Schimmer BP, Funder W. Adrenocorticotropic Hormone, Adrenal Steroids, and the Adrenal Cortex. In: Brunton LL, Hilal-Dandan R, Knollmann BC. eds.Goodman & Gilman's: The Pharmacological Basis of Therapeutics, 13e. McGraw-Hill; 2018.)

In the zonae fasciculata and reticularis, pregnenolone is first converted by two microsomal enzymes and three subsequent hydroxylation reactions at the 17, 21, and 11 positions producing cortisol. In the zona glomerulosa, pregnenolone is converted to aldosterone by a series of enzyme-catalyzed reactions like those involved in cortisol formation; because the cells of this zone lack the 17α-hydroxylase the initial hydroxylation product is corticosterone. The corticosterone is acted on by 18-hydroxylase, and then 18-hydroxysteroid dehydrogenase to form aldosterone.

Glucocorticoids

Physiological effects include (a) hepatic glucose production, gluconeogenesis (liver), (b) protein catabolism (skeletal muscle), (c) fat catabolism (adipose tissue), (d) increased bone resorption, (e) altered mood (CNS), (f) increased gastric acidity (GI tract), and (g) PNMT synthesis (adrenal medulla). At pharmacological doses, cortisol can (a) prevent vascular collapse during overwhelming stress, (b) provide an anti-inflammatory effect, and (c) invoke immunosuppression.

Adrenocortical Toxicity

Compounds that frequently produce necrosis in the zonae fasciculata and reticularis include 7,12-dimethylbenz[*a*]anthracene (DMBA), acrylonitrile, thioacetamide, hexadimethrine bromide, polyanethole sulfonate, and basic polyglutamic acid. Chemicals that directly target glucocorticoid secretion include dimethoate, ketoconazole, spironolactone, efonidipine, mibefradil, 1-aminobenzotriazole, and various PCBs. Lesions may be classified as endothelial damage (e.g., acrylonitrile), mitochondrial damage (e.g., DMNM, *o,p′*-DDD, amphenone), endoplasmic reticulum disruption (e.g., triparanol), lipid aggregation (e.g., aniline), lysosomal phospholipid aggregation (e.g., chlorphentermine), and secondary effects due to embolization by medullary cells (e.g., acrylonitrile). Tricresyl phosphate (TCP) blocks cholesterol uptake from serum and storage pathways.

Biologically active cationic amphiphilic compounds produce a generalized phospholipidosis that involves primarily the zonae fasciculata and reticularis and produce microscopic phospholipid-rich inclusions. Compounds known to induce phospholipidosis include chloroquine, triparanol, and chlorphentermine.

Adrenocortical toxicity involving increased secretion of endogenous glucocorticoids may include ethanol, chlordecone, carbon disulfide, cannabinoids, cocaine, amitriptyline, and cytotoxic anticancer drugs. Furthermore, pharmacological treatment with glucocorticoid agonists that have been widely used as anti-inflammatory drugs can produce symptoms that resemble Cushing’s syndrome.

In Vitro Testing

The human adrenocortical carcinoma–derived NCI-H295R cell line expresses all key enzymes necessary for steroidogenesis, and it produces all of the major steroids including progesterone, androgens, estrogens, glucocorticoids, and aldosterone. These cells express functional receptors for CRH, angiotensin II, vasoactive intestinal peptide, atrial natriuretic peptide, LH, human chorionic gonadotropin (hCG), tumor necrosis factor, and activin A. They also express functional receptors for ACTH. This cell line has proven useful for identification of specific steroidogenic enzymes that are targeted by xenobiotics. It is also very worthwhile for hazard risk assessment.

Serum Binding Proteins

Cortisol and corticosterone are transported in blood by corticosteroid binding globulin (CBG), also called transcortin. When bound to CBG, the steroid is biologically inactive. Thus, a chemical affecting CBG could alter the balance between free and bound hormone, impacting its availability in target tissues. Nonsteroidal anti-inflammatory drugs (NSAIDs) decrease the binding capacity of CBG by a mechanism other than simple displacement of bound glucocorticoid.

Target Tissue Receptors

Adrenocortical steroids exert their effects through receptors located in target tissues throughout the body. These receptors can be upregulated or downregulated by the action of xenobiotic compounds. Spironolactone, an antimineralocorticoid, is an example of a peripherally acting adrenocortical hormone antagonist.

Neuroendocrine Regulation

The zonae fasciculata and reticularis of the adrenal cortex are under trophic control by the pituitary hormone ACTH, which stimulates them to produce cortisol. Increased cortisol then provides long-loop negative feedback on the hypothalamus and anterior pituitary and decreases CRH and ACTH secretion, respectively (Fig. 20–4). Stress is a major factor that can override the negative feedback control system and stimulate cortisol secretion. Persistent exposure of the adrenal cortex to high levels of ACTH during chronic stress can result in adrenocortical hypertrophy. Conditions of reduced exposure of the cortex to ACTH can result in adrenal atrophy.

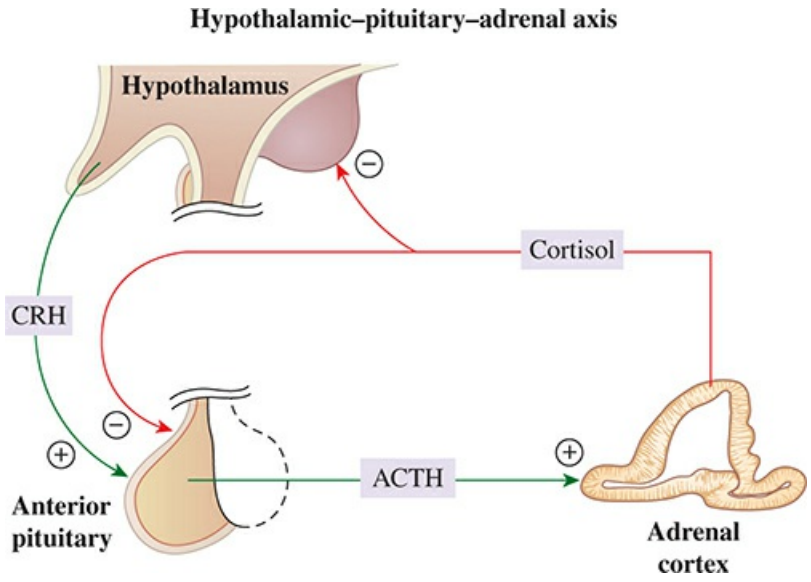

FIGURE 20–4 *Hypothalamic–pituitary–adrenal axis of regulation.* CRH from the hypothalamus stimulates release of ACTH from the anterior pituitary. ACTH stimulates synthesis and secretion of cortisol from the adrenal cortex. Cortisol provides inhibitory (negative) feedback on the hypothalamus and pituitary. Stress, the major stimulator of the axis, can override the feedback regulatory loop. +, stimulatory; −, inhibitory. (Adapted from Hedge GA, Colby HD, Goodman RL, eds. *Clinical Endocrine Physiology*. Philadelphia, PA: W.B. Saunders; 1987.)

Synthetic glucocorticoids, valproic acid, bromocriptine, cyproheptadine, ketanserin, ritanserin, somatostatin analogs, 4′-thio-β-d-arabinofuranosylcytosine, hexachlorobenzene, alcohol, and caffeine suppress ACTH or CRH secretion. ACTH secretion can be stimulated by caffeine, methylxanthines, adenosine analogs, 3,4-methylenedioxymethamphetamine, and di-2-ethylhexyl phthalate. Exogenous steroids can disrupt normal function and structure of the adrenal cortex. Agonists will cause negative feedback inhibition of ACTH secretion in the pituitary and will result in atrophy of the zonae fasciculata and reticularis. In contrast, antagonists of steroids will block hormone action in target, tissues and on negative feedback, which will lead to increased ACTH secretion, and differential hyperplasia of the cortex.

Mineralocorticoids

The adrenals are essential to life, mainly because of the salt-retaining function of the zona glomerulosa. Loss of mineralocorticoid production by the cortex results in a life-threatening retention of potassium and hypovolemic shock associated with the excessive urinary loss of sodium, chloride, and water. Aldosterone promotes sodium reabsorption and increases the excretion of potassium and hydrogen ions by the kidney. Chemicals that target steroidogenic enzymes in the pathway to aldosterone secretion could affect both aldosterone and glucocorticoid (steroidogenic acute regulatory protein, CYP11A1, and CYP21) or only aldosterone synthesis and secretion.

The renin–angiotensin system is the primary regulator of aldosterone secretion in the adrenal cortex. Renin is a proteolytic enzyme synthesized and secreted in the renal juxtaglomerular

apparatus (JGA). Release of renin from the JGA is stimulated by decreases in blood pressure or volume, or reduced plasma sodium. In the blood stream, renin cleaves angiotensinogen to become angiotensin I, which is modified by angiotensin converting enzyme to angiotensin II. Although a potent vasoconstrictor, angiotensin II also acts as a tropic hormone for the zona glomerulosa cells to stimulate synthesis and secretion of aldosterone.

Fetal Adrenal

A specialized fetal adrenal cortex exists in primates during late gestation that produces abundant cortisol and estrogen precursors. The hormones secreted by the cortex are important for normal development of the fetus. After birth, there is a rapid regression, apoptosis, and lysis of the fetal cortex with dilation of cortical capillaries and replacement by the typical three cortical zones. It is important not to misinterpret this as a lesion in neonatal primates since it represents physiological replacement of the fetal cortex with the postnatal adrenal cortex.

X-Zone

The X-zone in the mouse adrenal cortex is also a unique physiological phenomenon that develops postnatally in the inner cortex of mice and is fully formed at weaning. Its function is unknown. In male mice, the X-zone undergoes degeneration at puberty. In females, the zone undergoes slow regression and degeneration during the first pregnancy.

ADRENAL MEDULLA

Because it is classified as a specialized postganglionic (sympathetic) neuron, the adrenal medulla is a functional extension of the nervous system. The medulla is composed of cells called pheochromoblasts, also known as chromaffin cells, which are the site of catecholamine synthesis and secretion. These cells are true neuroendocrine cells: sympathetic, cholinergic stimulation of the cell bodies results in secretion of catecholamines into the circulation and producing true endocrine effects throughout the body. Chromaffin cells also contain chromogranins, enkephalin, neuropeptide Y, substance P, vasopressin, oxytocin, galanin, and neurotensin.

Sympathetic Response

The sympathetic division of the autonomic nervous system functions to (a) ensure reciprocity to counteract and balance the tonic effects of parasympathetic stimulation, (b) assist in the maintenance of steady-state functions of digestion, secretion, vasomotor tone, etc., and (c) assist in the mobilization of body reserves to meet emergency situations–fright, fight, or flight.

Catecholamines

The adrenal medulla is the major site of epinephrine production with a tyrosine precursor and dopamine intermediate. Norepinephrine can be converted to epinephrine by PNMT. Release of

catecholamines is stimulated by acetylcholine from preganglionic neurons. Physiological activators of release include decreased blood pressure, decreased blood glucose, decreased oxygen availability, stress or anxiety, cold, exercise, and postural hypotension. Catecholamines generally affect all tissues. The most pronounced effects are on the heart, liver, skeletal muscle, adipocytes, vascular smooth muscle, and bronchial smooth muscle.

Adrenergic Receptors

The two major types of these receptors are known as α and β adrenoceptors, with further division into at least two subtypes of each, α_1 and α_2, and β_1 and β_2. The relative number of each receptor type in each target organ determines, in part, the nature of the response of the organ to the catecholamines. Therefore, receptor type variation on target tissues contributes to the diversity with which the sympathetic response exerts its specific effects.

General Toxicity

Examples of specific chemicals that target chromaffin cells include toxins that block voltage-gated ion channels and bacterial toxins, which block exocytosis of secretory granules, thereby preventing catecholamine release. The most common pathological changes seen in the adrenal medulla in toxicological studies involve proliferative lesions classified as nodular hyperplasia, although degenerative changes can occasionally be observed.

Pheochromocytoma

Larger benign adrenal medullary proliferative lesions are designated pheochromocytomas that are composed of chromaffin cells with variable numbers of hormone-containing secretory granules. In humans, pheochromocytomas are uncommon except in patients with inherited clinical syndromes of multiple endocrine neoplasia (MEN). These tumors in rats usually do not secrete excess amounts of catecholamines, whereas human pheochromocytomas episodically secrete increased amounts of catecholamines, leading to hypertension and other clinical disturbances.

Pheochromocytomas in rats differ from those in all other species in that they are common, often bilateral, and can be induced by many chemicals. Vitamin D is the most powerful mitogenic stimulus to cause chromaffin cell proliferation in the adrenal medulla in rats. Because the vitamin D effect has been seen in vivo only, it may result from impaired calcium homeostasis, resulting in hypercalcemia. Many of the chemicals that induce pheochromocytoma in animals are uncouplers of oxidative phosphorylation, for example, acrylamide or furan.

The human adrenal medulla, as in mice, has a low spontaneous incidence of proliferative lesions of chromaffin cells. Human chromaffin cells also failed to respond to a variety of mitogenic stimuli in culture.

In Vitro Testing

PC12 pheochromocytoma cells derived from a rat adrenal medullary tumor are widely used in

neurotoxicological studies. The PC12 cell line has been useful in determining intracellular mechanisms at the molecular level that are involved in chromaffin cell signaling and proliferation. Substances that inhibit mitochondrial function (cyanide, rotenone) or uncouple oxidative phosphorylation (dinitrophenol, *p*-trifluoromethoxyphenylhydrazone) stimulate catecholamine secretion. This is thought to be dependent upon Ca^{++} influx through voltage-gated channels.

THYROID GLAND

The thyroid gland consists of two lobes of endocrine tissue located just below the larynx on each side of the trachea with an isthmus connecting the two lobes. The C cells, or parafollicular cells composing the intrafollicular spaces, synthesize and secrete calcitonin (CT), a hormone involved in calcium homeostasis.

The thyroid secretes two hormones known as thyroxine (T_4) and triiodothyronine (T_3). The basic functional unit of the thyroid is the follicle, which consists of a sphere of epithelial cells surrounding a colloidal core, which is composed of thyroglobulin (TGB), which acts as a storage depot for T_4 and T_3. In humans, about 3 months of thyroid hormone is stored as TGB in the colloid. T_4 and T_3 are important regulators of overall metabolism, and the primary target tissues include the liver, kidney, heart, brain, pituitary, gonads, and spleen.

Some studies indicate that xenobiotics directly affect the structure of the thyroid gland. Heavy metals and red dye #3 decrease the size of the colloid space. These morphological changes are thought to reduce the space required for storing hormones, leading to an impaired ability of the thyroid gland to synthesize and store thyroid hormones.

Thyroid Hormone Structure and Synthesis

Thyroid hormones are composed of two modified, covalently linked tyrosine amino acids (Fig. 20–5). Each of the aromatic rings of the tyrosines contains one or two iodides. T_4 contains two iodides on each aromatic ring for a total of four, whereas T_3 contains two iodides on the tyrosine closest to the amino acid moiety (amino and carboxy groups), and one iodide on the outer aromatic ring. The iodide derived from dietary intake is required for biologic activity.

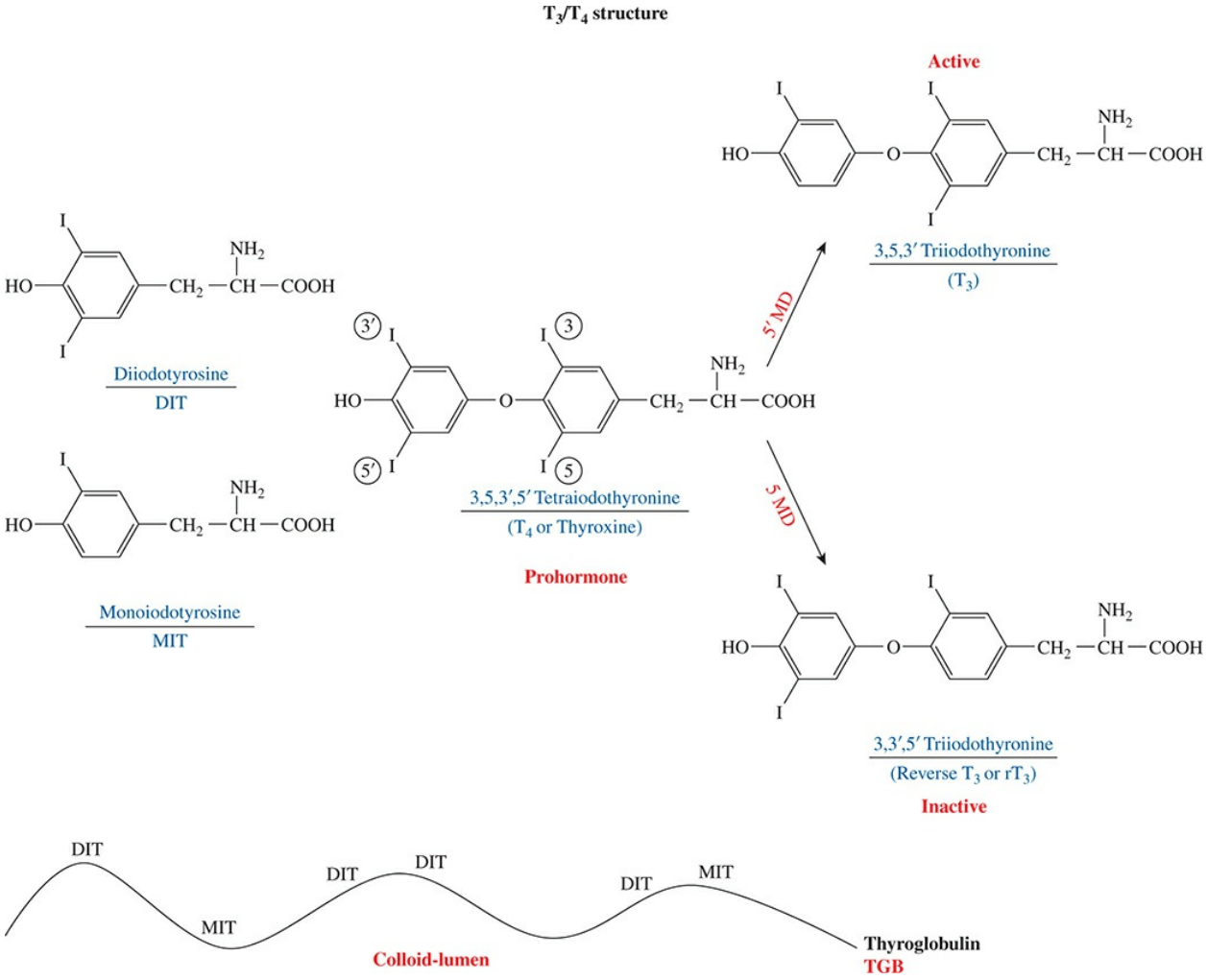

FIGURE 20–5 *Structure of thyroid hormone (T_3 and T_4).* The schematic shows the structures required to make T_3, T_4, and reverse T_3 (rT_3). At the apical membrane of the follicular cells, I_2 combines with tyrosine residues on thyroglobulin (TGB) to form monoiodotyrosine (MIT) and diiodotyrosine (DIT). Coupling between MIT and DIT occurs such that combined MIT and DIT forms T_3, whereas combined DIT and DIT forms T_4. T_4 from the thyroid gland can be peripherally converted to T_3 (active hormone) or rT_3 (inactive metabolite), then successively deiodinated by the monodeiodinases. (Adapted from Hedge GA, Colby HD, Goodman RL, eds. *Clinical Endocrine Physiology*. Philadelphia, PA: W.B. Saunders; 1987.)

Thyroglobulin (TGB), a glycoprotein containing numerous tyrosine amino acid residues, is synthesized in the epithelial cell and serves as the backbone for thyroid hormone synthesis. Iodine in the form of iodide (I^-) is actively transported into the epithelial cell, where it is oxidized to I_2 by thyroid peroxidase. At the apical membrane, I_2 combines with tyrosine residues on TGB to form monoiodotyrosine (MIT) and diiodotyrosine (DIT), which remain attached to TGB. Coupling between MIT and DIT occurs such that a combined MIT and DIT forms T_3, whereas a combined DIT and DIT forms T_4. Upon stimulation, iodinated TGB is endocytosed into the epithelial cell and transported in the direction of the basal membrane where lysosomal

enzymes hydrolyze peptide bonds to release T_3 and T_4 for passive diffusion into the circulation.

T_4 from the thyroid gland can be peripherally converted to T_3 (active hormone) or reverse T_3 (rT_3, the inactive metabolite) then successively deiodinated by the monodeiodinases. Circulating T_4 levels provide a "sink" of prohormone that can serve as a ready supply for peripheral conversion to T_3 (the active form).

Xenobiotics can interfere with thyroid gland function by adversely affecting the process of thyroid hormone synthesis. For example, perchlorate, chlorate, and bromate inhibit uptake of iodide and thus decrease thyroid hormone synthesis. Triclosan and triclocarban inhibit iodide uptake and thyroperoxidase activity. Genistein and daidzein in soy products, thionamides, and substituted phenols can inhibit thyroid peroxidase, blocking incorporation of iodide into TGB. Further, red dye #3 and propylthiouracil inhibit 5′-monodeiodinase, leading to reduced serum levels of T_3.

Thyroid Hormone Binding Proteins

Once released into the blood, thyroid hormones are rapidly bound to high-affinity serum binding proteins. The result is that less than 1% of the T_3 (99.7% bound) and less than 0.1% of the T_4 (99.97% bound) are free in circulation. Only the small unbound fraction of the total hormone pool has access to receptors in target cells, and exerts biological activity.

There are three types of thyroid hormone binding proteins: thyroid binding globulin binds about 80% of the thyroid hormones, thyroxine-binding prealbumin (TBPA) binds about 10%, and albumin binds about 10% of the thyroid hormones. Environmental chemicals such as the PCBs and PBDEs displace thyroid hormones from serum binding proteins. The displacement of thyroid hormones from the binding proteins often leads to a rapid decline in serum thyroid hormone levels.

Thyroid Hormone Receptors

Thyroid hormone receptors (TRs) are members of the nuclear receptor superfamily of ligand-inducible transcription factors. In humans, thyroid hormone receptors are the products of two genes that encode three thyroid hormone receptor isoforms known as TRα, TRβ1, and TRβ2. Environmental chemicals can interfere with thyroid hormone binding to TRs and thyroid hormone–related transcription at multiple levels. First, some chemicals such as PBDEs bind directly to TRs and induce either agonistic or antagonist effects. Second, environmental chemicals may interfere with thyroid hormone binding to receptors via indirect mechanisms exerting their effects by promoting coactivators or inhibiting corepressors. Third, some xenobiotics can interfere with cross talk between TRs and other nuclear receptors. Fourth, some chemicals such as BPA, phthalates, and acetochlor can inhibit expression of TRs.

Thyroid Hormone Clearance

The main pathway for clearance of thyroid hormones from the serum is via conjugation to glucuronic acid or sulfate. The metabolites are transported across plasma membranes for elimination by multidrug resistance protein 1 and the multidrug resistance–associated protein 2

(MRP2). Coplanar and noncoplanar PCB congeners induce glucuronsyl- and sulfo- transferases. Xenobiotics such as pentachlorophenol or triclosan may inhibit sulfotransferases, increasing thyroid hormone activity. Dioxin, rifampicin, and phenobarbital may decrease the transport of thyroid hormones.

Regulation of Thyroid Hormone Release

Thyroid hormone secretion is regulated by thyroid-stimulating hormone (TSH, thyrotropin) from the anterior pituitary gland. The release of TSH is under a hypothalamic–pituitary–thyroid regulatory axis involving negative feedback (Fig. 20–6). Specifically, hypothalamic thyroid releasing hormone (THRH) directly stimulates release of TSH from the anterior pituitary. TSH then increases secretion of T_4 and T_3 from the thyroid gland. In turn, T_4 and T_3 can feed back primarily to the anterior pituitary to inhibit TSH release and they can feed back to some degree to the hypothalamus to inhibit TRH release. Interestingly, most inhibition by thyroid hormones is by T_4.

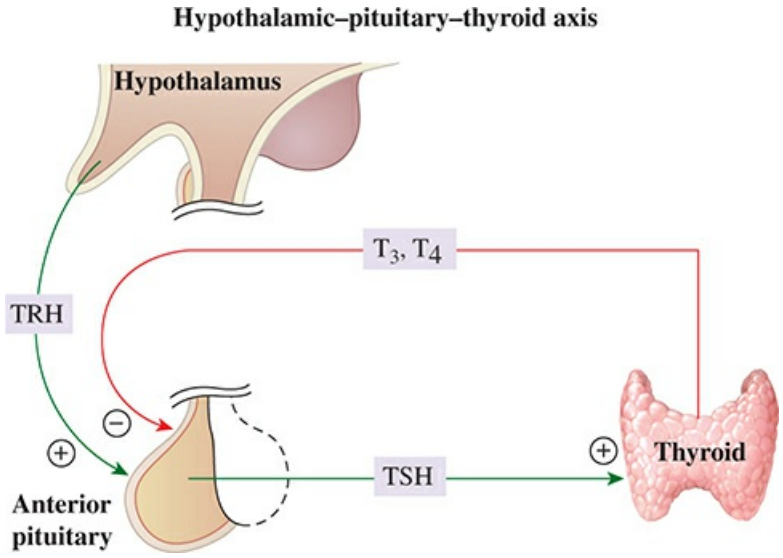

FIGURE 20–6 *Hypothalamic–pituitary–thyroid axis.* The hypothalamus synthesizes and secretes thyroid-releasing hormone (TRH). TRH travels to the anterior pituitary via the portal plexus and stimulates the thyrotropes to synthesize and secrete TSH. TSH acts on the thyroid gland to stimulate production and/or release of T_3 and T_4. T_3 and T_4 can then exert negative feedback control at the level of the anterior pituitary to inhibit further release of TSH. (Adapted from Hedge GA, Colby HD, Goodman RL, eds. *Clinical Endocrine Physiology*. Philadelphia, PA: W.B. Saunders; 1987.)

There is a circadian rhythm of TRH and TSH release, with a decrease following the onset of sleep. Overall, the thyroid axis responds rapidly at the hypothalamo-pituitary unit. The long half-lives of thyroid hormones in circulation dampen the diurnal rhythm that is obvious in TSH levels. As a result, this rhythm is not reflected in circulating thyroid hormone concentrations.

Chemicals such as PBDEs may increase TSH levels, leading to increased levels of T_4 and T_3. Alternatively, xenobiotics may inhibit TRH or TSH levels, leading to decreased T_4 and T_3.

Physiological Effects

Thyroid hormones influence nearly every tissue in the body, with their primary function being determination of the overall metabolic rate of the body. Thyroid hormone stimulates both anabolic and catabolic biochemical pathways; however, its overriding effect is catabolism (energy mobilization). Thyroid hormone also produces effects on growth and development, including normal development of the CNS and maturation of the skeleton in early life.

Thyroid Toxicity

Xenobiotics that affect thyroid hormone levels often cause symptoms of hypothyroidism, hyperthyroidism, or lead to significant impairment in brain development and function. Below are some specific examples of environmental chemicals that affect thyroid hormone levels, resulting in adverse physiological outcomes.

PCBs—PCBs interfere with the thyroid system in a manner that leads to serious neurocognitive effects. PCB exposure in prenatal life is associated with lower full-scale and verbal IQ scores and less short-term and long-term memory and attention in postnatal life. Also, PCBs decrease the levels of thyroid hormone by inhibiting its synthesis and/or increasing its metabolism by inducing phase II enzymes. Other studies indicate that PCBs interfere with thyroid hormone action by inhibiting the binding of thyroid hormones to binding proteins or TRs.

PBDEs—PBDEs are also well-known thyroid-disrupting chemicals that inhibit thyroid hormone levels and action by inducing hepatic phase II enzymes, leading to serious neurocognitive deficits. They also can downregulate transport proteins and even bind to TRs.

Perchlorate—Perchlorate inhibits thyroid hormone synthesis primarily by reducing iodide uptake.

Pesticides—Pesticides such as dichlorodiphenyltrichloroethane (DDT) and hexachlorobenzene (HCB) increase thyroid volume and induce antibodies that attack the thyroid gland, resulting in autoimmune thyroid disease. HCB blocks thyroid hormones binding to TR. Organochlorine pesticides have been associated with increased TSH and decreased T_4 in a male agricultural population.

Perfluorinated Chemicals—Perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) alter T3 and T4 levels by affecting phase II metabolic enzymes in the liver and increasing deiodinases in the thyroid gland.

Bisphenol A—Bisphenol A (BPA) blocks T_3 binding to its receptor. BPA exposure is associated with an increase in anti-thyroperoxidase antibodies in men and women. BPA may inhibit sulfotransferase activity, disrupt thyroid-specific gene expression, and/or suppress thyroid hormone receptor transcription.

Phthalates—Phthalate exposures may alter the levels of T_4 and T_3 levels in adult men and pregnant women by inhibiting thyroperoxidase, resulting in low thyroid hormone levels and symptoms of hypothyroidism in humans. Phthalates may activate the Ras/Akt/TRH receptor

pathway and/or disrupt the hypothalamic–pituitary–thyroid axis.

PARATHYROID GLAND

Humans have four parathyroid glands, which are located on the back side of the thyroid gland. The parathyroid glands are comprised mainly of chief cells, which produce parathyroid hormone (PTH). PTH helps maintain normal plasma calcium levels (Fig. 20–7). Because calcium is required in optimal concentrations for many of life's fundamental processes, the concentrations of calcium in cellular and extracellular fluids must be maintained at a constant value. When the parathyroid glands are removed or damaged, PTH levels drop, causing a major drop in circulating calcium levels. In turn, this can lead to tetanic convulsions and death.

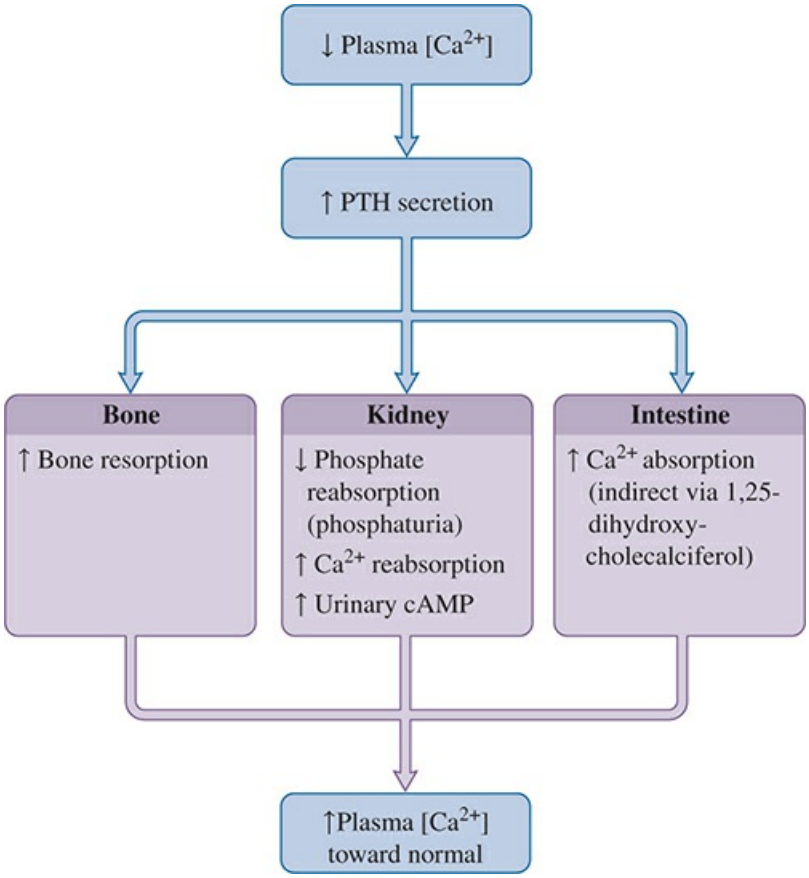

FIGURE 20–7 *Effects of calcium on target tissues.* Low levels of circulating Ca^{2+} stimulate an increase in secretion of parathyroid hormone (PTH). PTH helps restore normal plasma Ca^{2+} levels by acting on bone, the kidney, and the intestine. (Reprinted from Boron WF, Boulpaep EL, eds. *Medical Physiology*. Philadelphia, PA: Elsevier; 2005.)

Parathyroid Toxicity

Xenobiotic exposures may alter the structure of the parathyroid gland. Chemicals such as the

anticancer drug L-asparaginase and heroin cause degenerative changes that reduce the size and limit PTH release. Other xenobiotics increase the size of the parathyroid gland (lead, rotenone, malathion, malaoxon, hexachlorobenzene, ethylene glycol, hydrochlorothiazide).

PTH Structure and Synthesis

PTH is a polypeptide hormone that is derived from a precursor molecule called preproparathyroid hormone (Fig. 20–8). Xenobiotics may interfere with the normal synthesis of PTH. Aluminum and cadmium have been shown to inhibit PTH secretion. Similarly, alcohol consumption has been shown to decrease PTH levels in pregnant rats. Lithium and lead have been associated with a rise in PTH levels and calcium levels. Prenatal exposure to nicotine reduces the activity of parathyroid chief cells, leading to hypertrophy and increased levels of calcitonin in parafollicular cells.

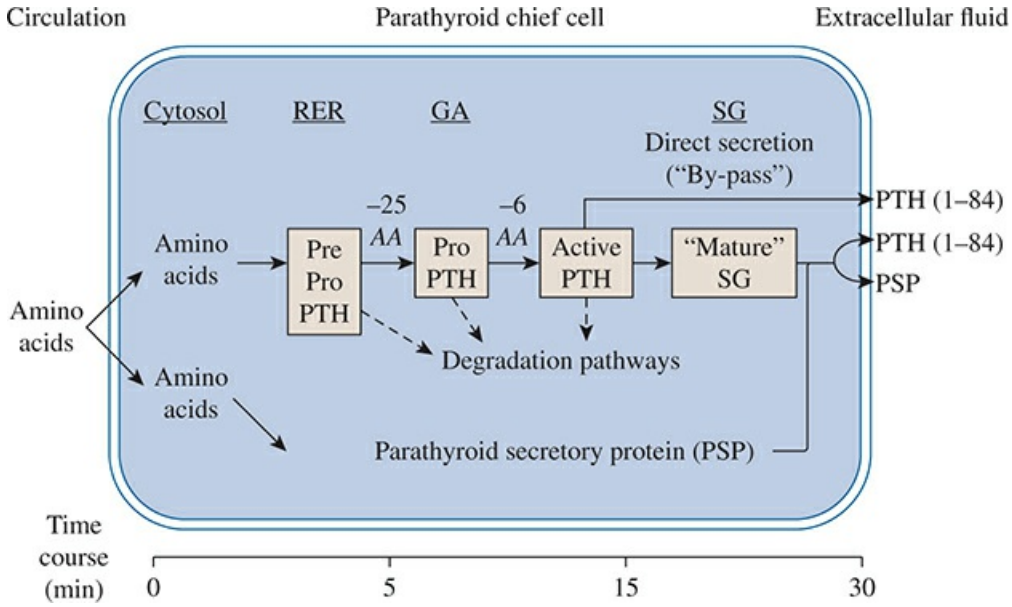

FIGURE 20–8 *Biosynthesis of PTH.* Active PTH is synthesized as a larger biosynthetic precursor molecule (preproPTH) that undergoes rapid posttranslational processing to proPTH prior to secretion as active PTH (amino acids 1 to 84) from chief cells in the parathyroid glands.

PTH Receptors

The PTH receptor is a single G protein–coupled receptor called the PTHR1. PTHR1 is extensively localized in bone and kidney cells. Xenobiotics may alter the expression of PTHR1. Specifically, binge alcohol drinking significantly decreased expression of PTHR1 in male rats.

PTH Clearance

PTH is primarily metabolized by the liver and kidney. It is unclear whether xenobiotics directly affect PTH metabolism.

Physiological Effects

The main physiological role of the parathyroid gland is to control circulating calcium levels. PTH works in concert with calcitonin (CT) and vitamin D. PTH serves to increase circulating calcium levels by increasing the release of calcium from bone demineralization. PTH also increases tubular reabsorption of calcium by the kidney. Further, it inhibits the renal reabsorption of phosphate, which aids in increasing the solubility of calcium. PTH also enhances magnesium reabsorption, inhibits bicarbonate ion reabsorption, and blocks exchange of sodium ions by the tubules. These actions of PTH result in metabolic acidosis, which favors removal of calcium from plasma proteins and bones, which increases circulating levels of ionized calcium.

CT reduces circulating calcium levels by reversing the action of PTH on bone resorption. CT serves to prevent hypercalcemia by shutting down efflux of calcium from bone. It also negatively regulates PTH to prevent kidney calcification. Vitamin D_3 (cholecalciferol) inhibits PTH actions, builds bone, and is essential for absorption of calcium from the GI tract.

Some xenobiotics such as pesticides and fungicides can cause excessive PTH secretion by the parathyroid gland and lead to hyperparathyroidism. Xenobiotic exposures such as those to heavy metals may cause low PTH secretion and lead to hypoparathyroidism.

Regulation of PTH Release

The calcium sensor or calcium receptor on the parathyroid cell belongs to the 7-transmembrane class of G protein–coupled receptors linked to phospholipase C. When the calcium receptors in the parathyroid gland sense low calcium levels, they stimulate the parathyroid gland to release PTH, which functions to raise plasma calcium primarily by stimulating bone resorption and secondarily by enhancing renal calcium reabsorption. Further, PTH stimulates renal metabolism of vitamin D to its active form, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) or 1,25-dihydroxycholecalciferol. This last effect of PTH shifts calcium recovery from the skeletal reserve and the kidney in the acute situation to intestinal absorption mediated by $1,25(OH)_2D_3$ in the chronic situation, thereby sparing mineralized bone.

ENDOCRINE PANCREAS

Scattered among the pancreatic acini are the endocrine units, the Islets of Langerhans. The endocrine pancreas serves as the primary homeostatic regulator of fuel metabolism, particularly circulating glucose. Islet cells are sensors of glucose homeostasis that respond to changes in their nutrient and hormonal environment. The most abundant cell type is the beta cell (β), the site of synthesis and secretion of insulin. Glucagon is produced by the alpha cell (α) and the delta cell (δ) synthesizes somatostatin.

Role of the Liver in Glucose Production

Energy for cellular metabolism can be derived from fatty acids (β oxidation) or glucose (glycolysis, TCA cycle) in the blood. The liver is the primary contributor to increasing blood glucose levels. Figure 20–9 summarizes physiological sources of energy for cellular metabolism.

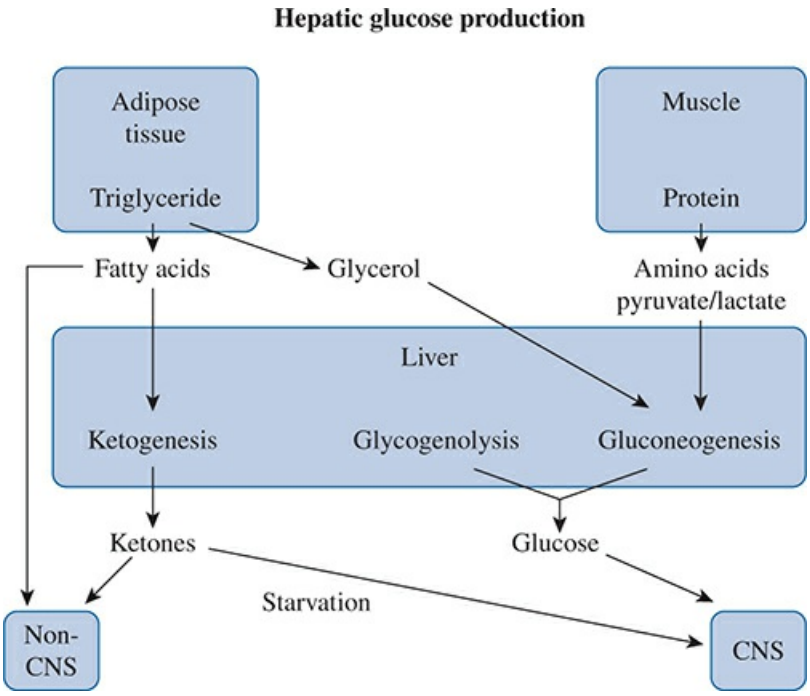

FIGURE 20–9 *Hepatic production of glucose.* The liver provides most of the circulating glucose in the fasting state by glycogen breakdown (glycogenolysis) and de novo synthesis (gluconeogenesis). Substrates for gluconeogenesis are provided by adipose tissue (glycerol from triglyceride breakdown) and muscle (amino acids from protein breakdown). Fatty acids from triglyceride breakdown are used to produce ketones, which can be used by the CNS for energy as an alternate to glucose during starvation.

Pancreatic Hormones

Insulin—The overall effects of insulin are to stimulate anabolic processes (energy storage). Specifically, insulin functions to lower blood levels of glucose, fatty acids, and amino acids and to promote their conversion to the storage form of each: glycogen, triglycerides, and protein, respectively. Physiological responses to insulin include (a) increased cellular glucose uptake (in most tissues), (b) lower blood glucose levels, (c) stimulated glycogen synthesis (liver, muscle), (d) stimulated glycerol production (adipose tissue), (e) increased amino acid uptake (liver, muscle), (f) inhibited lipolysis (adipose tissue), and (g) stimulated protein synthesis (replication, transcription, and translation), a mitogenic response.

Glucagon—Counter-regulatory to insulin, glucagon stimulates catabolic processes (energy mobilization) to prevent hypoglycemia. The release of glucagon is stimulated by epinephrine and norepinephrine (via β-adrenergic receptor stimulation of cAMP production), and by the amino acids, arginine, leucine, and alanine (unless accompanied by glucose ingestion). Conversely, glucagon secretion is inhibited by insulin and somatostatin. Physiological responses to glucagon occur mostly in the *liver* with stimulation of glycogenolysis, gluconeogenesis, lipolysis, and ketogenesis (over a long time). Additionally, glucagon stimulates the secretion of insulin from pancreatic β cells.

Somatostatin—Somatostatin was first isolated from the hypothalamus; its role in regulation of neuroendocrine function is to inhibit secretion of growth hormone in the anterior pituitary. Somatostatin inhibits secretion of insulin and glucagon, and itself.

Interactions of Release

By exerting opposing effects on carbohydrate metabolism, glucagon and insulin act in concert to preserve normoglycemia in the face of any perturbations that might elevate or lower blood glucose. Therefore, many investigators like to think of the insulin-to-glucagon ratio in blood as an important determinant of overall metabolic status. When there is a high ratio of insulin to glucagon, the effects of insulin dominate, producing a relatively anabolic state. When the ratio of insulin to glucagon is low, a catabolic state exists.

Metabolic Responses in Diabetes

Diabetes mellitus is the result of inadequate insulin action. Type 1 (insulin-dependent) results from autoimmune-based destruction of pancreatic β cells. Type 2 involves end-organ insensitivity or resistance to insulin (non-insulin-dependent). In type 2 diabetes, insulin levels also eventually drop due to extended stress placed on pancreatic β cells. As a result of the insufficient insulin action, reduced glucose removal from plasma causes hyperglycemia and various resulting metabolic alterations. These effects are summarized in Fig. 20–10.

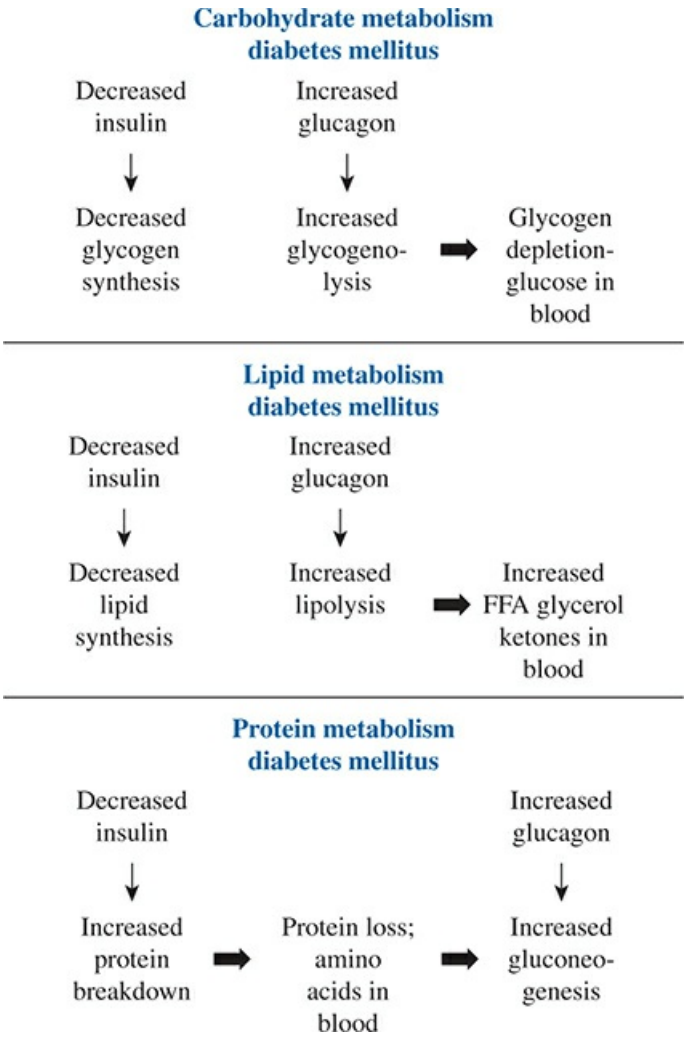

FIGURE 20–10 *Effects of diabetes mellitus on metabolism.* Decreased insulin (type 1) or insulin action (type 2) inhibits glycogen, lipid, and protein synthesis. Increased glucagon stimulates glycogenolysis, lipolysis, and protein breakdown. Glycogenolysis increases circulating glucose. Increased glycerol and amino acids serve as substrates for gluconeogenesis to further increase circulating glucose.

Pancreatic Toxicity

The insulin-secreting β cells are particularly sensitive to chemical attack, compared with the glucagon-secreting α and somatostatin-secreting δ cells. The clinical consequences of insulin deficiency are physiologically more severe than those resulting from glucagon deficiency because other counterregulatory hormones that oppose insulin action can compensate for reduced glucagon regulation. Two chemicals widely used to generate animal models of diabetes are alloxan and streptozotocin (STZ). These agents selectively destroy pancreatic β cells, thereby causing insulin insufficiency. Alloxan and STZ cause DNA damage, activation of poly (ADP-ribose) synthetase, increased polyadenylation, and decreased NAD. Multiple exposure of rats to low doses of STZ triggers an immune system response with destruction of β cells 10 to 15 days after initiation of treatment. Unlike in rats, STZ, when used as chemotherapy, has not caused

diabetes in humans.

Insulin Resistance

Insulin resistance and defective function of pancreatic β cells usually occur sometime before the development of type 2 diabetes. Nondiabetic residents living near a deserted pentachlorophenol and chloralkali factory in Taiwan had insulin resistance associated with increased circulating levels of dioxins and mercury. In addition, bisphenol A exposure of pregnant mice resulted in increased insulin, leptin, triglyceride, and glycerol levels and greater insulin resistance.

In Vitro Testing

Several cell lines are available for testing insulin secretion. Pancreatic β-cell-derived RINm5F cells that produce insulin were exposed to a combination of the cytokines, IL-1β, TNF-α, and IFNγ to simulate type 1 diabetes. Hydrogen peroxide produced by these cytokines reacted in the presence of trace metal Fe^{++} with nitric oxide to form highly toxic hydroxyl radicals. RINm5F cells were also used to investigate the role of oxidative stress in inorganic arsenic exposure. Several proapoptotic mitochondrial and cytosolic markers were investigated and found to be elevated during β-cell toxicity.

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QUESTIONS

1. The inability to release hormones from the anterior pituitary would NOT affect the release of which of the following?
   - **a.** LH.
   - **b.** PRL.
   - **c.** ADH.
   - **d.** TSH.
   - **e.** ACTH.

2. Which of the following statements regarding pituitary hormones is TRUE?
   - **a.** The hypothalamic–hypophyseal portal system transports releasing hormones to the neurohypophysis.
   - **b.** Dopamine enhances prolactin secretion from the anterior pituitary.
   - **c.** Somatostatin inhibits the release of GH.
   - **d.** The function of chromophobes in the anterior pituitary is unknown.
   - **e.** Oxytocin and ADH are synthesized by hypothalamic nuclei.

3. 21-Hydroxylase deficiency causes masculinization of female genitals at birth by increasing androgen secretion from which region of the adrenal gland?
   a. zona glomerulosa.
   b. zona reticularis.
   c. adrenal medulla.
   d. zona fasciculata.
   e. chromaffin cells.

4. Which of the following statements regarding adrenal toxicity is TRUE?
   a. The adrenal cortex and adrenal medulla are equally susceptible to fat-soluble toxins.
   b. Adrenal cortical cells lack the enzymes necessary to metabolize xenobiotic chemicals.
   c. Pheochromocytomas of the adrenal medulla can cause high blood pressure and clammy skin due to increased epinephrine release.
   d. Xenobiotics primarily affect the hydroxylase enzymes in the zona reticularis.
   e. Vitamin D is an important stimulus for adrenal cortex steroid secretion.

5. Chemical blockage of iodine transport in the thyroid gland:
   a. affects export of T_3 and T_4.
   b. prevents reduction to I_2 by thyroid peroxidase.
   c. decreases TRH release from the hypothalamus.
   d. interrupts intracellular thyroid biosynthesis.
   e. mimics goiter.

6. Chromaffin cells of the adrenal gland are responsible for secretion of which of the following?
   a. aldosterone.
   b. epinephrine.
   c. corticosterone.
   d. testosterone.
   e. estradiol.

7. The parafollicular cells of the thyroid gland are responsible for secreting a hormone that:
   a. increases blood glucose levels.
   b. decreases plasma sodium levels.
   c. increases calcium storage.
   d. decreases metabolic rate.
   e. increases bone resorption.

8. Parathyroid adenomas resulting in increased PTH levels would be expected to cause which of the following?
   a. hypocalcemia.
   b. hyperphosphatemia.
   c. increased bone formation.

d. osteoporosis.
e. rickets.

9. Which of the following vitamins increases calcium and phosphorus absorption in the gut?
a. vitamin D.
b. niacin.
c. vitamin A.
d. vitamin B_{12}.
e. thiamine.

10. All of the following statements regarding glucose control are true EXCEPT:
a. Glucagon stimulates glycogenolysis, gluconeogenesis, and lipolysis.
b. Insulin stimulates glycogen synthesis, gluconeogenesis, and lipolysis.
c. Glucagon stimulates catabolic processes (mobilizes energy) to prevent hypoglycemia.
d. Insulin promotes storage of glucose, fatty acids, and aminoacids by their conversion to glycogen, triglycerides, and protein, respectively.
e. Insulin and glucagon exert opposing effects on blood glucose concentrations.

CHAPTER 21

Toxic Responses of the Reproductive System

Paul M.D. Foster and L. Earl Gray Jr.

KEY POINTS

- The gonads possess a dual function: an endocrine function involving the secretion of sex hormones and a nonendocrine function relating to the production of germ cells (gametogenesis).
- Gametogenic and secretory functions of either the ovary or testes are dependent on the secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary.
- The blood–testis barrier between the lumen of an interstitial capillary and the lumen of a seminiferous tubule impedes or prevents the free exchange of chemicals/drugs between the blood and the fluid inside the seminiferous tubules.
- Xenobiotics can act directly on the hypothalamus and the adenohypophysis, leading to alterations in the secretion of hypothalamic-releasing hormones and/or gonadotropins.
- Steroid hormone biosynthesis can occur in several endocrine organs including the adrenal cortex, ovary, and the testes.
- Female reproductive processes of oogenesis, ovulation, the development of sexual receptivity, coitus, gamete and zygote transport, fertilization, and implantation of the

conceptus may be sites of xenobiotic interference.

- Xenobiotics may influence male reproductive organ structure, spermatogenesis, androgen hormone secretion, and accessory organ function.

INTRODUCTION

Chemicals can adversely affect reproduction in males and females, impacting the viability and quality of life of their potential offspring and even later generations. Recent trends in human fertility, fecundity—changing social influences (age at which women have their first child), and the knowledge that populations in many western countries are no longer self-sustaining, point to potential declines in normal human reproduction. Underlying these issues with human reproductive performance is the concept that exposure to environmental chemicals and drugs may be contributing to these declines. The reproductive cycle is outlined in Fig. 21–1.

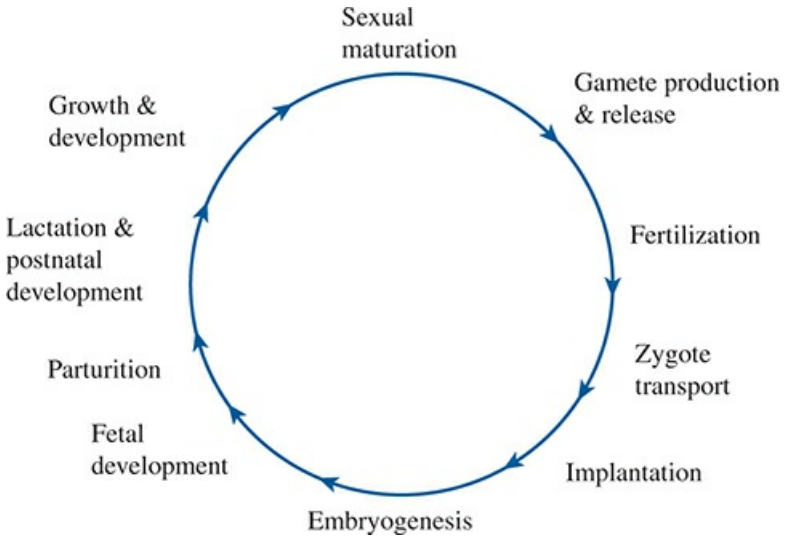

FIGURE 21–1 *The reproductive cycle.*

THE REPRODUCTIVE CYCLE

Numerous complex processes must be orchestrated in a precise, sequential order for optimal performance at different stages of the life cycle of animals and humans. Following fertilization of an egg by a sperm, the resulting zygote must be transported along the oviduct while maturing into an early embryo. This embryo must implant in the uterus successfully, such that the developing conceptus can differentiate, produce a placenta, and undergo normal embryogenesis and fetal development. Parturition must occur at the correct time with birth of the neonate, lactation, and weaning following sequentially.

Acquisition of sexual maturity involves generation of gametes by the gonads. For the parental animals, once their reproductive life span has finished, the process of reproductive senescence occurs. These processes involve complex interplay between tissues and cells, under hormonal

control that provides the critical signals and precise timing of these events. All these processes can be targets for chemicals that can disturb these events leading to adverse effects on reproduction. The dose of the toxic chemical and its resultant effects will depend on when in the life stage of the organism that the chemical is administered and evaluated. Table 21–1 provides basic reproductive parameters for experimental animals and humans.

TABLE 21–1 Reproductive Parameters for Various Species

			Ovulation		Gestation	
Species	**Age at Puberty/ Period**	**Sexual Cycle Duration (Days)**	**Time**	**Type**	**Implantation (Days)**	**Parturition (Days)**
Mouse	5–6 weeks	4	2–3 hours	S	4–5	19 (19–21)
Rat	6–11 weeks	4–6	8–11 hours	S	5–6	21–22
Rabbit	6–7 months	Indefinite	10 hours	I	7–8	31 (30–35)
Hamster	5–8 weeks	4	Early estrus	S	5+	16 (15–18)
Guinea Pig	8–10 weeks	16–19	10 hours	S	6	67–68
Ferret	8–12 months	Seasonal	30–36 hours	I	12–13	42
Cat	6–15 months	Seasonal	24–56 hours	I	13–14	63 (52–69)
Dog	6–8 months	9	1–3 days	S	13–14	61 (53–71)
Monkey	3 years	28	9–20 days	S	9	168 (146–180)
Human	12–16 years	27–28	13–15 days	S	7.5	267 (ovulation)

Abbreviations: I, induced; S, spontaneous.

REPRODUCTIVE DEVELOPMENT AND SEXUAL DIFFERENTIATION

During the seventh week of human gestation, male and female morphological characteristics begin to develop. Gonadal differentiation is dependent on signals from the Y chromosome, which contains the genes necessary to induce testicular morphogenesis. One of these signals is the SRY gene, the sex-determining region on the short arm of the Y chromosome, which acts as a "switch" to initiate transcription of other genes, which contribute to testicular organogenesis. In the absence of the SRY protein, the gonad remains indifferent for a short time before differentiating into an ovary.

Interstitial Leydig cells produce the male sex hormone testosterone, which induces masculine differentiation of the Wolffian duct and external genitalia. Figure 21–2 provides a diagrammatic representation of sexual differentiation in the human male. In rodent and human species, fetal testicular androgen production is necessary for proper testicular development, normal male sexual differentiation, and differentiation of the Wolffian ducts into the epididymides, vasa deferentia, and seminal vesicles.

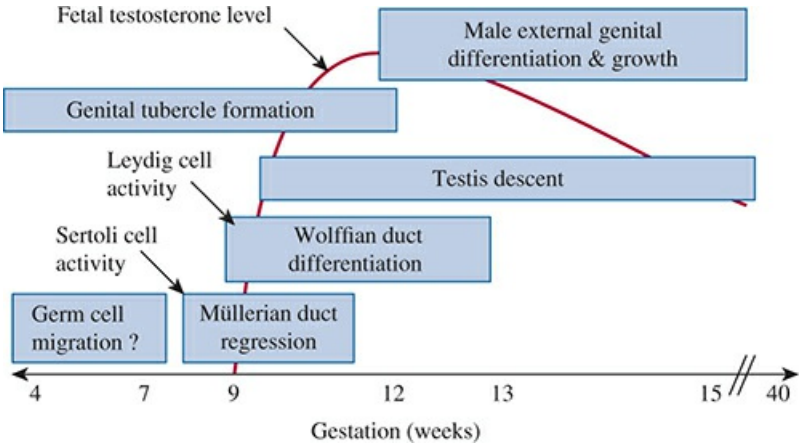

FIGURE 21–2 *Male sexual differentiation in humans during gestation.* (Reprinted from Klonisch T, Fowler PA, Hombach-Klonisch S. Molecular and genetic regulation of testis descent and external genitalia development. *Dev Biol*. 2004;270:1–18.)

Androgens from the Leydig interstitial cells stimulate the mesonephric (or Wolffian) ducts to form the male genital ducts, while Sertoli cells produce Müllerian inhibiting substance (MIS or Anti-Müllerian Hormone, AMH), which suppresses development of the paramesonephric (Müllerian) ducts, or female genital ducts.

In humans, the external genitalia are indistinguishable until the ninth week of gestation, and not fully differentiated until the twelfth week of development. Development of the external genitalia coincides with gonadal differentiation. Fetal testicular androgens are responsible for the induction of masculinization of the androgynous external genitalia. Thus male, but not female, reproductive tract development is totally hormonally dependent and inherently more susceptible to endocrine disruption.

GAMETOGENESIS

The mammalian oocyte begins meiosis during fetal development but arrests part-way through meiosis I and does not complete the first division until ovulation; the second division is completed only if the egg is fertilized (Fig. 21–3). In contrast, male meiosis begins at puberty and is a continuous process, with spermatocytes progressing from prophase through the meiotic second division in little more than a week. This difference has implications for the action of toxicants and critical time periods when these cells may be vulnerable to attack. The complement of oocytes available to the mammalian female is complete at birth, whereas there is significant stem cell (spermatogonial) renewal to maintain the higher number of germ cells available in males.

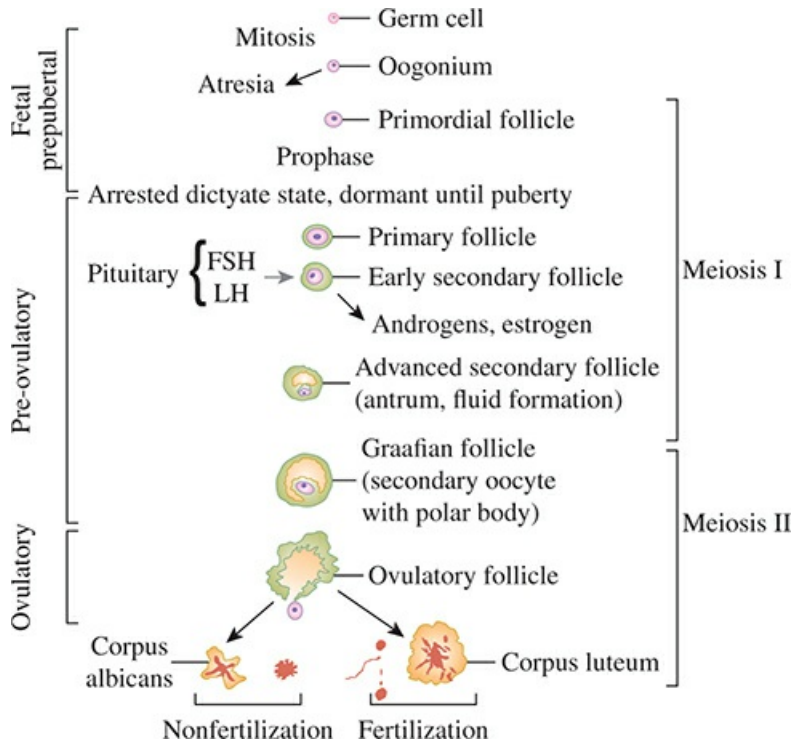

FIGURE 21–3 *Development of the oocyte.*

NEONATAL DEVELOPMENT

Late in gestation, male rats display longer anogenital distances (AGD) than do female rats, with neonatal male AGD being more than twice that of females. There are homologous sex differences in humans. In many mammalian species, including humans and rats, males of the species engage in more aggressive play than do females. Both AGD and behavior can be altered by exposure to hormonal and antihormonal agents.

INFANTILE DEVELOPMENT

During the infantile period of development, emergence of the nipple buds and areolae in females and maturation of the hypothalamic–pituitary axis occurs. Emergence of the nipple buds is prevented in males by prenatal androgen-induced atrophy of the nipple anlagen.

PUBERTAL DEVELOPMENT

Puberty is the stage of life when an individual matures from a child, through adolescence to full maturity. The process is marked by dramatic development of hormone-dependent sexual characteristics, somatic growth, and sexual and social behaviors eventually resulting in full reproductive capacity.

Puberty is initiated by activation of the HPG and hypothalamic–pituitary–adrenal (HPA) axes (Fig. 21–4). At the onset, the HPG axis releases gonadotropin-releasing hormone (GnRH) pulses with increasing frequency and amplitude, which induces complementary pulsatile secretions of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary. In turn, LH and FSH stimulate the gonads inducing gonadarche and the onset of gonadal hormone production. In females, secretion of androgens from theca cells is controlled by LH whereas estradiol production from granulosa cells of maturing follicles prior to ovulation is controlled by FSH. In males, LH stimulates the testicular synthesis and secretion of androgens and insulin-like 3 peptide hormone from the Leydig cells.

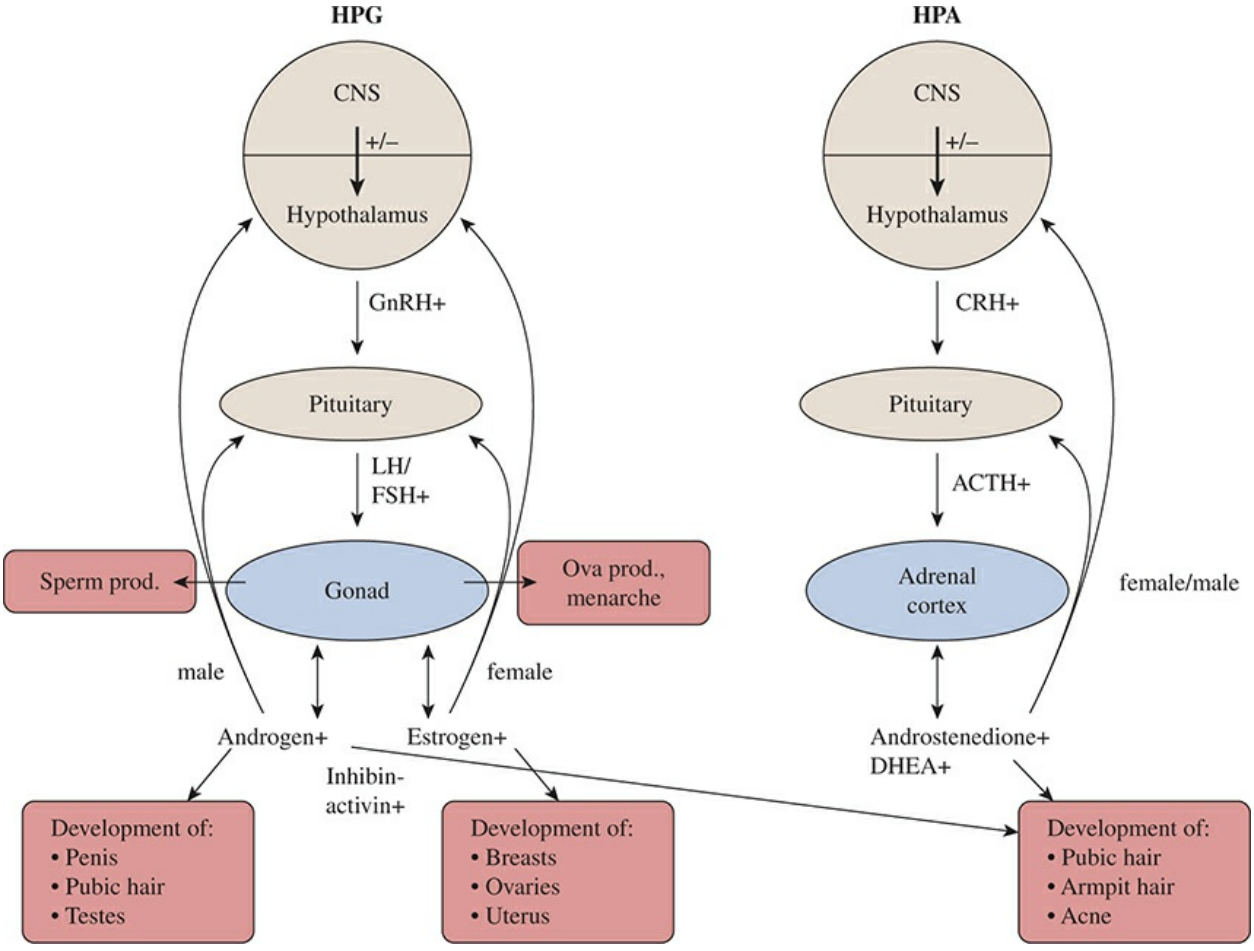

FIGURE 21–4 *Endocrine control of puberty in males and females.* Prod, production

In humans, adrenarche, the maturation of adrenal endocrine function, occurs early in pubertal development, resulting in the growth of pubic hair, acne, and other secondary sex traits. These physical changes result from increasing adrenal synthesis and secretion of steroids including dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS), and androstenedione, steroids with weak androgenic activities. Adrenarche is independent of gonadarche and typically occurs between 6 and 8 years of age in both sexes. Adrenarche occurs only in primates.

Precocious puberty is defined as the onset of sexual traits before 8 and 9 years of age in girls and boys, respectively, whereas puberty is considered as delayed in girls if thelarche (the onset

of breast development) is not displayed by 13 years and at 14 years of age in boys when testicular volume is less than 4 mL. Precocious puberty may result from early onset of HPG function or via gonadotropin-independent alterations.

Premature thelarche and premature adrenarche are often referred to as pseudoprecocious puberty when the full spectrum of pubertal changes does not occur. Premature thelarche in girls and gynecomastia in boys result from direct exposure to estrogen-containing personal care and "natural" products. Prolonged exposure may shorten stature due to effects of estrogens on the growth plates of the long bones and cause sexual–social behavior inappropriate for the child's age. Concerns also have been expressed that premature thelarche may enhance the likelihood of developing diseases such as breast cancer and endometriosis.

Rodent Models of Puberty

Rodents provide important animal models in the study of the genetic and environmental factors that regulate puberty. In laboratory rats, the standard landmarks of puberty are the age of male preputial separation (PPS), an androgen-mediated event, and the ages of vaginal opening (VO), an estrogen-mediated event, and first estrus. Toxicants can alter puberty as a consequence of in utero, lactational, or pubertal exposures.

Selected Examples of Chemicals That Alter the Onset of Pubertal Landmarks in Rats After Acute In Utero and/or Lactational Exposures—2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), busulfan, androgens, antiandrogens, estrogens such as ethinyl estradiol or methoxychlor, and endocrine disrupting chemicals (EDCs) can alter the onset of pubertal landmarks in male and female rats. A measurable delay in PPS, a landmark of puberty in the rat, provides an endpoint to evaluate chemicals for this form of endocrine activity.

Selected Examples of Chemicals That Alter the Onset of Pubertal Landmarks in Rats After Peripubertal Exposures—The "pubertal female rat assay" monitors vaginal estrous cycles until necropsy. Necropsy measurements include serum thyroid hormones, and uterine and ovarian weight and histology. This assay detects alterations in thyroid hormone status, HPG function, and inhibition of steroidogenesis, estrogens, and antiestrogens. The "pubertal male assay rat" detects alterations of thyroid function, HPG maturation, steroidogenesis, and altered steroid hormone function (androgen).

SEXUAL MATURITY

Hypothalamic–Pituitary–Gonadal Axis

FSH and LH are glycoproteins synthesized and released from the pituitary gland. Hypothalamic neuroendocrine neurons secrete specific releasing or release-inhibiting factors into the hypophyseal portal system, which carries them to the adenohypophysis, where they act to stimulate or inhibit the release of anterior pituitary hormones. GnRH acts on gonadotropic cells, thereby stimulating the release of both FSH and LH.

The neuroendocrine neurons have nerve terminals containing monoamines (norepinephrine, dopamine, and serotonin) that impinge on them. Reserpine, chlorpromazine, and monoamine

oxidase (MAO) inhibitors modify the content or actions of brain monoamines that affect gonadotropin production.

In the female (Fig. 21–5), LH acts on thecal cells of the ovary to induce steroidogenesis, particularly the production of progesterone and androgens that are transferred to the granulosa cells which can be stimulated by FSH to produce estradiol. These steroids then provide feedback on the hypothalamus and pituitary to regulate gonadotropin production.

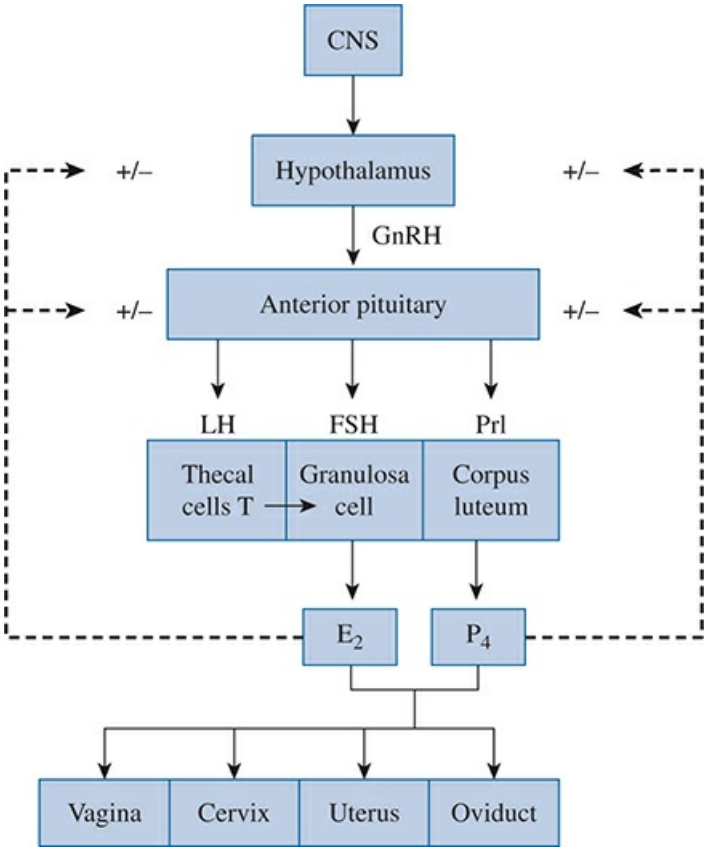

FIGURE 21–5 *Endocrine control of the female reproductive cycle.* CNS, central nervous system; GnRH, gonadotropin releasing hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; Prl, Prolactin; T, testosterone; E_2, estradiol; P_4, progesterone.

In the male (Fig. 21–6), FSH acts primarily on the Sertoli cells, but it also appears to stimulate the mitotic activity of spermatogonia. LH stimulates steroidogenesis in the interstitial Leydig cells. A defect in the function of the testis (in the production of spermatozoa or testosterone) will tend to be reflected in increased levels of FSH and LH in serum because of the lack of negative feedback of testicular hormones.

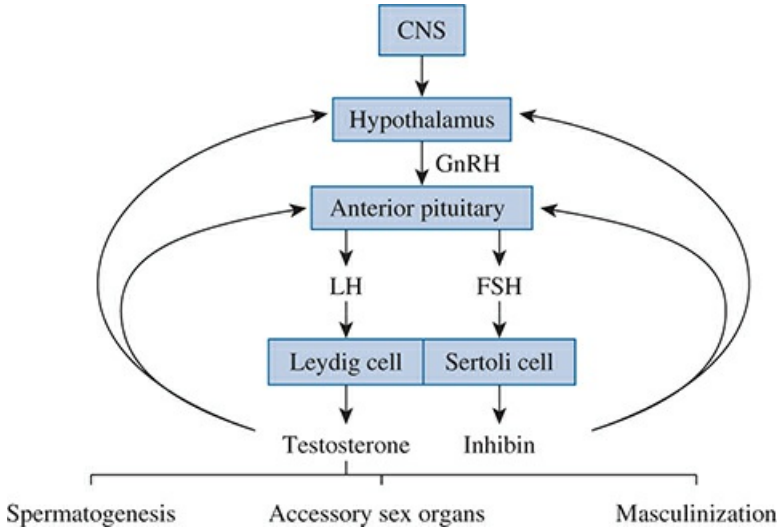

FIGURE 21–6 *Endocrine control of male reproduction.*

The HPG feedback system is a very delicately modulated hormonal process. Several sites in the endocrine process can be perturbed by different chemicals.

Ovarian Function

Oogenesis—About 400,000 follicles are present at birth in each human ovary. After birth, many undergo atresia, and those that survive are continuously reduced in number. Any chemical that damages the oocytes will accelerate the depletion of the pool and can lead to reduced fertility in females. About one-half of oocytes present at birth remain at puberty; the number is reduced to about 25,000 by 30 years of age. About 400 primary follicles will yield mature ova during a woman's reproductive life span. During the approximately three decades of fecundity, follicles in various stages of growth can always be found. After menopause, follicles are no longer present in the ovary.

Ovarian weight, unlike uterine weight, in the rat does not fluctuate during the estrous cycle, thus providing useful information about the effects of toxicants on the female reproductive system. Ovarian weight can be reduced by either depletion of oocytes or disruption of the HPG axis. Toxicants affect ovarian histology by inducing polyovular follicles, oocyte depletion, interstitial cell hyperplasia, corpora albanicans, and absence of corpora lutea.

Case Study—Busulfan—An alkylating drug used to treat chronic myelogenous leukemia, severe thrombocytosis, and polycythemia vera, busulfan may interfere with normal menstrual cycles in women and block sperm production in men. In addition, busulfan causes ovarian failure and prevents or delays the onset of puberty in girls.

In the rat, busulfan inhibits germ cell development. The most severely affected females do not display estrous cycles or spontaneous sexual behavior as a consequence of exposure. Even though the gonads of both sexes were affected at similar dosage levels, fertility and gonadal hormone production were much more easily disrupted in female than male offspring because the steroid producing cells in the ovary fail to differentiate in the absence of the oocyte.

In addition to busulfan, occupational exposure to some of the benzidine-based dyes and 4-

vinylcyclohexene causes follicle destruction presumably upregulating the rate of atresia through activation of proapoptotic signaling events. Methoxychlor (MXC) is an organochlorine pesticide that produces antral follicle atresia, in part by altering apoptotic regulators (Bcl-2 and Bax). MXC directly inhibits follicle growth partly by Bcl-2 and Bax pathways, and increases atresia partly through Bcl-2 pathways by inducing oxidative stress.

Ovarian Cycle

The cyclic release of pituitary gonadotropins determines ovulation and prepares the female accessory sex organs to receive the male sperm. This axis can be disrupted, resulting in infertility at any level of the endocrine system. For example, chemicals that block the LH surge transiently can prevent or delay ovulation resulting in infertility or lower fecundity due to delayed fertilization of ova.

Postovarian Processes

Female accessory sex organs function to bring together the ovulated ovum and the ejaculated sperm. The chemical composition and viscosity of reproductive tract fluids, as well as the epithelial morphology of these organs, are controlled by ovarian (and trophoblastic) hormones.

Oviducts—The progression of the fertilized eggs through the oviduct and uterus is under hormonal regulation and chemicals such as the estrogens can stimulate oviductal transport and interfere with uterine endometrial function, precluding implantation.

Uterus—Uterine endometrium reflects the cyclicity of the ovary as it is prepared to receive the conceptus. The myometrium's major role is contractile. In primates, at the end of menstruation, all but the deep layers of the endometrium are sloughed and a new cycle begins.

Other mammals have estrous cycles rather than menstrual cycles. Figure 21–7 diagrams the human menstrual and rat estrous cycles regarding timing and endocrine control.

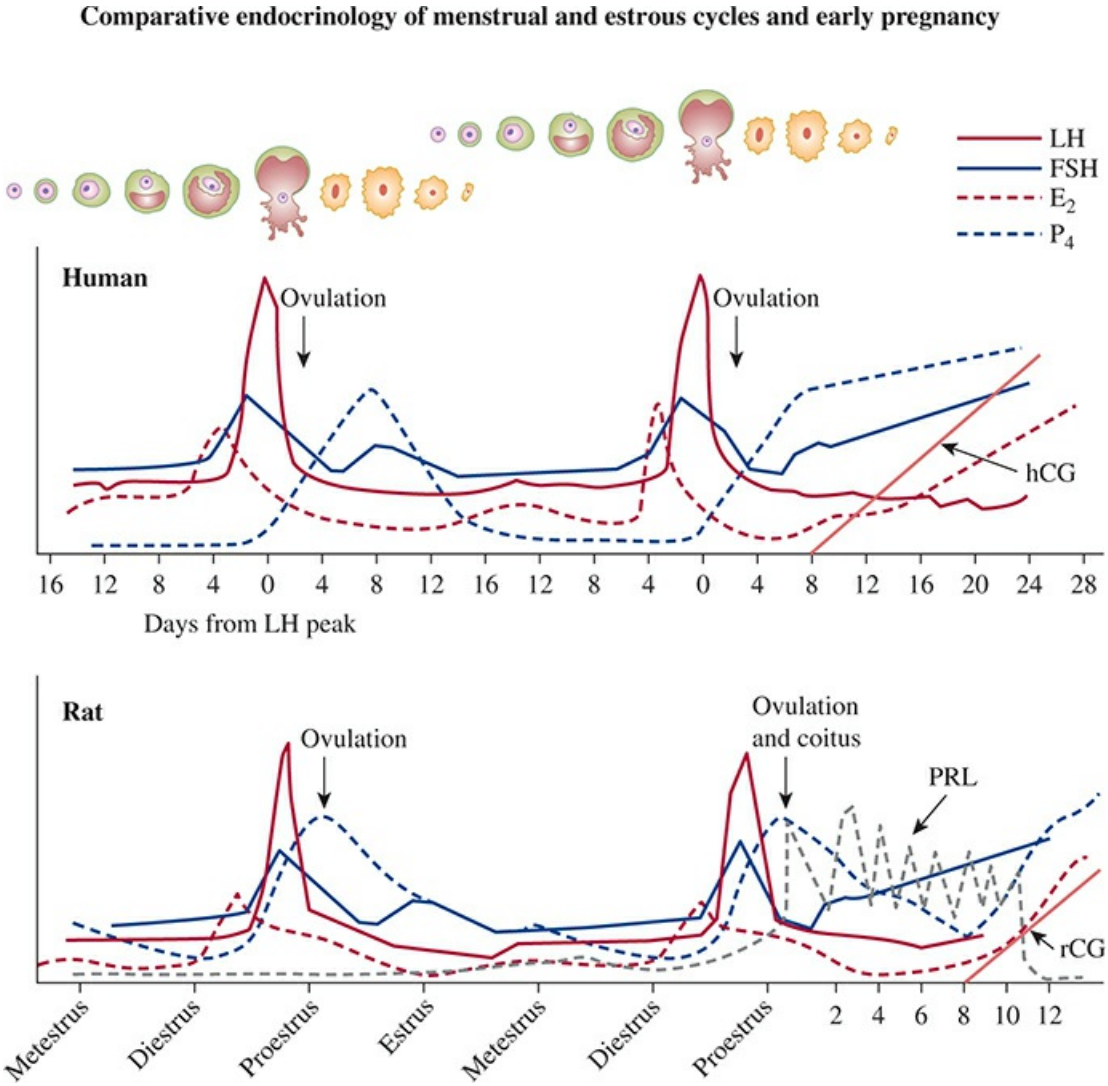

FIGURE 21–7 *Comparison of the timing of the human and rat cycles.* LH, luteinizing hormone; FSH, follicle-stimulating hormone; PRL, prolactin; E_2, estradiol; P_4, progesterone; hCG, human chorionic gonadotropin; rCG, rat chorionic gonadotropin.

TESTICULAR STRUCTURE AND FUNCTION

Targets for Toxicity

For the adult male, there are numerous potential targets for the action of chemicals upon the system (Fig. 21–8). Dopamine analogs interrupt GnRH secretion and estrogens reduce normal gonadotropin (LH and FSH) production directly affecting. However, perturbing the homeostasis of nutrients can lead to direct effects on spermatogenesis and subsequent issues with fertility. Similarly, chemicals with direct effects on the liver (e.g., CCl_4) can disturb the normal metabolism and clearance of sex steroids (predominantly glucuronide and sulfate conjugates of hydroxytestosterones in the male), indirectly affecting the HPG axis and impacting male

reproduction.

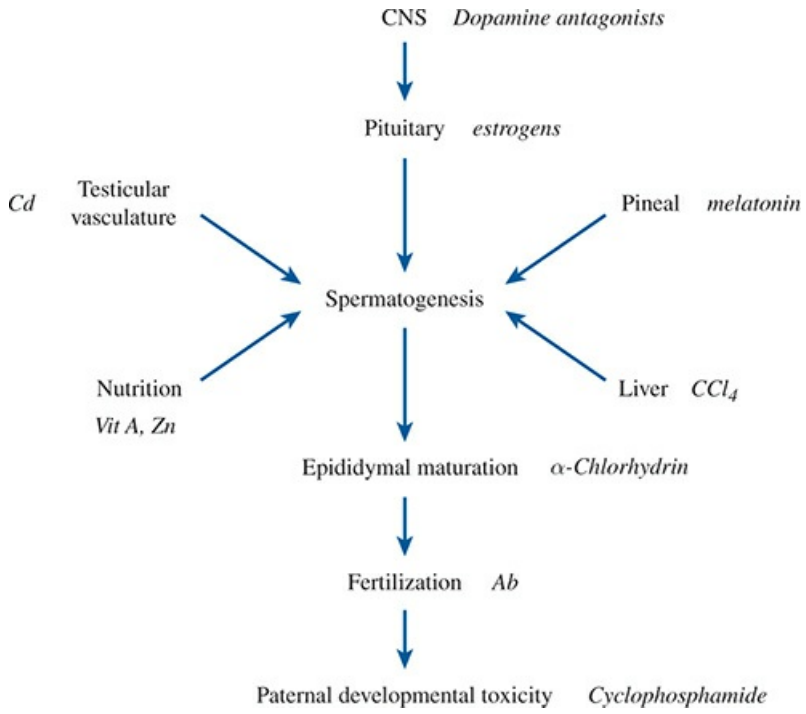

FIGURE 21–8 *Potential target sites for male reproductive toxicants.* Examples of agents shown in italics.

The testis also has a finely tuned circulatory system in mammals, termed the pampiniform plexus, designed to shunt the arterial venous blood supply and aid in scrotal cooling. Some chemicals (e.g. cadmium) actually target this structure and the testis circulatory system to induce ischemic shock to the testis resulting in injury and reduced fertility.

Testicular Structure and Spermatogenesis

Numerous chemicals affect the male reproductive system by directly affecting the testis and the process of spermatogenesis. Spermatogenesis is an extremely ordered process in the rat. The spermatogonia have populations that act as the stem cells for the seminiferous tubules and a proportion of these cells undergo mitosis to increase numbers, move into meiotic prophase, and are then committed to becoming spermatozoa that mature and are released into the seminiferous tubule lumen.

Different biochemical events happen during the various stages, and this can provide clues as to the potential mode of action of chemicals producing stage-specific lesions. Such occurrences occur regularly with certain phthalate esters, glycol ethers, and antiandrogenic agents.

Once sperm are released into the seminiferous tubule lumen and proceed to the epididymis, they can be the target of toxicant action. Chlorosugars and epichlorohydrin (an organochlorine insecticide) inhibit energy metabolism in sperm, preventing them from functioning normally. The number of known environmental chemicals that produce adverse responses in human males is not large. All of these have been shown to induce effects in rodents and especially the rat, although there may be differences in sensitivity based on dose.

Posttesticular Processes

The efferent ducts then empty into the caput (head) of the epididymis that is a single highly coiled tube derived from the Wolffian duct in utero. The epididymis can be divided into three anatomical portions: the caput, corpus (body), and cauda (tail). Sperm undergo maturation in the caput and corpus and begin to acquire motility, whereas sperm are principally stored in the cauda. During the movement along the epididymal tubule, fluid is removed by active transport. This stage can be interfered with by toxicants, resulting in an inappropriate environment for normal sperm development.

Erection and Ejaculation

Pesticides, particularly the organophosphates, are known to affect neuroendocrine processes involved in erection and ejaculation. Many drugs act on the autonomic nervous system and affect potency. Impotence, the failure to obtain or sustain an erection, is rarely of endocrine origin.

Case Studies for Effects on the Male

m*-Dinitrobenzene—m*-Dinitrobenzene (*m*-DNB) produces rapid deleterious effects on the rat testis. Testicular weight remained reduced for many weeks after the treatment period with significant dose-related effects on fertility (measured by pregnancy rate and implantation success).

Ethylene Glycol Monomethyl Ether—EGME has been shown to produce testicular toxicity in several species, including rat, mouse, rabbit, and dog with reasonable evidence that it is likely to have effects in humans should exposure be high enough. Sertoli cell vacuoles, swollen germ cell mitochondria, and a breakdown of the membrane between the Sertoli cell and the pachytene spermatocyte have been described. Within hours, death of (probably) those pachytene spermatocytes follows. EGME is metabolized to active intermediates methoxyacetaldehyde and methoxyacetic acid (MAA). Treating animals with MAA produces identical testicular lesions as that of the parent compound.

MATING BEHAVIOR IN THE RAT

Male and female rat mating behavior is sufficiently stereotyped that it can be easily quantified to assess the effects of toxicants on these behaviors. In spontaneously ovulating rodents, the endocrine events are comparable with those in the menstrual cycle. In the rabbit, the LH surge and ovulation is a neural reflex produced by copulation.

Cervix

There are regular changes in cervical mucus. Estrogen, which makes the mucus thinner and more alkaline, promotes the survival and transport of sperm. Progesterone makes the mucus thick, tenacious, and cellular. The mucus is thinnest at the time of ovulation and dries in an arborizing,

fernlike pattern on a slide. After ovulation and during pregnancy, it becomes thick and fails to form the fern pattern. Disruptions of the cervix may be expressed as disorders of differentiation (including neoplasia), disturbed secretion, and incompetence. Oral contraceptives can affect the extent and pattern of cervical mucus.

Vagina

Estrogen produces a growth and proliferation of vaginal epithelium and the layers of cells become cornified. Progesterone stimulation produces thick mucus and the epithelium proliferates, becoming infiltrated with leukocytes. Cyclic changes in the vaginal smear in rats are easily recognized. Exposure to estrogenic toxicants can induce persistent vaginal cornification.

FERTILIZATION

To reach the oocyte, the sperm must penetrate the outer cumulus cell layer and inner zona pellucida layer. To facilitate these activities, sperm must be capacitated and the secretion of enzymes (hyaluronidases) allows the sperm to penetrate through the cumulus cells to the zona pellucida. This special extracellular matrix is composed of three glycoproteins and cell surface factors that cause the sperm to release secretory enzymes that enable the sperm to penetrate the zona pellucida and fuse with the oocyte plasma membrane. Genetic material in the male pronucleus eventually combines with the genetic material from the female to form the zygote.

IMPLANTATION

Implantation is an intricately timed event that can only occur when the embryo reaches the blastocyst stage and gains implantation competency and the uterus, through steroid hormone-dependent changes, attains a receptive state. This reciprocal interaction must occur between the blastocyst and uterus together with an increase in uterine vascular permeability at the site of blastocyst attachment. There are four stages that comprise early implantation in mammals: (1) apposition and adhesion of the blastocyst to the uterine lumen, (2) penetration of the epithelium, (3) decidualization of the stromal cells, and (4) trophoblastic invasion into the stromal vasculature. These four stages vary in length and in precise order in a species-specific manner.

PLACENTA

The placenta mediates exchanges between the mother and fetus and maternal tolerance of antigens produced by the fetus. There are a huge number of different placental types across eutherian mammals. Primates, including humans, have three layers of cells in the placenta that a substance must pass across.

Generally, the placenta is quite impermeable to chemicals with molecular weights of 1000 Da or more. Because most medications and xenobiotics have molecular weights of 500 Da or less,

molecular size is rarely a factor in a drug's movement across the placenta and into the embryo/fetus. Placental permeability of a chemical is affected by placental thickness, surface area, carrier systems, and lipid-protein concentration of the membranes. Inherent characteristics of the chemical such as its degree of ionization, lipid solubility, protein binding, and molecular size also affect its transport across the placenta. Various transporter families in the placenta contribute to the passage of xenobiotics across the placenta.

PREGNANCY

Because the transition from early to mid-pregnancy in the rat requires hormones from the feto-placental unit, if implantation or uterine decidualization is blocked by a chemical, then the female would resume her estrous cycles and the corpora lutea would regress. Chemicals that induce whole-litter loss at mid-to-late pregnancy may cause abortions in some of the females, whereas others fail to deliver and appear pregnant for an unusually long period of time. Many abortifacients induce pregnancy loss by reducing progesterone levels in the rat. Generally, reducing mid-pregnancy progesterone levels by half or more is sufficient to terminate pregnancy.

PARTURITION

Parturition is a complex process involving fetal, placental, and maternal signals, that is best thought of as a release from the inhibitory effects of pregnancy on the myometrium of the uterus rather than an active process, although the timing and order of the precise events is an active process. For most mammals, the uterus is held in a quiescent state by high levels of progesterone and it is the decrease of progesterone that provides the trigger for parturition. In humans, this does not seem to be the case.

LACTATION

Endocrine control of lactation is very complex. Mammogenesis, lactogenesis, galactopoiesis, and galactokinesis are all essential to assure proper lactation. Prolactin seems to be the single most important galactopoietic (milk synthesis) hormone. Oxytocin, serotonin, opioids, histamine, substance P, and arginine–leucine modulate prolactin release by means of an autocrine/paracrine mechanism, whereas estrogen and progesterone hormones can act at the hypothalamic and adenohypophysial levels. Human placental lactogen and growth factors work to assure successful lactation, with oxytocin being the most powerful galactokinetic (milk ejection) hormone.

SENESCENCE

Reproductive senescence is usually preceded by a dysregulation of the HPG axis, which leads to alterations in serum HPG hormones, accompanied by an upregulation in GnRH, LH, and activin

activities and a decrease of steroids in the brain. In females, reproductive senescence is associated with a transition from regular to irregular estrus (menstrual) cycles leading to acyclicity and ultimately a loss of fertility. A decrease in androgen is noted in around 20% of fit 60-year-old men, but the value of androgen supplementation is not clear with regard to reproductive senescence.

ENDOCRINE DISRUPTION (INCLUDING SCREENING AND PUBERTY)

Currently, the potential effects of “endocrine disrupting chemicals” (EDCs) on human health and the proven effects of EDCs on wildlife are a major focus among the scientific community. It has been suggested that in utero exposure to environmental estrogens, antiandrogens or chemicals like phthalates, or 2,3,7,8-TCDD could be responsible for the reported 50% decline in sperm counts in some areas and the apparent increase in cryptorchid testes, testicular cancer, and hypospadias.

Phthalate ester exposures have been associated with reduced AGD in boys and lower testosterone levels in men. In females, exposure to EDCs during development could contribute to earlier age at puberty and to increased incidences of endometriosis and breast cancer. In wildlife toxicology and ecosystem health, clear-cut cause and effect relationships exist between exposure to EDCs and adverse effects in several vertebrate classes from fish to mammals. Subtle, low-dose reproductive effects of endocrine disrupters will be difficult to detect in typical epidemiological studies because of the high variability normally seen in human reproductive function, the delayed appearance of the reproductive lesions and a lack of high quality exposure data. The list of EDCs includes phytosterols, estrogens, antibiotics, β-blockers, antiepileptics, and lipid-regulating agents.

Are Nonmonotonic Dose–Response Curves (NMDRs) Common After Estrogen or Androgen Signaling Pathway Disruption?

Reports of U-shaped (nonmonotonic), ultra-low-dose effects and nonthreshold effects for EDCs are challenging some of the basic assumptions of risk assessment for noncancer endpoints. While the focus of this debate has centered on the low-dose effects of bisphenol A, well-documented U-shaped dose–response curves are known from many in vitro and some in vivo studies. For some EDCs, the timing of exposure dictates not only the effect, but also whether the effects are adverse or beneficial. Even when administered during adult life, drugs with EDC-activity can simultaneously have a beneficial effect on one tissue and an adverse effect on another. The shape of the dose–response curve can be affected by factors such as (a) life stage, (b) route of exposure, (c) target tissue, (d) species differences in E and A pathways or ADME, (e) gut microbiome, (f) concurrent exposure to other chemicals or nonchemical stressors, and (g) background contaminant levels of ubiquitous chemicals.

Known Effects of EDCs in Humans and Animals

Chemicals known to affect humans, domestic animals, and/or wildlife via functional developmental toxicity or endocrine mechanisms include 2,3,7,8-TCDD, PCBs and polychlorinated dibenzofurans (PCDFs), methylmercury, EE, alkylphenols, plant sterols, fungal estrogens, androgens, chlordecone, DBCP, *o*,*p*′-DDD (Mitotane), *o*,*p*′-DDT, and *p*,*p*′-DDE. Additionally, over 30 different drugs taken during pregnancy alter human development through endocrine disruption. EDCs alter human development via several mechanisms besides the estrogen receptor (ER): this includes binding to AR or retinoic acid (RAR, RXR) receptors, and by inhibition of steroidogenic enzymes or the synthesis of thyroid hormones.

Effects of Drugs on Human Sexual Differentiation—Exposure to hormonally active chemicals during sex differentiation can produce pseudohermaphroditism. Androgenic drugs such as danazol and methyltestosterone can masculinize human females causing “female pseudohermaphroditism.” The drug aminoglutethimide also masculinizes human females following in utero exposure.

Transplacental exposure of the developing fetus to DES causes clear cell adenocarcinoma of the vagina, as well as gross structural abnormalities of the cervix, uterus, and fallopian tube. DES-exposed women are more likely to have an adverse pregnancy outcome, including spontaneous abortions, ectopic pregnancies, and premature delivery. Pathological effects that develop in males following fetal DES exposure may result from an inhibition of androgen action (underdevelopment or absence of the vas deferens, epididymis, and seminal vesicles) and anti-Müllerian duct factor (persistence of the Müllerian ducts). DES may also have caused epididymal cysts, hypotrophic testes, and infertility in males.

Effects of Plant and Fungal Products in Animals and Humans—Over 400 species of plants contain potentially estrogenic isoflavonoids or coumestans. Although most naturally occurring environmental estrogens are relatively inactive, the phytoestrogen miroestrol is almost as potent as estradiol in vitro and even more potent than estradiol when administered orally. Many plant estrogens occur in such high concentrations that they induce reproductive alterations in domestic animals. “Clover disease,” which is characterized by dystocia, prolapse of the uterus, and infertility, is observed in sheep grazed on highly estrogenic clover pastures. Permanent infertility (defeminization) can be produced in ewes by much lower amounts of estrogen over a longer time period than are needed to produce “clover disease.” Feeds contaminated with the zearalenone-producing fungus (*Fusarium* spp.) induce adverse reproductive effects in domestic cows, swine, and turkeys.

Effects of Organochlorine Compounds in Humans—Several pesticides and toxic substances have been shown to alter human reproductive function. An accidental high-dose in utero exposure to PCBs and PCDFs has been associated with reproductive alterations in boys, increased stillbirths, low birth weight, malformations, and IQ and behavioral deficits. In addition, subtle adverse effects were seen in infants and children exposed to relatively low levels of PCBs and PCDFs.

One metabolite of DDT, *o*,*p*′-DDD (mitotane), is used to treat adrenal steroid hypersecretion associated with adrenal tumors because it reduces adrenal androgen production. In addition, lower doses of mitotane restored menstruation in women with spanomenorrhea associated with hypertrichosis.

Occupational Exposures—Occupational exposure to pesticides and other toxic substances (i.e.,

chlordecone and DBCP) in the workplace have been associated with reduced fertility, lowered sperm counts, and/or endocrine alterations in male workers. Workers exposed to high levels of chlordecone, an estrogenic and neurotoxic organochlorine pesticide, displayed severe neurotoxicity and abnormal testicular function. Male workers involved in the manufacture of 4,4′-diaminostilbene-2,2′-disulfonic acid (DAS), a key ingredient in the synthesis of dyes and fluorescent whitening agents, had lower serum testosterone levels and reduced libido as compared to control workers.

Environmental Androgens

Androgenic activity has been detected in several complex environmental mixtures. There are pulp and paper mill effluents (PME) include a chemical mixture that binds to the AR and induces androgen-dependent gene expression in vitro. This mode of action is consistent with the masculinized female mosquitofish (*Gambusia holbrooki*) collected from contaminated sites. Male-biased sex ratios of fish embryos have been reported in broods of eelpout (*Zoarces viviparus*) in the vicinity of a large kraft pulp mill on the Swedish Baltic coast, suggesting that masculinizing compounds in the effluent were affecting gonadal differentiation. Effluents from beef cattle concentrated animal feeding operations have been shown to display androgenicity.

Environmental Antiandrogens

Fungicides—Vinclozolin and procymidone act as AR antagonists. These pesticides, or their metabolites, competitively inhibit the binding of androgens to AR, leading to an inhibition of androgen-dependent gene expression in vitro and in vivo. Administration of vinclozolin during sexual differentiation demasculinizes and feminizes the male rat offspring such that treated males display female-like AGD at birth, retained nipples, hypospadias, suprainguinal ectopic testes, a blind vaginal pouch, and small to absent sex accessory glands.

Procymidone induces shortening of the AGD in male pups, and older males display retained nipples, hypospadias, cryptorchidism, cleft phallus, a vaginal pouch, and reduced sex accessory gland size. Procymidone also induces fibrosis, cellular infiltration, and epithelial hyperplasia in the dorsolateral and ventral prostatic and seminal vesicular tissues in adult offspring.

Prochloraz disrupts reproductive development and function by inhibiting the steroidogenic enzymes 17,20-lyase and aromatase and it is an AR antagonist. Prenatal prochloraz reduces fetal testis testosterone and increases progesterone without affecting Leydig cell insulin-like 3 (insl3) mRNA levels. Prenatal prochloraz treatment delayed parturition and altered reproductive development in male offspring in a dose-related manner. Treated males displayed reduced AGD and female-like areolas and high-dose males displayed hypospadias.

Linuron (Herbicide)—The herbicide binds rat and human AR and inhibits DHT-hAR induced gene expression in vitro. In utero linuron exposure produces male rat offspring that display epididymal and testicular abnormalities. The syndrome for linuron more closely resembles the effects seen with in utero phthalates that inhibit fetal Leydig cell insl3 hormone levels. Fetal testosterone production is significantly reduced in linuron-treated fetal males demonstrating that linuron is antiandrogenic via dual mechanisms of action.

p,—p′*-DDE (Pesticide Metabolite)—p,p′*-DDE displays AR antagonism both in vivo and in

vitro and inhibits androgen-dependent gene expression. In vivo, *p,p′*-DDE delays pubertal development in male rats and inhibits androgen-stimulated tissue growth. *p,p′*-DDE administered to Long-Evans Hooded and Sprague–Dawley male rats in utero reduces AGD, induces nipples, and permanently reduces androgen-dependent organ weights.

Phthalates (Plasticizers and Solvents)—In utero, some phthalate esters alter development of the male rat reproductive tract at relatively low dosages. Prenatal exposure to DBP, benzyl butyl phthalate (BBP), di-isononyl phthalate (DINP), and DEHP treatment cause a syndrome of effects, including underdevelopment and agenesis of the epididymis and other androgen-dependent tissues and testicular abnormalities. The phthalates are unique in their ability to induce agenesis of the gubernacular cords, a tissue whose development depends on the peptide hormone insulin-like peptide-3 (insl3). Phthalates reduce both insl3 mRNA and testosterone levels during sexual differentiation of the male rat.

DBP also disrupts reproductive function in the rabbit. After in utero exposure, male offspring exhibited reduced numbers of ejaculated sperm, testis weight, and accessory sex gland weight.

Environmental Estrogens

Methoxychlor (M) produces estrogen-like effects on the male and female rats. This pesticide requires metabolic activation in order to display full endocrine activity in vitro. The active metabolites bind ER and activate estrogen-dependent gene expression in vitro and in vivo in the female rat, thereby stimulating a uterotrophic response, accelerating VO and inducing constant estrus, and reducing infertility. In the ovariectomized female rat, M also induces estrogen-dependent reproductive and nonreproductive behaviors including female sex behaviors, running wheel activity, and food consumption.

EE is a synthetic derivative of estradiol that is in almost all modern formulations of combined oral contraceptive pills. EE is found in many aquatic systems contaminated by sewage effluents, originating principally from human excretion. Thus, EE plays a major role in causing widespread endocrine disruption in wild populations of fish species and other lower vertebrate species.

EDC Screening Programs

The Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) proposed a tiered screening and testing strategy for EDCs, including a process to prioritize chemicals for (a) evaluation and recommendations, (b) screening (Tier 1), and (c) testing (Tier 2) batteries. The recommended screening battery was designed to detect alterations of HPG function; estrogen, androgen, and thyroid hormone synthesis; and androgen receptor (AR) and estrogen receptor (ER)-mediated effects on mammals and other taxa.

In Vivo Mammalian Assays—EDSTAC recommended the laboratory rat as the species of choice for the endocrine screening and testing assays. The EDSTAC proposed three short-term in vivo mammalian assays for the Tier 1 Screening (T1S) Battery: the uterotrophic, Hershberger, and pubertal female rat assays.

***Uterotrophic Assay*—**Estrogen agonists and antagonists are detected in a 3-day uterotrophic assay using subcutaneous administration of the test compound. The selected uterotrophic assays

for estrogens and antiestrogens use either the intact juvenile or the castrated ovariectomized adult/juvenile female rat.

***Hershberger Assay*—**In this assay, weights of the ventral prostate, Cowper's glands, seminal vesicle (with coagulating glands and fluids), glans penis, and levator ani/bulbocavernosus muscles are quantified in castrated, testosterone-treated (or untreated) male rats after 10 days of oral treatment with the test compound. This assay is very sensitive for detection of androgens and antiandrogens.

***Pubertal Female Rat Assay*—**In the pubertal female rat assay, weanling female rats are dosed daily by gavage for 21 days while the age at VO (puberty) is monitored. The females are necropsied at about 42 days of age. This assay detects alterations in thyroid hormone status, HPG function, inhibition of steroidogenesis, estrogens, and antiestrogens, and has been found to be highly reproducible and very sensitive to certain endocrine activities including estrogenicity, inhibition of steroidogenesis, and antithyroid activity.

Alternative Screening Assays—Alternative in vivo assays, if they are of sufficient sensitivity, specificity, and relevance, might replace or augment current Tier 1 assays.

***Pubertal Male Rat Assay*—**This assay detects alterations of thyroid function, HPG maturation, steroidogenesis, and altered steroid hormone function (androgen). Intact weanling males are exposed to the test substance for approximately 30 days. The age at puberty is determined by measuring the age at PPS, and reproductive tissues are evaluated, and serum taken for hormonal analyses.

***In Utero Lactational Assay*—**The EDSTAC recommended that the EPA develop and evaluate an in utero lactational assay due to the unique sensitivity of the fetal reproductive system to disruption by some toxicants. One version of the assay takes about 80 days and uses approximately 10 litters per group (120 to 150 pups). In this protocol, androgens and antiandrogens can be detected in approximately 2 to 3 weeks, and EDCs with antithyroid activity can be detected in infant or weanling offspring after 4 to 5 weeks of maternal treatment.

Mixtures of EDCs

Humans and wildlife are exposed to mixtures of chemicals in the exposome, including pesticides, toxic substances, drugs, natural products and food additives in our diet, and endogenous hormones. Some of these chemicals are known reproductive toxicants.

Because we cannot test the toxicity of every mixture in the exposome, another approach is to develop statistical models that accurately predict how untested mixtures will behave. This approach involves mixing chemicals whose individual reproductive toxicity is well characterized to determine what mixture model provides the most accurate prediction of how the chemicals in the mixture interact.

A significant preponderance of data from several laboratories studying the effects of mixtures on male rat reproductive development indicates that chemicals that disrupt common target organs like the genitalia, epididymis, or testis are accurately predicted using dose addition models, regardless of the mechanism of toxicity. In contrast, response addition models may severely underestimate the effects of mixtures on the male reproductive tract. Additional studies are

warranted to determine how low-dose levels of mixtures behave and to examine additional pathways beyond those studied to date.

TESTING FOR REPRODUCTIVE TOXICITY

Screens and Multigeneration Studies

Comprehensive testing for reproductive toxicity normally involves simultaneous exposures of both males and females. Only with specific protocol amendments will the affected sex(es) be determined (e.g., exposure of one sex, or a cross-over mating study design in which treated males and females are mated with the corresponding control animals). The rat is the most common species employed in reproductive toxicity studies. Suitable amendments can be made for other species (the mouse is sometimes used) and nonhuman primates occasionally employed —particularly for testing drugs.

Significant attention has focused on the development of "screens" for reproductive toxicity to evaluate the 80,000 chemicals used in commerce. The screens currently employed have been developed to prioritize chemicals for more comprehensive testing. Such approaches can identify chemicals that have adverse effects on reproductive function. The outcomes from such screens can be summarized as "*a positive response is a positive, but a negative response is a maybe.*"

The most comprehensive assessment of reproductive toxicity would be provided by a protocol that exposes the animal model throughout the reproductive cycle (Fig. 21–1) and involves the assessment of multiple endpoints at different life stages during this continuous exposure. The protocol and guideline coming closest to this ideal is the multigeneration reproduction study used for the assessment of chemicals, pesticides, and some food additives. (See Fig. 21–9.) In general, parental (F_0) animals are exposed for approximately 10 weeks prior to mating (based on the duration of the spermatogenic wave of 8 weeks and the passage of sperm through the epididymis and the availability of mature sperm for fertilization). Exposure continues through mating (and after the mating pairs are separated), gestation, birth, and lactation. Litters may be "standardized" to ensure equal lactational demand on the dams and normalize the growth of pups (litters are usually reduced to four males and four females per litter on PND 4). At weaning, F_1 litters are usually culled to one male and one female that are raised and exposed until adulthood and the exposure continues through the same processes in the second breeding generation, which usually halts at weaning of the F_2 pups. These studies normally have at least three dose levels (with the highest dose level designed to induce some toxicity) and at least 20 litters produced per dose group. Measurements of reproductive performance include number of pregnant females from the number of pairs mated, number of females producing a litter, litter size, number of live pups with their birth weights, and sex. Measurement of growth and analysis of the reproductive organs in the F_0 parental generation are conducted (including specific evaluations of ovarian follicles, estrous cyclicity, and sperm parameters). Similar measurements to those undertaken for the F_0 are made on the F_1 parents, but in addition offspring are normally carefully examined at birth (and sexually dimorphic endpoints may be collected such as AGD), at weaning, and at puberty (particularly the assessment of VO and time of first estrus in females and balanopreputial separation in males) in addition to the adult measurements of reproductive performance, organ weights, histology, etc.

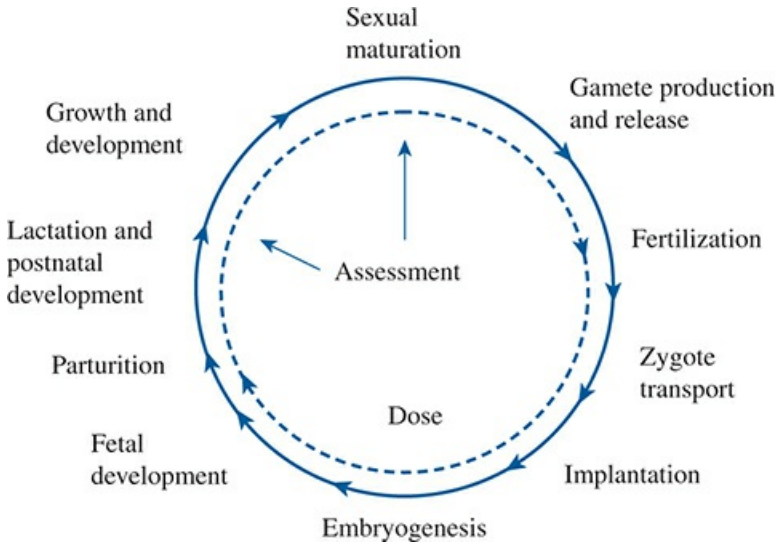

FIGURE 21–9 *Multigeneration reproduction study.*

Testing for Endocrine Disrupting Chemicals

In a tiered screening and testing approach, only chemicals that display positive reproducible responses in Tier 1 screening (T1S) would continue evaluation. In Tier 2 testing (T2T), issues of dose–response, relevance of the route of exposure, sensitive life stages, and adversity are resolved. AGD at birth and nipple/areola retention in infant female and male rats should be included in testing of androgens and antiandrogens, respectively, because they are sensitive, potentially permanent effects that are highly correlated with malformations and reproductive organ weight changes later in life. These early alterations constitute part of the antiandrogen-induced developmental syndromes.

Developmental Syndromes and Tailored Testing—A careful evaluation of the male rat offspring allows one to distinguish the "phthalate syndrome," in which effects on reproductive development involve a decrease in fetal testicular testosterone and insl-3 peptide hormone biosynthesis from the "AR antagonist syndrome," induced by vinclozolin or flutamide. The main distinction between the two syndromes is that the phthalate syndrome includes agenesis of the testis, epididymis, and gubernacular cord. These lesions are rarely seen in the AR antagonist syndrome, even when all the males display hypospadias. In addition, of all chemicals that interfere with the androgen-signaling pathway in the fetal male rat, only the phthalates affect Leydig cell insl-3 hormone synthesis and cause undescended testes due to gubernacular agenesis.

Data should be summarized in a manner that clearly delineates the proportion of animals that are affected. In teratology studies, data are typically presented and analyzed in this manner, indicating the number of malformed/number observed on an individual and litter basis, whereas multigenerational studies are frequently presented and analyzed differently, even when clear teratogenic and other developmental responses are noted after birth. Multigenerational protocols are used in T2T because only these protocols expose the animals during all critical stages of development and examine reproductive function of offspring after they mature.

Although the new EPA multigenerational test provides for a comprehensive evaluation of the F_0 or parental generation, too few F_1 animals (offspring with developmental exposure) are

examined after maturity to detect anything but the most profound reproductive teratogens. F_0 animals within a dose group typically respond in a similar fashion to the chemical exposure; however, the response to toxicants in utero can vary greatly even within a litter with only a few animals displaying severe reproductive malformations in the lower dosage groups.

"Transgenerational" protocols typically use fewer litters (7 to 10 per dose group), but examine all animals in each litter. These protocols use fewer animals but provide enhanced statistical power to detect reproductive effects in the F_1 generation. The lifelong exposure of both males and females in the F_1 generation, which allows one to detect effects induced in utero, during lactation, or from direct exposure after puberty, can confound the identification of when the effect was induced (i.e., during adulthood vs. development) or even the affected sex.

Test Design and Numbers of F1 Animals—Testing should be tailored based on the pharmacological activity demonstrated in T1S. In addition, the developing fetus is not always the most sensitive life stage. Some EDCs disrupt pregnancy by altering maternal ovarian hormone production in F_0 dams at dosage levels that appear to be without direct effect on the offspring. In such cases, the standard EPA multigenerational protocol with minor enhancements would be recommended, or a transgenerational protocol with exposure continued after weaning. The transgenerational or in utero lactational protocols fill a gap in the testing program for EDCs that should be used only on a case-by-case basis, as indicated by the results of T1S and any Tier 1 repeat study.

Testing Pharmaceuticals

In the case of pharmaceuticals, it is rare for multigeneration studies to be conducted, because it is not common for all the population to use a specific drug and that exposure to the drug is not necessarily chronic and over many different life stages. Typically, three specific studies are undertaken:

1. *A study of fertility and early embryonic development* (Fig. 21–10). Parental adults are exposed to the test chemical for 2 weeks (females) or 4 weeks (males) prior to breeding and then during breeding. Females then continue their exposure till implantation. Males can be necropsied for the endpoints noted for the multigeneration studies after pregnancy has been confirmed, and for the pregnant females, necropsy takes place any time after mid-gestation. As with the multigeneration study, reproductive and target organs are weighed and examined histologically, sperm parameters are assessed in males and in females, the uterine implantation sites and ovarian *corpora lutea* are counted, as well as live and dead embryos.

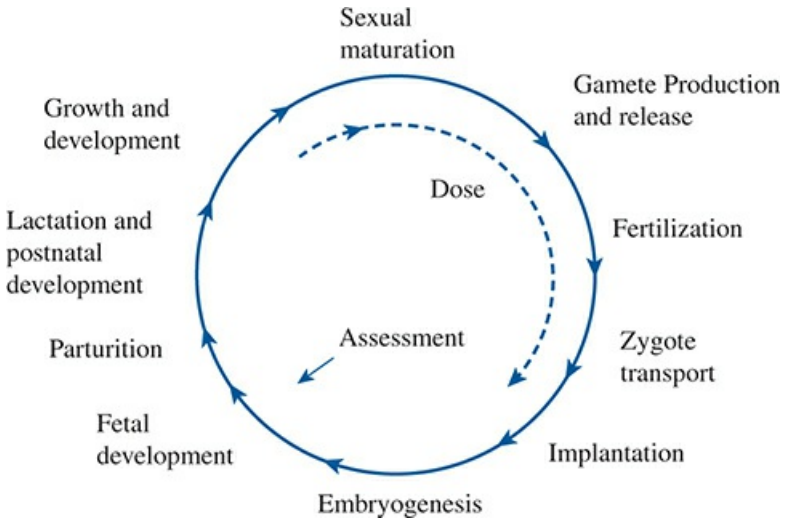

FIGURE 21–10 *Fertility and early embryonic study.*

2. *A study of effects on pre- and postnatal development including maternal function* (Fig. 21–11). In this study, pregnant females are exposed from implantation until weaning of their offspring (usually PND 21 in the rat). After cessation of exposure, selected offspring (one male and one female per litter) are raised to adulthood and then mated to assess reproductive competence. These animals are observed for maturation and growth (but are not exposed). Puberty indices, as employed in the multigeneration study, are quantified. In addition, sensory function, reflexes, motor activity, learning, and memory are also evaluated.

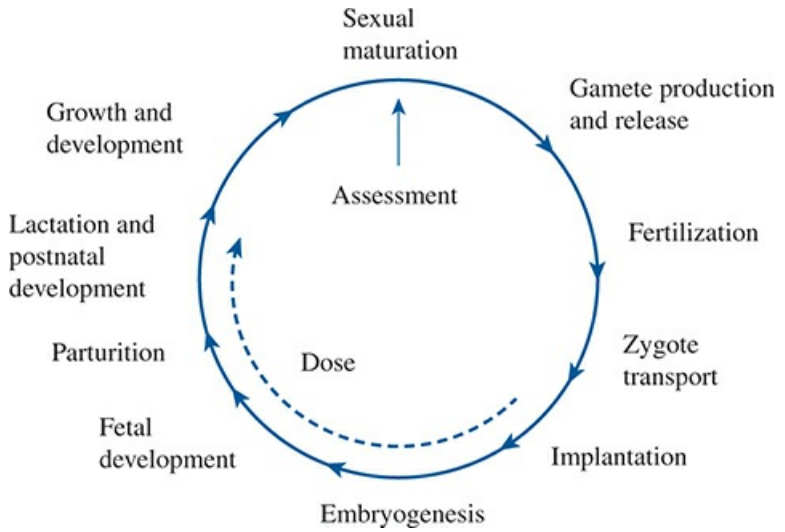

FIGURE 21–11 *Pre- and postnatal developmental toxicity study.* Dosing is from implantation until the litters are weaned.

3. *A study of embryo–fetal development* (Fig. 21–12). This study tests for enhanced toxicity relative to that noted in pregnant females and is normally conducted in two species (typically the rat and rabbit). Exposure occurs between implantation and closure of the hard palate (GD 6 to 15 in the rat), and females are killed just prior to parturition. At necropsy, dams are observed for any affected organs and *corpora lutea* are counted. Live and dead fetuses are

counted and examined for external, visceral, and skeletal abnormalities.

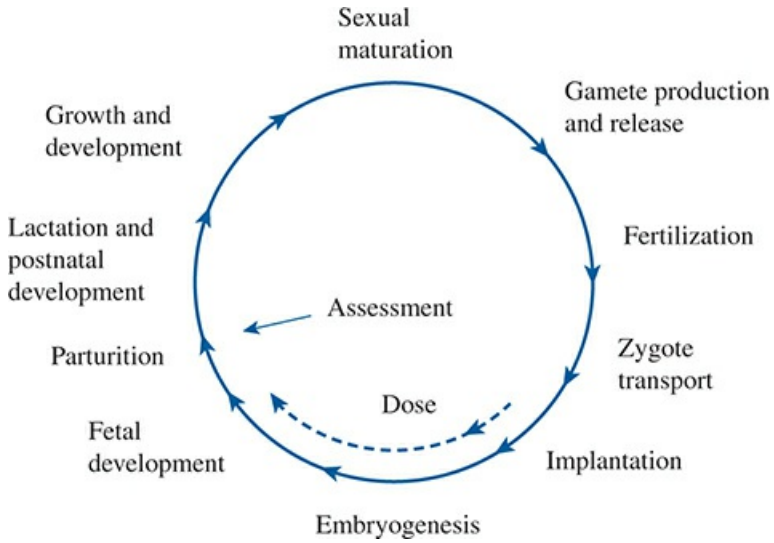

FIGURE 21–12 *Embryo–fetal developmental toxicity study as used by FDA guidelines.* Dosing starts at implantation and continues to closure of the hard palate (GD 6 to 15 in the rat) with an assessment of fetuses just prior to parturition.

One of three summary risk conclusions would be applied to the drug label: (1) the drug is not anticipated to produce reproductive and/or developmental effects above the background incidence for humans when used in accordance with the dosing information on the product label; (2) the drug may increase the incidence of adverse reproductive and/or developmental events; or (3) the drug is expected to increase the incidence of adverse reproductive and/or developmental effects in humans when used according to the product label.

Newer Guidelines and Approaches

As knowledge of critical windows of exposure has increased, particularly with the increased focus on chemicals that may have endocrine-like activity, there has been a larger focus on the evaluation of potential postnatal adverse outcomes. More functional endpoints (e.g., sperm and oocyte analysis, vaginal cytology, indices of puberty, and sexual differentiation) have been incorporated to improve the detection of chemicals affecting reproduction and the endocrine status of animals.

The new NTP design employs pregnant animals, with dosing commencing at implantation (gestation day 6 in the rat) and continually exposes the dams throughout gestation and lactation. After weaning (usually postnatal day 21 in the rat), the offspring would continue to be administered the test article at the same dose level as their respective dam. These cassettes are essentially protocols used on other standard studies and would normally include:

- Evaluation of target organ toxicity, pathology, clinical pathology, etc., similar to a current 90-day toxicity protocol—*a subchronic toxicity cohort*. This would normally require 10 animals to be evaluated per sex, per dose group.
- Evaluation of prenatal developmental toxicity—*a teratology cohort*. One male and female

offspring from each litter would be selected and nonsibling matings would be performed in each group on reaching sexual maturity (~PND 110). Just prior to expected delivery, a Cesarean section would be performed on the pregnant dams for a standard evaluation of external, visceral, and skeletal abnormalities of the fetuses.

- Evaluation of breeding performance—*a littering cohort*. One male and female offspring from each litter would be selected and nonsibling matings would be performed in each group on reaching sexual maturity (~PND 110). The pregnant dams would be allowed to deliver their litters and raise them to weaning.

This design emphasizes a full evaluation of the F_1 animals in the study. The design generates important information on both reproduction and postnatal development, together with a pathological evaluation of all the offspring (after PND 4) when they reach adulthood. A major addition will be the information achieved on prenatal developmental toxicity. The teratology and littering cohorts of animals allow the evaluation of fertility and fecundity and importantly, to maintain the relationship between structural changes in the reproductive organs and functional outcomes in the same animals. In addition, the design will be able to maintain a 10-week exposure period prior to mating of the F_1 animals to ensure that any potential male germ cell effects could be reflected in a functional outcome.

EVALUATION OF TOXICITY TO REPRODUCTION

Concordance of Endpoints

There are general points that an investigator should note in any estimation of potential reproductive toxicity:

- Adequacy of experimental design and conduct. Was there sufficient statistical power in the evaluation(s)?
- Occurrence of common versus rare reproductive deficits. Biological versus statistical significance.
- Use of historical control data to place concurrent control data into perspective and to estimate population background incidence of various reproductive parameters and deficits.
- Known structure–activity relationships for inducing reproductive toxicity.
- Concordance of reproductive endpoints (e.g., did a decrease in litter size relate to ovarian histology and changes in vaginal cytology?).
- Did the reproductive deficits become more severe with increases in dose? For example, did histological changes at one dose level become decrements in litter size and then reductions in fertility at higher dose levels in any generation?
- Did the reproductive deficits increase in prevalence (more individuals and/or more litters) with dose level in any generation?
- Decrements in reproductive parameters noted in the F_1 generation (and potentially later generations) that were not seen in the F_0 generation may suggest developmental, as well as reproductive, toxicity. Likewise, findings in an F_1 generation animal may (or may not) be

reproduced in F_2 offspring. For example, effects in the F_1 generation on reproductive parameters may have resulted in the selection out of sensitive animals in the population, thus not producing F_2 offspring for subsequent evaluation.

Consistency Across Generations

The F_0 reproductive parameters can differ markedly from those noted in the F_1 and similarly from the F_2 generation in a multigeneration study. Because exposure in a multigeneration study typically starts with the F_0 generation as young adults, critical periods of reproductive development have already taken place. Differences between F_1 and F_2 generations can also arise. Here the exposure duration and critical windows of development are the same. However, since one normally only takes one male and female from each litter to generate the F_1 and F_2 parents, it is distinctly possible that a selection bias can exist. Other specific effects may increase severity in the F_2 versus the F_1.

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Waters MD, Hughes CL. *Translational Toxicology and Therapeutics: Windows of Developmental Susceptibility in Reproduction and Cancer*. Hoboken, NJ: John Wiley & Sons; 2018.

QUESTIONS

1. Which of the following cell types secretes anti-Müllerian hormone (AMH)?
   - **a.** spermatogonium.
   - **b.** Leydig cell.
   - **c.** Sertoli cell.
   - **d.** primary spermatocyte.
   - **e.** spermatid.

2. Penile erections are dependent on:
   - **a.** the CNS.
   - **b.** sympathetic nerve stimulation.
   - **c.** helicine (penile) artery constriction.
   - **d.** corpora cavernosa smooth muscle relaxation.
   - **e.** a spinal reflex arc.

3. The corpus luteum is responsible for the secretion of which of the following hormones

during the first part of pregnancy?

a. estradiol and hCG.
b. progesterone and estradiol.
c. progesterone and hCG.
d. FSH and LH.
e. FSH and progesterone.

4. All of the following statements regarding the hypothalamo-pituitary–gonadal axis are true EXCEPT:

a. FSH increases testosterone production by the Leydig cells.
b. FSH and LH are synthesized in the anterior pituitary.
c. Estradiol provides negative feedback on the hypothalamus and the anterior pituitary.
d. GnRH from the hypothalamus increases FSH and LH release from the anterior pituitary.
e. The LH spike during the menstrual cycle is responsible for ovulation.

5. Which of the following statements is FALSE regarding gametal DNA repair?

a. DNA repair in spermatogenic cells is dependent on the dose of chemical.
b. Spermiogenic cells are less able to repair damage from alkylating agents.
c. Female gametes have base excision repair capacity.
d. Meiotic maturation of the oocyte decreases its ability to repair DNA damage.
e. Mature oocytes and mature sperm no longer have the ability to repair DNA damage.

6. Reduction division takes place during the transition between which two cell types during spermatogenesis?

a. spermatogonium and primary spermatocyte.
b. primary spermatocyte and secondary spermatocyte.
c. secondary spermatocyte and spermatid.
d. spermatid and spermatozoon.
e. spermatozoon and mature sperm.

7. Which of the following cell types is properly paired with the substance that it secretes?

a. ovarian granulosa cells—progesterone.
b. Leydig cells—ABP.
c. ovarian thecal cells—estrogens.
d. Sertoli cells—testosterone.
e. gonadotroph—LH.

8. Which of the following statements regarding male reproductive capacity is FALSE?

a. Klinefelter’s syndrome males are sterile.
b. FSH levels are often measured in order to determine male reproductive toxicity of a particular toxin.
c. Divalent metal ions, such as Zn, Hg, and Cd, can affect male reproduction.
d. The number of sperm produced per day is approximately the same in all males.

e. ABP is an important biochemical marker for testicular injury.

9. Reduction of sperm production can be caused by all of the following diseases EXCEPT:
   a. hypothyroidism.
   b. measles.
   c. Crohn's disease.
   d. renal failure.
   e. mumps.

10. Of the following, which is LEAST likely to be affected by estrogen?
   a. nervous system.
   b. musculoskeletal system.
   c. digestive system.
   d. cardiovascular system.
   e. urinary system.

UNIT 5 TOXIC AGENTS

CHAPTER 22

Toxic Effects of Pesticides

Lucio G. Costa

KEY POINTS

- A pesticide may be defined as any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating any pest.
- Pesticide exposures include (1) accidental and/or suicidal poisonings; (2) occupational exposure (manufacturing, mixing/loading, application, harvesting, and handling of crops); (3) bystander exposure to off-target drift from spraying operations; and (4) the general public who consume food items containing pesticide residues.
- Chemical insecticides in use today poison the nervous systems of the target organisms.
- An herbicide is any compound that is capable of either killing or severely injuring plants.
- A fungicide is any chemical capable of preventing growth and reproduction of fungi.

INTRODUCTION

Pesticides can be defined as any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating pests. Pests can be insects, rodents, weeds, and a host of other unwanted organisms. The most common classification of pesticides relies on the target species

such as insecticides (insects), herbicides (weeds), fungicides (fungi, molds), rodenticides (rodents), acaricides (mites), molluscicides (snails, other mollusks), miticides (mites), larvicides (larvae), and pediculicides (lice). For regulatory purposes, plant growth regulators, repellents, and attractants (pheromones) often fall in this broad classification of chemicals.

ECONOMICS AND PUBLIC HEALTH

The use of pesticides must consider the balance of the benefits that may be expected versus the possible risks of injury to human health or degradation of environmental quality. Pesticides play a major role in the control of vector-borne diseases, which represent a major threat to the health of large human populations. When introduced in 1942, DDT appeared to hold immense promise. However, because of its bioaccumulation in the environment and its effects on bird reproduction, DDT was eventually banned in most countries by the mid-1970s. In South Africa, DDT was banned in 1996 and less than 10,000 cases of malaria were registered in that country. By 2000, cases of malaria had increased to 62,000, but with the reintroduction of DDT at the end of that year, cases were down to 12,500 thus indicating its utility in controlling disease.

Use of Pesticides

Introduction of integrated pest management approaches and the increased popularity of organic farming have contributed, at least in the developed countries, to a decrease and/or stabilization of pesticide use. Pesticides are often used as formulations, in which the active ingredient is present together with other ingredients to allow mixing, dilution, application, and stability. These other ingredients are lumped under the term “inert” or “other” and may be emulsifiers, solvents, carriers, aerosol propellants, fragrances, and dyes. Though non-pesticidal, inert ingredients may not always be devoid of toxicity.

Exposure

Exposure to pesticides can occur via the oral or dermal routes, or by inhalation. High oral doses, leading to severe poisoning and death, are achieved as a result of pesticide ingestion for suicidal intents, or of accidental ingestion, commonly due to storage of pesticides in improper containers. Chronic low doses are consumed by the general population as pesticide residues in food, or as contaminants in drinking water. Regulations exist to ensure that pesticide residues are maintained at levels below those that would cause any adverse effect. Workers involved in the production, transport, mixing and loading, application of pesticides, and harvesting pesticide-sprayed crops are at highest risk for pesticide exposure. Dermal exposure during normal handling or application of pesticides, or in case of accidental spillings, occurs in body areas not covered by protective clothing, such as the face or the hands. Furthermore, deposition of pesticides on clothing may lead to slow penetration through the skin and/or to potential exposure of others, if clothes are not changed and washed upon termination of exposure.

Human Poisoning

Pesticides are not always selective for their intended target species, and adverse health effects can occur in nontarget species. In the general population and in occupationally exposed workers, recurring concerns relate to a possible association between pesticide exposure and increased risk of cancer, neurodegenerative diseases, endocrine disruptors, and reproductive and developmental toxicity. Globally, the major problem with pesticides remains that of acute human poisoning.

The World Health Organization (WHO) has recommended a classification of pesticides by hazard, where acute oral or dermal toxicities in rats are considered (Table 22–1), which is similar to the "Globally Harmonized System of Classification and Labelling of Chemicals" (GHS). As a class, the insecticides are the most acutely toxic followed by herbicides and fungicides.

TABLE 22–1 WHO-Recommended Classification of Pesticides by Hazard

Class		Ld_{50} In Rat (Mg/Kg Body Weight)	
		Oral	Dermal
Ia	Extremely hazardous	<5	<50
Ib	Highly hazardous	5–50	50–200
II	Moderately hazardous	50–200	200–2000
III	Slightly hazardous	Over 200	Over 2000
U	Unlikely to present hazard	Over 5000	Over 5000

Data from IPCS (International Programme on Chemical Safety). *The WHO Recommended Classification of Pesticides by Hazard and Guidelines to Classification: 2009*. Geneva: World Health Organization; 2010:78.

Regulatory Mandate

The awareness that pesticides may pose potential health hazards has led to regulatory measures to ensure their safe use and the protection of the population. In the United States, the Environmental Protection Agency (EPA) registers pesticides for use under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) and establishes maximum allowable levels of pesticide residues (tolerances) in foods and animal feeds under the Federal Food, Drug and Cosmetic ACT (FFDCA).

The Food Quality and Protection Act (FQPA) provided the statutory mandate, under FIFRA, for continuing the expedited consideration of applications for pesticides that may provide reduced risks for human health, nontarget species, and the environment. Under FQPA, pesticide residues are excluded from the definition of food additive, and the Delaney clause no longer applied to residues in food. Also, the EPA was mandated to assess aggregate risks based on dietary consumption patterns of children, possible susceptibility of infants and children to pesticides, and cumulative effects of compounds that share the same mechanism of toxicity. In the absence of adequate data, an additional default 10-fold safety factor was applied to ensure

children's safety.

Pesticides sold or distributed in the United States must be registered by the EPA. To register a formulated product, about 140 studies are required, a process that can take about 10 years and anywhere between $100 and $250 million. The database should include information on product and residue chemistry, environmental fate, toxicology, biotransformation/degradation, occupational exposure and reentry protection, spray drift, environmental impact on nontarget species (birds, mammals, aquatic organisms, plant, and soils), environmental persistence and bioaccumulation, and product performance and efficacy (Table 22–2).

TABLE 22–2 Basic Toxicology Testing Requirements for Pesticide Registration

Test	Animal Species*
Acute lethality (oral, dermal, inhalation)	Rat, mouse, guinea pig, rabbit
Dermal irritation	Rabbit, rat, guinea pig
Dermal sensitization	Guinea pig
Eye irritation	Rabbit
Acute delayed neurotoxicity	Hen
Genotoxicity studies (in vitro, in vivo)	Bacteria, mammalian cells, mouse, rat, *Drosophila*
Teratogenicity	Rabbit, rodent (mouse, rat, hamster)
Two-four week toxicity study (oral, dermal, inhalation)	Rat, mouse
90-Day toxicity study (oral)	Rat
Chronic toxicity study (oral; 6 months to 2 years)	Rat, dog
Oncogenicity study	Rat, mouse
Reproductive/fertility study	Rat
Developmental neurotoxicity study	Rat

**Substantial efforts are being devoted to develop alternative nonanimal test systems. Nevertheless, only limited in vitro tests (for primary skin irritation) have been validated and accepted by regulatory bodies.*

Canada, Japan, and most European countries have legislated procedures for registration of pesticides. The European Union (EU) has created a framework for pesticide regulation. The WHO provides guidance, particularly with the setting of acceptably daily intake (ADI) values for pesticides.

INSECTICIDES

Insecticides play a most relevant role in the control of insect pests, particularly in developing countries. All chemical insecticides in use today are neurotoxicants that poison the nervous systems of the target organisms (Table 22–3). The central nervous system (CNS) of insects is highly developed and not unlike that of mammals. As a class, insecticides have higher acute toxicity toward nontarget species compared to other pesticides. Some of them, notably the organophosphates, are involved in a great number of human poisonings and deaths each year.

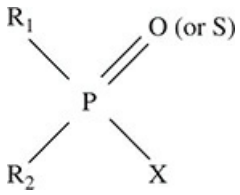
TABLE 22–3 Molecular Targets of the Major Classes of Insecticides

Target	Insecticide	Effect
Acetylcholinesterase	Organophosphates	Inhibition
	Carbamates	Inhibition
Sodium channels	Pyrethroids (Type I and II)	Activation
	DDT	Activation
	Dihydropyrazoles	Inhibition
Nicotinic acetylcholine receptors	Nicotine	Activation
	Neonicotinoids	Activation
GABA receptors–gated chloride channels	Cyclodienes	Inhibition
	Phenylpyrazoles	Inhibition
	Pyrethroids (Type II)	Inhibition
Glutamate-gated chloride channels*	Avermectins	Activation
Octopamine receptors†	Formamidines	Activation
Mitochondrial complex I	Rotenoids	Inhibition
Ryanodine receptors	Diamides	Activation

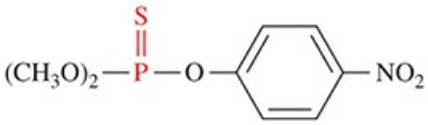
*Found only in insects. In mammals avermectins activate $GABA_A$ receptors.
†In mammals, formamidines activate α_2-adrenoceptors.

Organophosphorus Compounds

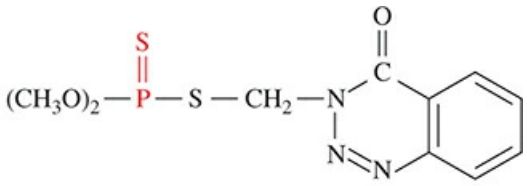
The general structure of OP insecticides can be represented by



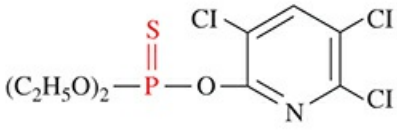
where X is the so-called "leaving group," which is removed when the OP phosphorylates acetylcholinesterase (AChE), and is the most sensitive to hydrolysis; R_1 and R_2 are commonly alkoxy groups (i.e., OCH_3 or OC_2H_5) or other chemical substitutes; either an oxygen or a sulfur (in this case the compound should be defined as a phosphorothioate) is also attached to the phosphorus with a double bond. Based on chemical differences, OPs can be divided into several subclasses, which include phosphates, phosphorothioates, phosphoramidates, phosphonates, and others. Figure 22–1 shows the chemical structures of some commonly used OPs.



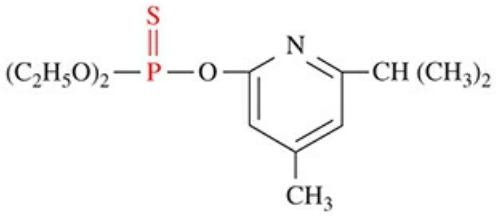
Methylparathion



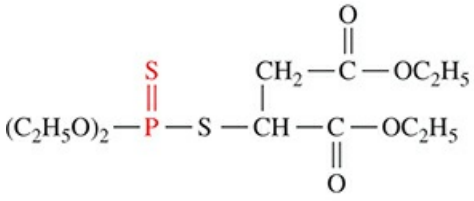
Azinphos-methyl (Guthion)



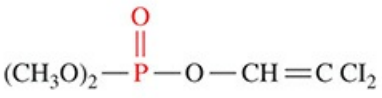
Chlorpyrifos



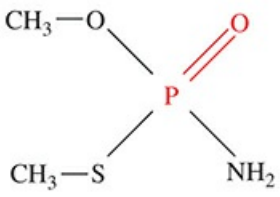
Diazinon



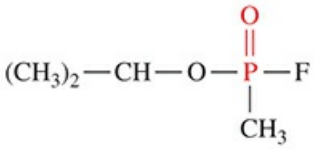
Malathion



Dichlorvos



Metamidophos



Sarin

FIGURE 22–1 *Structures of some organophosphorus insecticides and of the nerve agent sarin.* Note that most compounds used as insecticides are organophosphorothioates (i.e., have

a P=S bond), though some (as well as sarin) have a P=O bond and do not require metabolic activation.

Biotransformation—For compounds containing a sulfur bound to the phosphorus, bioactivation is necessary for their biological activity, as only compounds with a P=O moiety are effective inhibitors of AChE. Oxidative desulfuration by cytochrome P450 enzymes (CYPs) leads to the formation of an oxygen analog of the parent insecticide. Thioether oxidation (formation of a sulfoxide, S=O, followed by the formation of a sulfone, O=S=O) is also catalyzed by CYPs. Several other detoxication reactions lead to metabolites of lesser or no toxicity. Catalytic hydrolysis by phosphotriesterases, known as A-esterases (which are not inhibited by OPs), can detoxify certain OPs. Noncatalytic hydrolysis of OPs also occurs when these compounds phosphorylate serine esterases classified as B-esterases.

Signs and Symptoms of Toxicity, Mechanism of Action, and Treatment of Poisoning—OP insecticides have high acute oral toxicity. Inhibition of AChE by OPs causes accumulation of acetylcholine at all cholinergic synapses in the body. With overstimulation of muscarinic and nicotinic cholinergic receptors, a “cholinergic syndrome” ensues, which includes increased sweating and salivation, profound bronchial secretion, bronchoconstriction, miosis, increased gastrointestinal motility, diarrhea, tremors, muscular twitching, and various CNS effects (Table 22–4). When death occurs, this is thought to be due to respiratory failure as a result of inhibition of respiratory centers in the brain. While respiratory failure is a hallmark of severe OP poisoning, mild poisoning may display no clear-cut signs and symptoms.

TABLE 22–4 Signs and Symptoms of Acute Poisoning with Anticholinesterase Compounds

Site and Receptor Affected	Manifestations
Exocrine glands (M)	Increased salivation, lacrimation, perspiration
Eyes (M)	Miosis, blurred vision
Gastrointestinal tract (M)	Abdominal cramps, vomiting, diarrhea
Respiratory tract (M)	Increased bronchial secretion, bronchoconstriction
Bladder (M)	Urinary frequency, incontinence
Cardiovascular system (M)	Bradycardia, hypotension
Cardiovascular system (N)	Tachycardia, transient hypertension
Skeletal muscles (N)	Muscle fasciculations, twitching, cramps, generalized weakness, flaccid paralysis
Central nervous system (M, N)	Dizziness, lethargy, fatigue, headache, mental confusion, depression of respiratory centers, convulsions, coma

Abbreviations: M, muscarinic receptors; N, nicotinic receptors.

OPs with a P=O moiety phosphorylate a hydroxyl group on serine in the active (esteratic) site of the enzyme, thus impeding its action on the physiological substrate (Fig. 22–2). Phosphorylated AChE is hydrolyzed by water very slowly, and the rate of "spontaneous reactivation" depends on the chemical nature of the R substituents. Reactivation decreases in the order dimethoxy, diethoxy, and diisopropoxy. Reactivation of phosphorylated AChE does not occur once the enzyme–inhibitor complex has "aged." Aging consists of the loss (by nonenzymatic hydrolysis) of one of the two alkyl (R) groups. When phosphorylated AChE has aged, the enzyme is irreversibly inhibited, and the only means of replacing its activity is through synthesis of a new enzyme, a process that may take days.

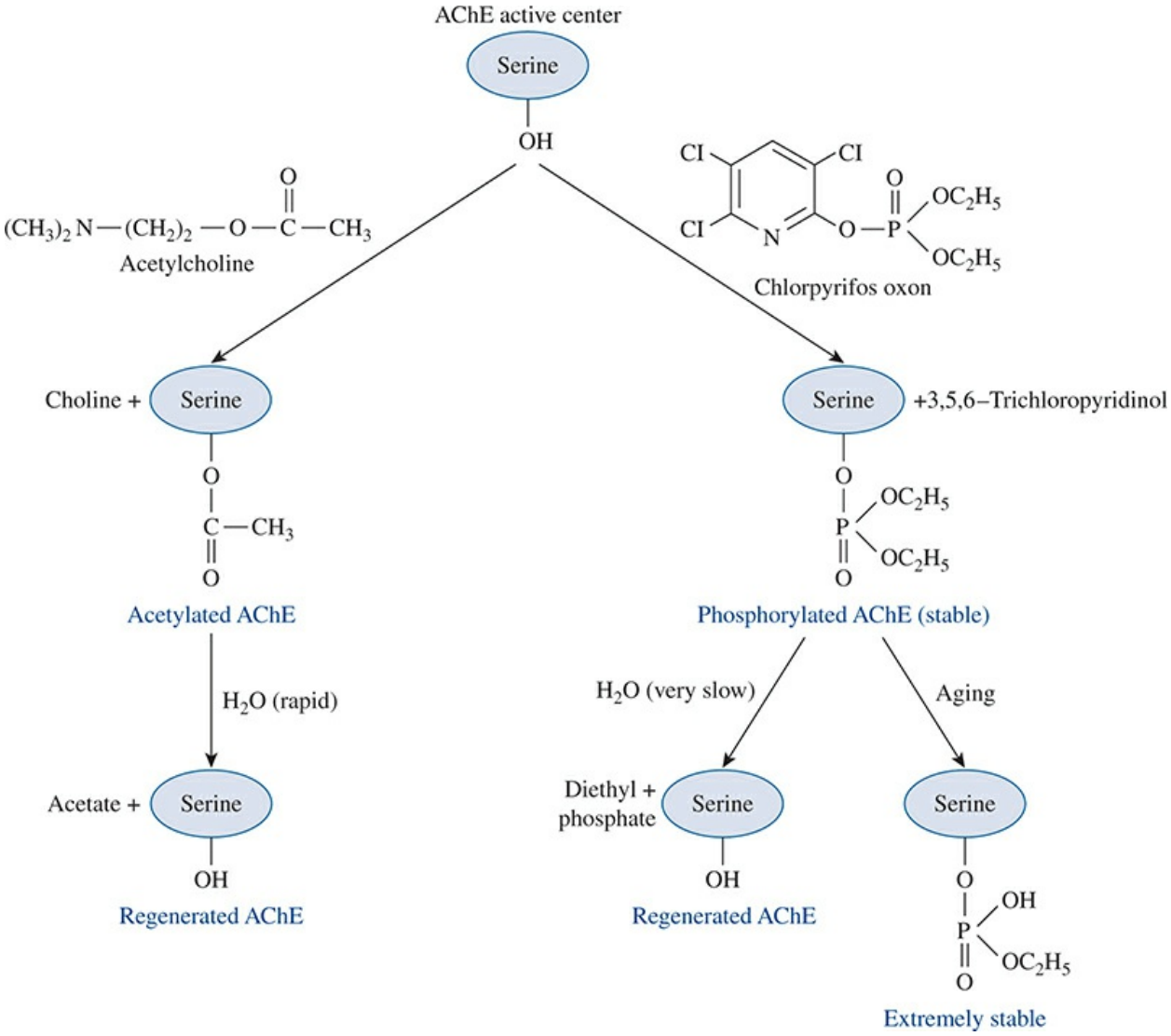


FIGURE 22–2 *Scheme of hydrolysis of acetylcholine by acetylcholinesterase (AChE) and reaction of chlorpyrifos oxon with AChE (see text for details).*

Procedures aimed at decontamination and/or at minimizing absorption depend on the route of exposure. In case of dermal exposure, contaminated clothing should be removed, and the skin washed thoroughly with alkaline soap. Special attention should be exercised by medical personnel, because passive contamination may occur. In case of ingestion, procedures to reduce absorption from the gastrointestinal tract do not appear to be effective. The muscarinic receptor antagonist atropine prevents the action of accumulating acetylcholine on these receptors. The best clinical approach is to administer doses of atropine large enough to achieve evidence of atropinization, that is, flushing, dry mouth, changes in pupil size, bronchodilation, and increased heart rate, which should be maintained for at least 48 hours. Administration of oximes such as pralidoxime (2-PAM) early after OP exposure can theoretically facilitate dephosphorylation of the enzyme, restoring catalytic function of AChE and preventing aging. Effectiveness of oximes is equivocal and there is potential for harm.

Biochemical Measurements—In addition to synapses, AChE present in red blood cells and pseudo-cholinesterase in plasma are usually inhibited upon exposure to OPs. Their measurements are a valid way to determine exposure to OPs, to confirm diagnosis of OP poisoning, or to monitor occupationally exposed workers. The parent compound is quantified in blood, while metabolites are measured in urine.

The Intermediate Syndrome—A second distinct manifestation of OP exposure, the so-called intermediate syndrome, is seen in 20% to 30% of acute OP poisoning cases. The syndrome develops one to several days after poisoning, during or after recovery from the initial cholinergic crisis. Prominent features of the intermediate syndrome are a marked weakness of respiratory, neck, and proximal limb muscles. Extrapyramidal symptoms may also be present. Mortality due to respiratory paralysis and complications ranges from 15% to 40%, and recovery in surviving patients usually takes up to 30 days. The muscle weakness may result from nicotinic receptor desensitization due to prolonged cholinergic stimulation.

Organophosphate-Induced Delayed Polyneuropathy—OPIDP causes signs and symptoms of tingling of the hands and feet, followed by sensory loss, progressive muscle weakness and flaccidity of the distal skeletal muscles of the lower and upper extremities, and ataxia. These may occur 2 to 3 weeks after a single exposure, when signs of both the acute cholinergic and the intermediate syndromes have subsided. OPIDP can be classified as a distal sensorimotor axonopathy. The primary lesion is a bilateral degenerative change in distal levels of axons and their terminals, primarily affecting larger/longer myelinated central and peripheral nerve fibers, leading to breakdown of neuritic segments and the myelin sheaths. OPIDP is not related to AChE inhibition. Epidemics like the so-called Ginger-Jake paralysis in the 1930s caused by TOCP (tri-*ortho*-cresyl phosphate) are rare today. The target for OPIDP is an esterase, present in nerve tissues as well as other tissues (e.g., lymphocytes), named neuropathy target esterase (NTE). Several OPs, depending on their chemical structure, can inhibit NTE, as do certain carbamates and sulfonyl fluorides. Phosphorylation of NTE by OPs is similar to that observed for AChE. However, only OPs whose chemical structure leads to aging of phosphorylated NTE (by a process analogous to that described for AChE) can cause OPIDP. Other compounds that inhibit NTE but cannot undergo the aging reaction are not neuropathic. For OPIDP to be initiated, phosphorylation and subsequent aging of at least 70% of NTE occurs within hours of poisoning. When the first clinical signs of OPIDP are evident some weeks later, NTE activity has recovered.

NTE is inhibited by OPs rather than hydrolyzing them, and has phospholipase activity. A member of a nine-protein family of patatin-like phospholipase domain–containing proteins (PNPLAs), NTE is PNPLA6. Mutations of the *PNPLA6* gene have been associated with alterations in the central and/or peripheral nervous systems. Though reductions in axonal transport precede overt clinical signs, the exact chain of events occurring between phosphorylation and aging of NTE and axonal degeneration is still unknown.

The occurrence of OPIDP in humans is now rare, since OPs must undergo specific neurotoxicity testing in the hen (one of the most sensitive species) to determine whether OPIDP is produced. For this test, high doses of OPs are used, and animals are protected from acute cholinergic toxicity with atropine.

Genetic Susceptibility—As CYPs are important for the activation and detoxication of OPs, polymorphisms of these enzymes may affect OP metabolism and toxicity. Also, at least 39 genetic variants of BuChE have been identified, with nucleotide alterations in the coding region.

Genetic variants of human AChE are mainly not associated with altered AChE activity.

Long-Term Toxicity—There is still controversy on possible long-term effects of these insecticides. Acute exposure to high doses of OPs may result, in some cases, in long-lasting adverse health effects (particularly CNS sequelae). The balance of evidence does not support the existence of clinically significant neuropsychological effects, neuropsychiatric abnormalities, or peripheral nerve dysfunction in humans chronically exposed to low levels of OPs. OPs as a class are not considered to be mutagenic. Tetrachlorvinphos, parathion, malathion, and diazinon are carcinogenic. Immunotoxicity of OPs has been suggested from in vitro or high-dose animal studies, but evidence in humans is lacking. Some OPs have also endocrine-disrupting activities in vitro, but in vivo studies are equivocal.

Developmental Toxicity and Neurotoxicity—Young animals are more sensitive to the acute cholinergic toxicity of OPs, perhaps owing to lower detoxication abilities. Studies in rodents indicate that OPs can affect various cellular processes (e.g., DNA replication, neuronal survival, and neurite outgrowth) and noncholinergic pathways (e.g., serotoninergic synaptic functions and the adenylate cyclase system), and cause various behavioral abnormalities. Such effects are at times seen at dose levels that produce no cholinergic signs of toxicity. These findings, together with results of biomonitoring studies that indicate exposure of children to OPs, have led to regulatory restrictions on the use of certain OPs, and to concern for their potential neurotoxic effects in children.

Carbamates

Carbamate insecticides derive from carbamic acids. Acute oral toxicity ranges from moderate to low toxicity with carbaryl to extremely high toxicity with aldicarb (Fig. 22–3). Dermal toxicity is lower, but skin penetration is increased by organic solvents and emulsifiers present in most formulations. Carbamates are susceptible to enzyme-catalyzed biotransformation reactions, principally oxidation and hydrolysis. The carbamates rapidly and reversibly inhibit AChE. The signs and symptoms of carbamate poisoning include miosis, urination, diarrhea, salivation, muscle fasciculation, and CNS effects (Table 22–4). Acute intoxication by carbamates is generally resolved within a few hours. Treatment involves atropine administration.

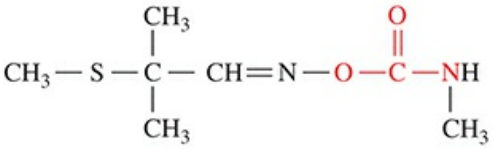
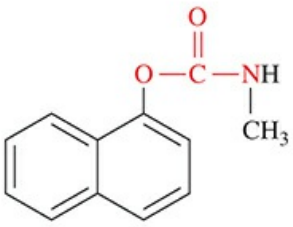
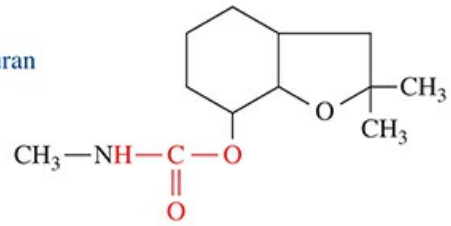
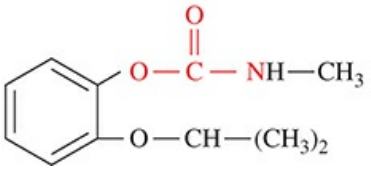
		LD_{50} in rats (mg/kg)		Water solubility (g/L)
		Oral	Dermal	
Aldicarb		0.8	3.2	6.0
Carbaryl		400	>5000	0.7
Carbofuran		10	>1000	0.7
Propoxur		85	~1500	2.0

FIGURE 22–3 *Structures of some carbamate insecticides, with indication of acute oral and dermal toxicity in the rat, and of water solubility.* Note that carbofuran has been banned in recent years in several countries.

Carbamates can inhibit NTE, but since carbamylated NTE cannot age, they are thought to be unable to initiate OPIDP. Additionally, when given before a neuropathic organophosphate, carbamates offer protection against OPIDP, but when given after, they can promote OPIDP. Methylcarbamates are not mutagenic or carcinogenic. Embryotoxicity or fetotoxicity are observed only at maternally toxic doses. Limited evidence suggests that carbamates (e.g., aldicarb) may be more acutely toxic to young animals than to adults, possibly because of lower detoxication.

Pyrethroids

Extracts of the flower heads of *Chrysanthemum cinerariaefolium* contain six insecticidal esters, referred to as pyrethrins. Pyrethrins and synthetic pyrethroids have high insecticidal potency, generally low mammalian toxicity, and a lack of environmental persistence. Pyrethroids are used

widely as insecticides both in the house and in agriculture, in medicine for topical treatment of scabies and headlice, and in tropical countries for malaria control, in soaked bed nets to prevent mosquito bites, as well as in indoor residual spraying. Pyrethroids alter the normal function of insect nerves by modifying voltage-sensitive sodium channels and thus the nerve action potential. Acute oral mammalian toxicity of pyrethroids is generally low. Despite extensive worldwide use, there are relatively few reports of human poisonings, and only a dozen deaths. Most deaths occurred following accidental or intentional exposure to pyrethroids. Dermal toxicity of pyrethroids is low, because of limited absorption through the skin.

Upon absorption, pyrethroids are very rapidly metabolized through two major biotransformation routes: hydrolysis of the ester linkage, which is catalyzed by hepatic and plasma carboxylesterases and oxidation of the alcohol moiety by CYPs. These initial reactions are followed by further oxidations, hydrolysis, and conjugation with sulfate or glucuronide.

Signs and Symptoms of Toxicity and Mechanisms of Action—These compounds have been divided into two classes (Table 22–5): Type I compounds produce a syndrome consisting of marked behavioral arousal, aggressive sparring, increased startle response, and fine body tremor progressing to whole-body tremor and prostration (Type I or T [tremor] syndrome). Type II compounds produce profuse salivation, coarse tremor progressing to choreoathetosis, and clonic seizures.

TABLE 22–5 Classification of Pyrethroid Insecticides Based on Toxic Signs in Rats

Syndrome	Signs and Symptoms	Examples
Type I (T syndrome)	Aggressive sparring	Allethrin
	Increased sensitivity to external stimuli	Bioallethrin
		Resmethrin
	Whole-body tremors	Phenothrin
	Prostration	
Type 2 (CS syndrome)	Pawing and burrowing	Deltamethrin
	Profuse salivation	Fenvalerate
	Coarse tremor	Cypermethrin
	Choreoathetosis	Cyhalothrin
	Clonic seizures	

Pyrethroids in mammals disrupt voltage-gated sodium channels by binding the α subunit and slowing activation (opening) as well as the rate of inactivation (closing), leading to a stable hyperexcitable state. Sodium channels then open at more hyperpolarized potentials and are held open longer, allowing more sodium ions to cross and depolarize the neuronal membrane. In general, Type II compounds delay the inactivation of sodium channels substantially longer (more

than 10 milliseconds) than do Type I compounds (less than 10 milliseconds). This variance in open channel time may explain the differences observed between the Type I and Type II syndromes.

The higher sensitivity of insects to pyrethroid toxicity, compared to mammals, is thought to result from a combination of higher sensitivity of insect sodium channels, lower body temperature (as pyrethroids show a negative temperature coefficient of action), and slower biotransformation. Type II pyrethroids, but not type I compounds, also bind to and inhibit $GABA_A$-gated chloride channels, which might contribute to the seizures that accompany severe Type II pyrethroid poisoning.

Upon occupational exposure, the primary adverse effect resulting from dermal contact with pyrethroids is paresthesia. Symptoms include continuous tingling or pricking or, when more severe, burning. The condition reverses in about 24 hours, and topical application of vitamin E has been shown to be an effective treatment. Paresthesia is presumably due to abnormal pyrethroid-induced repetitive activity in skin nerve terminals.

Chronic studies with pyrethroids indicate that at high dose levels they cause slight hepatomegaly. There is little or no evidence of teratogenicity, mutagenicity, or endocrine disruption. An increased lymphoma incidence in rodents has been reported for deltamethrin, but the effect was not dose-dependent. Pyrethroids are particularly toxic to fish, but not to birds.

Organochlorine Compounds

The organochlorine insecticides include the chlorinated ethane derivatives, such as DDT and its analogs; the cyclodienes, such as chlordane, aldrin, dieldrin, heptachlor, endrin, and toxaphene; the hexachlorocyclohexanes, such as lindane; and the caged structures mirex and chlordecone (Fig. 22–4). Their acute toxicity is moderate (less than that of OPs), but chronic exposure may be associated with adverse health effects particularly in the liver and the reproductive system.

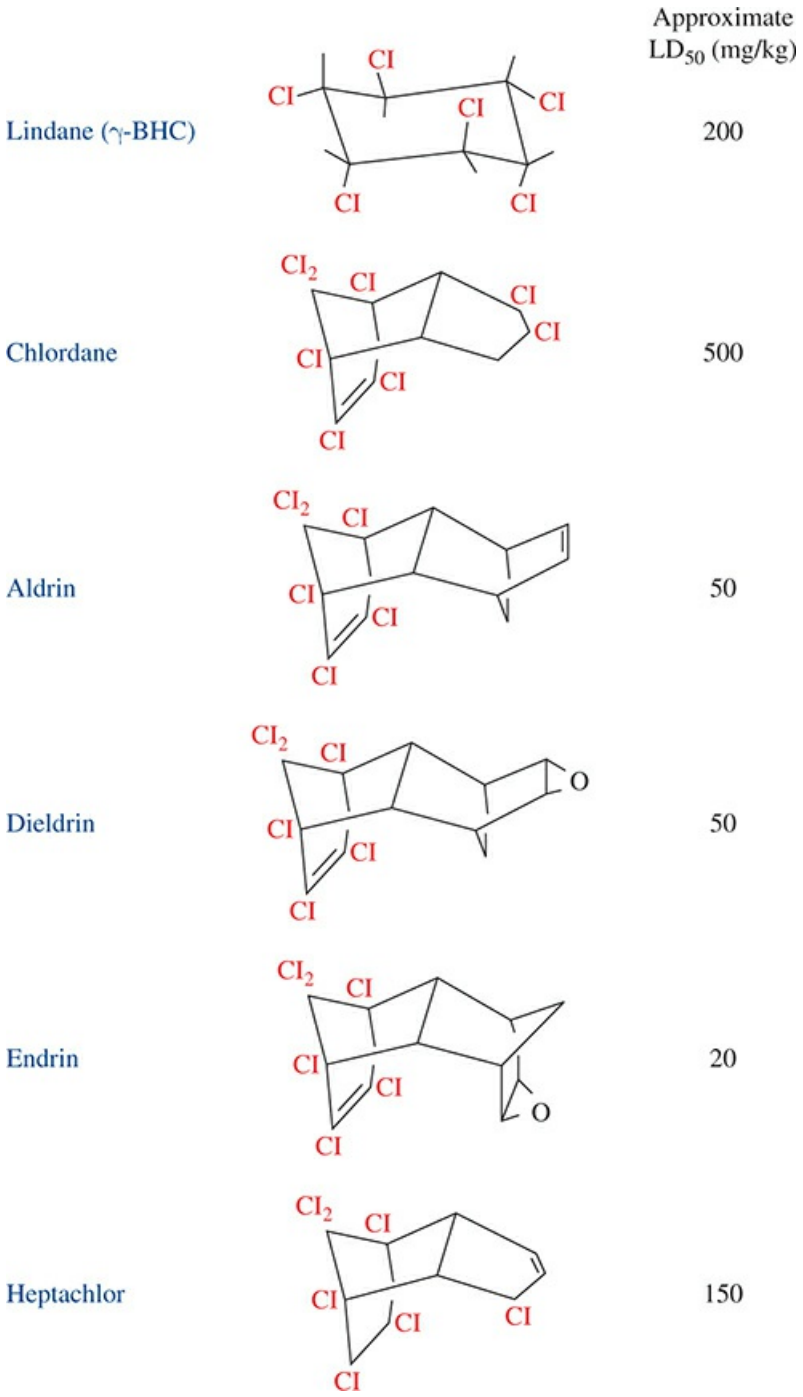


FIGURE 22–4 *Structure and acute toxicity (oral LD50 in rat) of selected organochlorine insecticides of different chemical classes (see text for details).*

DDT and Its Analogs—DDT (1,1,1-trichloro-2, 2-bis(4-chlorophenyl)ethane) was effective against many agricultural pests, as well as against insects that transmit some of the world's most serious diseases, such as typhus, malaria, and yellow fever. Technical-trade DDT is a mixture of several isomers, with *p,p′*-DDT being responsible for the insecticidal activity.

DDT has a moderate acute toxicity when given by the oral route, but dermal absorption of

DDT is limited. Upon absorption, DDT distributes in all tissues, with the highest concentrations found in adipose tissue. Excretion is through the bile, urine, and milk.

Acute exposure to high doses of DDT causes motor unrest, increased frequency of spontaneous movements, abnormal susceptibility to fear, and hypersusceptibility to external stimuli (light, touch, sound). In humans, the earliest symptom of DDT poisoning is hyperesthesia of the mouth and lower part of the face, followed by paresthesia of the same area including the tongue; this is followed by dizziness, tremor of the extremities, confusion, and vomiting, while convulsions occur only in severe poisoning. Death is usually due to respiratory failure.

Both in insects and in mammals, DDT interferes with the sodium channels in the axonal membrane by a mechanism similar to that of Type I pyrethroids.

While acute exposure to DDT is a rare event, chronic exposure targets the liver. DDT and DDE increase liver weight, cause hepatic cell hypertrophy and necrosis, and are potent inducers of CYPs, particularly CYP2B and CYP3A. The endocrine-disrupting actions of DDT and DDE give rise to some hormonally sensitive cancers, and positive associations were found for some (e.g., testicular cancer) but not for others (e.g., breast cancer). DDT is classified as a probable human carcinogen (Group 2A) by IARC.

Methoxychlor (2,2-bis (*p*-methoxyphenyl)-1,1,1-trichloroethane), the *p,p′*-dimethoxy analog of *p,p′*-DDT has a low acute toxicity. With chronic exposure, methoxychlor is a modest inducer of liver microsomal enzymes, causes chronic nephritis, and hypertrophy of kidneys, mammary glands, and uterus. Testicular atrophy and decreased spermatogenesis were also observed. Methoxychlor is demethylated by CYP2C19 and CYP1A2 to estrogenic compounds that are likely responsible for the reproductive system effects in both male and female animals.

Hexachlorocyclohexanes and Cyclodienes—These two families of organochlorine insecticides comprise many compounds that share a similar mechanism of neurotoxic action. Lindane, the γ isomer of benzene hexachloride (BHC; 1,2,3,4,5,6- hexachlorocyclohexane) (Fig. 22–4) has insecticidal activity. Cyclodiene compounds include chlordane, dieldrin, aldrin (which is rapidly metabolized to dieldrin), heptachlor, and endrin.

Lindane and cyclodienes have moderate to high acute oral toxicity with ready dermal absorption. The primary target for their toxicity is the CNS. Unlike DDT, tremor is essentially absent, but convulsions are a prominent aspect of poisoning. These compounds interfere with γ-aminobutyric acid (GABA)–mediated neurotransmission. Lindane and cyclodienes bind to the picrotoxin site on the chloride channel, thereby blocking its opening and thus antagonizing the "inhibitory" action of GABA. Cyclodienes induce microsomal biotransformation enzymes and cause liver enlargement upon chronic exposure. They act as tumor promoters and cause liver tumors in mice. Lindane is classified as carcinogenic to humans (Group 1) based on positive human and animal findings.

Mirex and Chlordecone—These organochlorine insecticides have a cage-like structure and were used against fire ants and leaf-eating insects, respectively. Chlordecone toxicity is manifested by tremors, which are observed in animals and in humans. Chlordecone neurotoxicity may involve inhibition of ATPases (both Na^+, K^+- and Mg^{2+}-ATPases), and ensuing inhibition of the uptake of catecholamines. In contrast to cyclodienes, chlordecone does not cause seizures. Chlordecone induces hepatic drug metabolizing enzymes and causes hepatosplenomegaly in rats and humans. It is not mutagenic but causes reproductive toxicity in animals, likely by mimicking the effects of excessive estrogens. Low or absent sperm count was found in chlordecone-exposed

workers.

Environmental Ubiquity and Persistence—The properties (low volatility, chemical stability, lipid solubility, slow rate of biotransformation, and degradation) that made organochlorine compounds effective insecticides also brought about their demise owing to their environmental persistence, bioconcentration, biomagnification in food chains, and the acquisition of biologically active body burdens at higher trophic levels. Adverse effects on bird reproduction (e.g., eggshell thinning) were among the first ecological effects to be identified. Because of their stability and high lipophilicity, organochlorines are present in adipose tissues of most individuals.

Endocrine Disruption—An endocrine, or hormone, disruptor can be defined as a xenobiotic that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body. The *o,p'*-isomer of DDT acts as an agonist at estrogen receptors. *p,p'*-DDE, in contrast, inhibits androgen binding to the androgen receptor. Methoxychlor metabolites are active as ERα agonists and ERβ antagonists and are antiandrogenic. Chlordecone also has estrogenic properties. Other organochlorine compounds with weak estrogenic activity are dieldrin, endosulfan, toxaphene, lindane, and the β isomer of hexachlorocyclohexane (β-BHC).

DDT and Public Health: Risk–Benefit Considerations—The Stockholm Convention on Persistent Organic Pollutants, ratified in 2004 by 50 countries, outlaws the use of several industrial chemicals (initially 12, the so-called “Dirty Dozen”), including several organochlorine insecticides. However, an exemption clause allows malaria-endemic nations to continue utilizing DDT for indoor residual wall spraying. About 25 countries use DDT under this exemption from its ban. This situation is keeping the debate on the risks and benefits of DDT usage very much alive. However, indoor residual spraying does expose humans to amounts of DDT that may cause adverse health effects. Preterm births and early weaning (decreased duration of lactation), which can lead to increased infant mortality, have been associated with DDT exposure.

Neonicotinoids

The neonicotinoids were developed by chemical modifications of nicotine and of other nicotinic acetylcholine receptor (nAChR) agonists. Nicotine is a systemic insecticide effective toward a wide range of insects, including aphids, thrips, and whiteflies. Nicotine exerts its pharmacological and toxic effects in mammals and insects by activating nAChRs. In vertebrates, nAChRs are expressed at neuromuscular junctions, in the peripheral nervous system and in the CNS, while in insects they are confined to the CNS. At high doses, parasympathetic stimulation and ganglionic and neuromuscular blockade predominate. Signs and symptoms of poisoning include nausea, vomiting, muscle weakness, respiratory effects, headache, lethargy, and tachycardia. Most cases of poisoning with nicotine occur after exposure to tobacco products, or gum or patches. Workers who cultivate, harvest, or handle tobacco may experience green tobacco sickness, caused by dermal absorption of nicotine.

Neonicotinoids include nithiazine, imidacloprid, nitenpyram, acetamiprid, and other compounds (Fig. 22–5). Signs and symptoms of toxicity in mammals are attributable to stimulation of nAChRs, particularly in the peripheral nervous system, given their poor penetration of the blood–brain barrier. Imidocloprid and thiacloprid are particularly toxic to

birds, and thiacloprid to fish. Imidacloprid, clothianidin, and thiamethoxan are highly acutely toxic to bees, and they are able to impair the immune system of bees. Most neonicotinoids are not mutagenic or carcinogenic, nor teratogenic. Neonicotinoids undergo limited biotransformation in mammals, involving mostly CYP-mediated oxidative reactions.

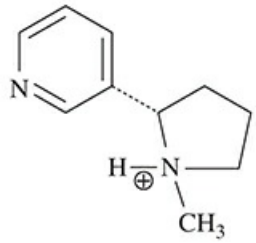
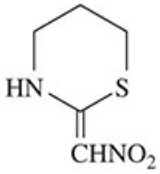
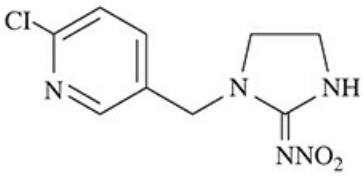
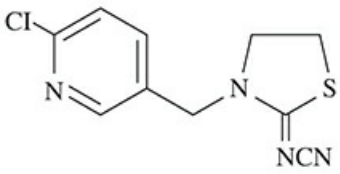
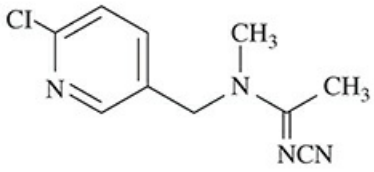
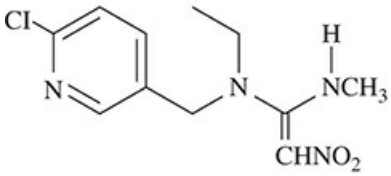
	Log *P*	Acute oral LD_{50} (mg/kg;rat)
 (–)-nicotine	0.93	50–60
 Nithiazine	–0.60	300
 Imidacloprid	0.57	450
 Thiacloprid	1.26	640
 Acetamiprid	0.80	182
 Nitenpyram	–0.66	1,628

FIGURE 22–5 *Structures of nicotine and of neonicotinoid insecticides with indication of their acute oral toxicity in rat and their octanol/water partition (P).* (Adapted from Tomizawa M, Casida JE. Neonicotinoid insecticide toxicology: mechanisms of selective action. *Annu Rev Pharmacol Toxicol*. 2005;45:247–268.)

Neonicotinoids display a high selectivity profile, which is largely attributable to their specificity toward insect versus mammalian nAChRs. There is an increasing number of acute neonicotinoid poisonings, primarily due to suicidal attempts; mortality is low (about 3%), because of their receptor selectivity and low lipophilicity.

Other Old and New Insecticides

Rotenoids—The roots of *Derris elliptica,* and those of *Lonchocarpus utilis* and *L. urucu*, contain at least six rotenoid esters; the most abundant is rotenone. Rotenone is used as an agricultural insecticide/acaricide. Rotenone toxicity in target and nontarget species is due to inhibition of the mitochondrial respiratory chain, by blocking electron transport at NADH-ubiquinone reductase, also known as complex I. Insect and fish mitochondria are particularly sensitive to complex I inhibition. Poisoning symptoms include initial increased respiratory and cardiac rates, clonic and tonic spasms, and muscular depression, followed by respiratory depression. Acute intoxication in humans is rare. Rotenone may play a role in the etiology of Parkinson's disease.

Formamidines—Formamidines, such as chlordimeform ((*N'*-(4-chloro-o-tolyl)-*N*,*N*-dimethylformamidine) or amitraz (*N'*-2,4-(dimethyl-phenyl)-*N*,*N*-((2,4-dimethylphenyl) imino) methyl-*N*-methanimidamide) exert their toxicity in invertebrates by activating an octopamine-dependent adenylate cyclase. In mammals, symptoms of formamidine poisoning are sympathomimetic, and include hypotension, bradycardia, and sedation. Formamidines act as rather selective agonists for α_2-adrenergic receptors. The *N*-demethylated metabolite (desmethylchlordimeform) is more acutely toxic than chlordimeform and displays a more than 400-fold higher potency toward α_2-adrenoceptors. Two other metabolites of chlordimeform, 4-chloro-toluidine and *N*-formyl-4-chloro-*o*-toluidine, are thought to be responsible for the haemangioendotheliomas in mice observed in carcinogenicity studies. Chlordimeform is classified as a probable human carcinogen (Group 2A).

Amitraz is used for the control of ectoparasites in farm animals and crops. Signs and symptoms of poisoning with amitraz mimic those of α_2-adrenergic receptor agonists such as clonidine, and include nausea, hypotension, hyperglycemia, bradycardia, and miosis. Amitraz is not genotoxic but is classified as a possible human carcinogen.

Avermectins—These macrocyclic lactones are isolated from the fermentation broth of *Streptomyces avermitilis*. Eight individual avermectins have antiparasitic activity. Abamectin is used primarily to control mites, while emamectin benzoate is effective at controlling lepidopteran species in various crops. Ivermectin an antihelmintic and antiparasitic agent in veterinary medicine, and in humans for infection of intestinal threadworms, onchocerciasis (river blindness), and lymphatic filariasis. In insects and nematodes, avermectins exert their toxic effects by activating invertebrate-specific glutamate-gated chloride channels. Signs and symptoms of intoxication include hyperexcitability, tremors, and incoordination, followed by ataxia and coma-like sedation.

Avermectins interact with P-glycoprotein, and P-glycoprotein-mediated efflux plays an important role in attenuating the neurotoxicity and developmental neurotoxicity of avermectins. P-glycoprotein polymorphisms may impair its function increase avermectin neurotoxicity in humans.

Phenylpyrazoles—Fipronil is a broad-spectrum insecticide with moderate mammalian toxicity. Fipronil blocks the $GABA_A$-gated chloride channel and has a much higher specificity for insect receptors over mammalian receptors. There is no evidence that fipronil is an eye or skin irritant, or has any mutagenic, carcinogenic, or teratogenic effects.

Diamides—Flubendiamide and chlorantraniliprole activate ryanodine receptors, a family of calcium channels located in the muscle sarcoplasmic reticulum, resulting in the release of stored pools of calcium and initiating muscle contraction. Ryanodine, a natural alkaloid present in the shrub *Ryania speciosa*, blocks these calcium channels, thus inhibiting muscle contraction. Diamides bind to a site different from ryanodine, and cause receptor activation in insects by prolonging channel opening. Both flubendiamide and chlorantraniliprole selectively activate insect over mammalian ryanodine receptors. Acute toxicity is low, and there is no evidence of genotoxicity, carcinogenicity, neurotoxicity, and reproductive and developmental toxicity.

Bacillus Thuringiensis—Biopesticides derived from plants, bacteria, and fungi are increasingly used in integrated pest management programs and also in organic productions, and generally have a favorable environmental and toxicological profile. Biopesticides fall into three major classes: (1) microbial pesticides that act as insecticides, (2) plant-incorporated-protectants are pesticidal substances that plants produce from genetic material that has been added to the plant, and (3) biochemical pesticides control pests by nontoxic mechanisms, such as sex pheromones that interfere with mating of insects, or various scented extracts that attract insect pests to traps.

Bacillus thuringiensis (Bt) is a soil microorganism that forms spores containing protein crystals that can be proteolytically processed to active toxins in the insect's midgut, where they bind receptors in the epithelial cells, insert into the cellular membrane, and form pores. Pores allow changes in K^+ fluxes across the epithelial cells, leading to changes in pH. Ultimately, cells of the midgut epithelium are destroyed by the high pH and by osmotic lysis. Insects eventually die as a result of gut paralysis, subsequent starvation and septicemia. Bt targets primarily leaf-feeding lepidoptera, breaks down rapidly in UV light, and exhibits low mammalian toxicity. The selective toxicity of Bt is attributed to the fact that crystalline Bt endotoxins require activation by alkalis and/or digestion, conditions absent in the mammalian stomach. Bt toxins have generally an unremarkable toxicological profile in mammals. Bt genes have been expressed in a variety of crop plants, most notably cotton and corn. Thus, the plant, instead of the Bt bacterium, produces one or more crystal toxins that affect the insect upon feeding.

INSECT REPELLENTS

Insect-transmitted diseases remain a major source of illness and death worldwide, as mosquitoes alone transmit disease to millions annually.

DEET

More than 200 formulations with varying concentrations (commonly 4.75% to 40%) of DEET (*N*,*N*-diethyl-*m*-toluamide or *N*,*N*-diethyl-3-methylbenzamide) are applied directly to the skin or on clothing. DEET is effective at repelling insects, flies, fleas, and ticks, and protection time increases with increasing concentrations. The repellent mechanism of DEET may be related to disturbances of the mosquito antennae that allow the insect to locate humans. DEET undergoes oxidative biotransformation catalyzed by various CYPs and is excreted mostly in the urine. Subchronic toxicity studies in various species did not reveal major toxic effects. No significant effects of DEET were seen in mutagenicity, reproductive toxicity, carcinogenicity, or acute and chronic neurotoxicity studies.

Picaridin

Picaridin (1-piperidine carboxylic acid, 2-(hydroxyl-ethyl), 1-methyl propylester) formulations (cream, aerosol, wipe) containing 5% to 20% are highly effective against arthropod pests, especially mosquitoes, ticks, and flies. Its action in insects is believed to be due to the interaction with specific olfactory receptors of the arthropod. It is hydroxylated and glucuronidated before excretion in the urine. The toxicological profile of picaridin is unremarkable. There is no evidence of genotoxicity, carcinogenicity, teratogenicity, reproductive toxicity, or neurotoxicity.

HERBICIDES

Herbicides are chemicals capable of killing or severely injuring plants. A classification of herbicides based on the mechanisms by which they exert their biological effects is shown in Table 22–6. *Preplanting* herbicides are applied to the soil before a crop is seeded; *preemergent* herbicides are applied to the soil before the time of appearance of unwanted vegetation; and *postemergent* herbicides are applied to the soil or foliage after the germination of the crop and/or weeds. *Contact* herbicides are those that affect the plant that was treated, while *translocated* herbicides are applied to the soil or to above-ground parts of the plant and are absorbed and circulated to distant tissues. Nonselective herbicides will kill all vegetation, while selective compounds are those used to kill weeds without harming the crops.

TABLE 22–6 Some Mechanisms of Action of Herbicides

Mechanism	Chemical Classes (Example)
Inhibition of photosynthesis	Triazines (atrazine), substituted ureas (diuron), uracils (bromacil)
Inhibition of respiration	Dinitrophenols
Auxin growth regulators	Phenoxy acids (2,4-D), benzoic acids (dicamba), pyridine acids (picloram)
Inhibition of cell division	Chloroacetanilides (alachlor)
Inhibition of protein synthesis	Dinitroanilines
Inhibition of lipid synthesis	Aryloxyphenoxypropionates (diclofop)
Inhibition of specific enzymes	
• Glutamine synthetase	Glufosinate
• Enolpyruvylshikimate 3-phosphate synthetase	Glyphosate
• Acetylase synthase	Sulfonylureas
Cell membrane disruptors	Bipyridyl derivatives (paraquat)

Herbicides, as a class, display relatively low acute toxicity except for paraquat. Some herbicides can cause dermal irritation and contact dermatitis, particularly in individuals prone to allergic reactions. Other compounds have suspected carcinogenicity or neurotoxicity. The principal classes of herbicides associated with reported adverse health effects in humans are discussed here in detail.

Chlorophenoxy Compounds

Chlorophenoxy herbicides are chemical analogs of auxin, a plant growth hormone, that produce uncontrolled and lethal growth in target plants. The most commonly used compound of this class is 2,4-dichlorophenoxyacetic acid (2,4-D). Because auxin is critical to the growth of many broad leaf plants, but is not used by grasses, chlorophenoxy compounds can suppress the growth of weeds (e.g., dandelions) without affecting the grass.

Upon oral exposure, 2,4-D is rapidly absorbed, binds extensively to serum albumin, but does not accumulate in tissues, and is excreted almost exclusively through the urine. Ingestion of 2,4-D has caused acute poisoning in humans, with vomiting, burning of the mouth, abdominal pain, hypotension, myotonia, and CNS involvement. Dermal exposure is the major route of unintentional exposure to 2,4-D in humans, but dermal absorption is usually less than 6%.

Chlorophenoxy herbicides elicit toxicity by the possible involvement of three actions: (1) cell membrane damage, (2) interference with metabolic pathways involving acetyl-coenzyme A, and (3) uncoupling of oxidative phosphorylation. There may be an association between exposure to 2,4-D and neurologic effects ranging from peripheral neuropathy to demyelination and ganglion degeneration in the CNS, to reduced nerve conduction velocity, myotonia, and behavioral

alterations. 2,4-D has little genotoxic potential. 2,4-D has been classified by IARC as possibly carcinogenic to humans (Group 2B).

Bipyridyl Compounds

Paraquat is a fast-acting, nonselective contact herbicide used to control broad-leaved weeds and grasses in plantations and fruit orchards, and for general weed control. The herbicide has a high acute toxicity. Paraquat is poorly metabolized and excreted in the urine. Paraquat accumulates in lung tissue, particularly, in Type II alveolar epithelial cells and Clara cells. It has minimal to no genotoxicity, is not carcinogenic in rodents, has no effect on fertility, is not teratogenic, and only produces fetotoxicity at maternally toxic doses.

Paraquat can be reduced to form a free radical with concomitant production of superoxide anion (Fig. 22–6). Intracellular redox cycling results in the oxidation of NADPH, leading to its cellular depletion, which is augmented by the detoxification of hydrogen peroxide formed in the glutathione peroxidase/reductase enzyme system to regenerate GSH.

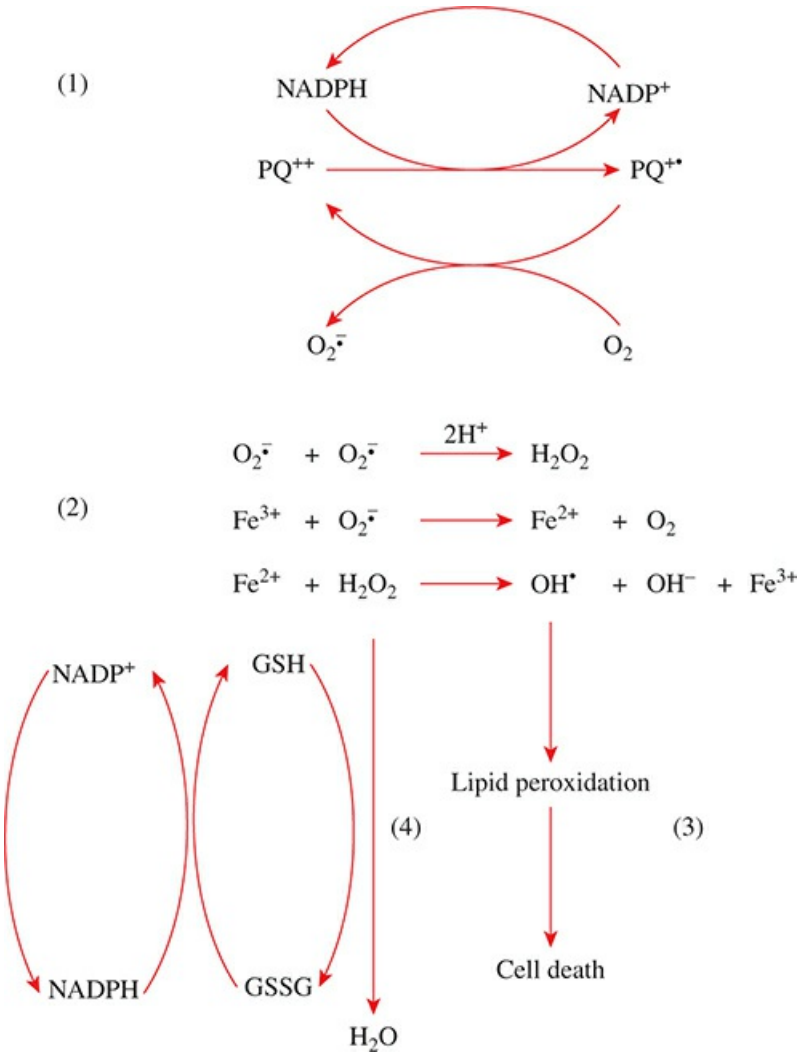


FIGURE 22–6 *Mechanism of toxicity of paraquat.* (1) Redox cycling of paraquat utilizing

NADPH; (2) formation of hydroxy radicals leading to lipid peroxidation (3); (4) detoxication of H_2O_2 via glutathione reductase/peroxidase couple, utilizing NADPH. (Reprinted from Smith LL. Mechanism of paraquat toxicity in the lung and its relevance to treatment. *Human Toxicol*. 1987;6:31–36.)

Upon acute exposure to lethal doses of paraquat, damage to alveolar epithelial cells is seen within 24 hours after exposure and progresses in the following 2 to 4 days with large areas of the alveolar epithelium completely lost. This is followed by alveolar edema, extensive infiltration of inflammatory cells into the alveolar interstitium, and finally death due to severe anoxia. Survivors of this destructive phase show a subsequent proliferative phase that is characterized by attempts of the alveolar epithelium to regenerate and restore normal architecture, and presents itself as an intensive fibrosis. Some individuals who survive the first phase may still die from the progressive loss of lung function several weeks after exposure.

Diquat presents a different toxicological profile. In contrast to paraquat, it does not accumulate in the lung, and there is no pulmonary toxicity. Upon chronic exposure, target organs for toxicity are the gastrointestinal tract, kidney, and eye. Like paraquat, diquat can be reduced to form a free radical and then reoxidized in the presence of oxygen, with the concomitant production of superoxide anion. This redox cycling occurs in the eye and may be the likely mechanism of cataract formation. Human clinical symptoms include nausea, vomiting, diarrhea, ulceration of mouth and esophagus, decline of renal functions, and neurologic effects, but no pulmonary fibrosis. Diquat is not a skin sensitizer, has minimal genotoxic activity, is not carcinogenic in rodents, has no effect on fertility, and is not teratogenic.

Chloroacetanilides

Alachlor, acetochlor, metolachlor, butachlor and propachlor are used to control herbal grasses and broad-leaf weeds in crops such as corn. They have been shown to inhibit the synthesis of proteins and to impact cell division. Most of the chloroacetanilides do not appear to be teratogenic or to cause reproductive or developmental toxicity. Extensive genotoxicity studies in bacterial and mammalian systems in vitro and in animals in vivo suggest that these compounds are not genotoxic. Yet, some compounds induce tumors at various sites in rats. Several mechanistic studies have provided evidence that tumors observed in rats may be species-specific, show a threshold, and are not due to genotoxic mechanisms.

Triazines

The triazine herbicides comprising atrazine, simazine, and propazine have been extensively used for preemergent and postemergent control of broad-leaf weeds and certain grasses. Their herbicidal action is due to inhibition of photosynthesis. There is no evidence that triazines are teratogenic, are developmental or reproductive toxicants, or are genotoxic. Atrazine is under scrutiny for its potential endocrine-disrupting effects. In addition, atrazine and its metabolites cause depletion of dopamine and disruption of motor functioning.

Phosphonomethyl Amino Acids

Glyphosate (*N*-phosphonomethyl glycine) and glufosinate (*N*-phosphonomethyl homoalanine) are broad-spectrum nonselective systemic herbicides used for post-emergent control of annual and perennial plants, and are marketed primarily as the isopropylamine salt (glyphosate) or ammonium salt (glufosinate). Though both compounds contain a P=O moiety, they are organophosphonates that do not inhibit AChE.

Glyphosate—Glyphosate is one of the most widely used herbicides worldwide, and the development of transgenic crops that can tolerate glyphosate treatment has expanded its utilization. Glyphosate inhibits 5-enolpyruvylshikimate-3-phosphate synthase, which is responsible for the synthesis of an intermediate in the biosynthesis of various amino acids important in plant growth. This metabolic pathway is not present in mammals. Given the low acute toxicity of glyphosate itself, the attention has focused on its formulation, which contains surfactants to aid its penetration. The most widely used glyphosate product is Roundup®, which is formulated as a concentrate containing water, 41% glyphosate (as isopropylamine salt), and 15% polyoxethyleneamine (POEA). The acute toxicity of this glyphosate formulation is due to the surfactant POEA, which may increase cell permeability to glyphosate. Mild intoxication results mainly in transient gastrointestinal symptoms, while moderate or severe poisoning presents with gastrointestinal bleeding, hypotension, pulmonary dysfunction, and renal damage. Glyphosate has no teratogenic, developmental, or reproductive effects. Glyphosate is classified a "probable human carcinogen" (Group 2A).

Glufosinate—Glufosinate is a nonselective contact herbicide that acts by irreversibly inhibiting glutamine synthetase. Plants die because of the increased levels of ammonia and deficiency of glutamine, leading to inhibition of photorespiration and photosynthetic processes. Mammals have other metabolizing systems that can cope with the effects on glutamine synthetase activity to a certain limit. There is no evidence of genotoxicity or carcinogenicity, or on reproductive performance and fertility. Developmental toxic effects found in rats, rabbits, and mice were considered not relevant to humans under normal use. The commonly used ammonium salt of glufosinate is formulated with an anionic surfactant. Symptoms include gastrointestinal effects, impaired respiration, neurological disturbance, and cardiovascular effects.

FUNGICIDES

Fungal diseases caused by thousands of species of fungi are virtually impossible to control without chemical application. Most fungicides are surface or plant protectants, and are applied prior to potential infection by fungal spores, either to plants or to postharvest crops. Other fungicides can be used therapeutically to cure plants when an infestation has already begun. Still others are used as systemic fungicides that are absorbed and distributed throughout the plant. With few exceptions, fungicides have low acute toxicity in mammals.

Captan and Folpet

Captan and folpet are broad-spectrum protectant chloroalkylthio fungicides, with side chains containing chlorine, carbon, and sulfur. They are potent eye irritants and moderate skin irritants. Dermal absorption is low. Captan, folpet, as well as their common metabolite thiophosgene are

mutagenic in in vitro tests; however, in vivo mutagenicity tests are mostly negative, possibly because of the rapid degradation of these compounds. Both fungicides induce the development of duodenal tumors in mice.

Dithiocarbamates

Many dithiocarbamates are associated with metal cations; there are maneb (Mn), ziram and zineb (Zn), mancozeb (Mn and Zn), and ferbam (Fe). Thiram is an example of dithiocarbamate without a metal moiety. The dithiocarbamates have low to moderate acute toxicity by the oral, dermal, and respiratory routes. These compounds are metabolized to a common metabolite, ethylenethiourea (ETU), which inhibits the synthesis of thyroxine (T4) and triiodothyronine (T3), leading to elevated serum levels of thyroid-stimulating hormone (TSH). ETU also causes liver tumors in mice. Neither dithiocarbamates nor ETU are genotoxic in testing.

Developmental toxicity and teratogenicity are observed with dithiocarbamates and ETU. A key concern with chemicals affecting thyroid functions is their potential developmental neurotoxicity. The structure of dithiocarbamate fungicides resembles that of disulfiram, which inhibits aldehyde dehydrogenase. Interactions of dithiocarbamates with alcohol may elevate acetaldehyde levels and with dopamine may elevate the toxic metabolite DOPAL in the striatum and contribute to Parkinson's disease.

Chlorothalonil

Chlorothalonil is a halogenated benzonitrile fungicide widely used to treat vegetable, ornamental, and orchard diseases. Oral and dermal toxicities are low. Chlorothalonil is metabolized through glutathione conjugation, with fecal excretion. Chlorothalonil is not mutagenic in in vitro and in vivo tests. Chlorothalonil is not a reproductive or developmental toxicant. Adverse effects in humans are limited to its irritant effects on eye and skin.

Benzimidazoles

Benomyl, the main representative of this class of fungicides, inhibits microtubule assembly in fungi, with minor effects in plants or mammals. Acute toxicity is low, while chronic studies have found effects in the liver, testes, bone marrow, and gastrointestinal tract. Benomyl causes chromosomal aberrations (aneuploidy) both in vitro and in vivo. The action on dividing cells has raised concern for benomyl's potential teratogenicity and developmental toxicity. Benomyl has been shown in animals to affect the male reproductive system (decreased testicular and epididymal weight and reduced sperm count). A metabolite, *S*-methyl *N*-butylthiocarbamate sulfoxide, inhibits aldehyde dehydrogenase, which raises DOPAL (dihydroxyphenylacetaldehyde) levels in the striatum, suggesting that benomyl may be an etiological factor in Parkinson's disease.

Other Old and New Fungicides

Inorganic and Organometal Fungicides—Copper sulfate has overall low toxicity and is a widely used fungicide. Triphenyltin acetate has moderate to high acute toxicity and may cause

reproductive toxicity and endocrine disruption. These effects are believed to be due to activation of RXR (retinoid X receptor) and of PPARγ (peroxisome proliferator–activated receptor γ) by triphenyltin.

Azoxystrobin—Azoxystrobin is a broad-spectrum fungicide obtained by chemical modification of strobilurins, antifungal compounds present in the mushroom *Strobilurus tenacellus*. Azoxystrobin inhibits mitochondrial respiration and energy production by blocking electron transfer at the quinone "outside" site (Qo), ultimately preventing generation of ATP. Azoxystrobin has low acute toxicity in mammals, though it is toxic to aquatic organisms.

Prothioconazole—Prothioconazole blocks the CYP51 sterol 14α-demethylase resulting in depletion of ergosterol. No significant mammalian toxicity has been identified.

RODENTICIDES

Rodents are vectors for several diseases (e.g., plague, endemic rickettsiosis, and spirochetosis), can occasionally bite people, can consume large quantities of postharvest stored foods, and can contaminate foodstuffs with urine, feces, and hair, which may cause diseases. To be effective, yet safe, a rodenticide must satisfy several criteria: (a) the poison must be very effective in the target species once incorporated into bait in small quantity; (b) baits containing the poison must not induce bait shyness, so that the animal will continue to eat it; (c) the manner of death must be such that survivors do not become suspicious of its cause; and (d) it should be species-specific, with considerably lower toxicity to other animals.

Anticoagulants

Coumarin derivatives, including warfarin, are used as rodenticides and as anticoagulants. Coumarins antagonize the action of vitamin K in the synthesis of clotting factors (factors II, VII, IX, and X). Their specific mechanism involves inhibition of vitamin K epoxide reductase, which regenerates reduced vitamin K necessary for sustained carboxylation and synthesis of relevant clotting factors. The "second generation" anticoagulants including brodifacoum, difenacoum, diphacinone, and chlorophacinone have prolonged half-lives and cause very long-lasting inhibition of coagulation. Hematuria, gingival bleeding, epistaxis, gastrointestinal bleeding, and spontaneous ecchymosis may be observed in cases of poisoning with long-acting anticoagulant rodenticides.

Bromethalin

Bromethalin is a "single dose" rodenticide. Its acute toxicity is linked to the ability of certain animal species (rats and cats) to metabolize bromethalin to the toxic desmethylbromethalin. Symptoms of poisoning include tremors, convulsions, and prostration, which are seen 2 to 36 hours after exposure. Mitochondrial oxidative phosphorylation is uncoupled resulting in decreased ATP synthesis. In the CNS this leads to retention of water causing edema of the myelin sheath.

Cholecalciferol

Vitamin D helps the body maintain calcium balance by enhancing its absorption from the gastrointestinal tract and the kidneys. Toxic doses of cholecalciferol cause an excessive increase in calcium levels, leading to renal, CNS, and muscle alterations. Several doses of cholecalciferol are needed to obtain the rodenticidal action.

Zinc Phosphide

Metal phosphides such as aluminum phosphide (AlP), magnesium phosphide (Mg_3P_2), and zinc phosphide (Zn_3P_2) are used globally as single-dose rodenticides. Their toxicity is due to the phosphine gas (PH_3) formed during ingestion by reactions with water or acids:

$$Zn_3P_2 + 6H_2O \rightarrow 3Zn(OH)_2 + 2PH_3;$$

$$Zn_3P_2 + 6H+ \rightarrow 3Zn^{2+} + 2PH_3.$$

Phosphine causes widespread cellular toxicity with injury to the heart, liver, kidney, and nervous system by generation of oxidative stress. Exposure to phosphides by accidental or intentional ingestion is often lethal, and there are no known antidotes.

Norbormide

Norbormide shows selective toxicity in rats but not mice. Toxicity may be accounted for by differences in response of the peripheral blood vessels to norbormide-induced vasoconstriction. At the cellular level, norbormide causes a rat-specific activation of the mitochondrial permeability transition pore, which requires an intact outer mitochondrial membrane, and is mediated by the 18-kDa translocator protein (TSPO).

Fluoroacetic Acid and Its Derivatives

Sodium fluoroacetate (Compound 1080) has high acute mammalian toxicity, targeting the CNS and the heart. Fluoroacetate is incorporated into fluoroacetyl-coenzyme A, which condenses with oxaloacetate to form fluorocitrate, which in turn inhibits mitochondrial aconitase. This results in inhibition of the Krebs cycle, leading to lowered energy production, reduced oxygen consumption, and reduced cellular ATP levels.

FUMIGANTS

Active toward insects, mites, nematodes, weed seeds, fungi, or rodents, fumigants are in the gaseous form at the time they exert their pesticidal action. They can be liquids that readily vaporize (e.g., ethylene dibromide), solids that can release a toxic gas on reaction with water (e.g., phosphine released by aluminum phosphide), or gases (e.g., methyl bromide). For soil fumigation, the compound is injected directly into the soil, then covered with plastic sheeting and

sealed. Compounds used as fumigants are usually nonselective, highly reactive, and cytotoxic. They provide a potential hazard, primarily for applicators, from the standpoint of inhalation exposure, and to a minor degree for dermal exposure or ingestion, in case of solids or liquids.

Methyl Bromide

Acute exposure results in respiratory, gastrointestinal, and particularly neurologic symptoms, including lethargy, headache, seizures, paresthesias, peripheral neuropathy, and ataxia. Methyl bromide is classified by IARC in Group 3 (not classifiable as to its carcinogenicity to humans). As methyl bromide is an odorless and colorless gas, another fumigant, *chloropicrin*, which has a pungent odor and causes eye irritation, was often used in conjunction with methyl bromide and other fumigant mixtures to warn against potentially harmful exposures. The main adverse effects of chloropicrin in humans are primarily lacrimation, irritation cough, burning, and chest pains.

1,3-Dichloropropene

1,3-Dichloropropene ($C_3H_4Cl_2$) is a soil fumigant, extensively utilized to control soil nematodes. It has a moderate to high acute toxicity in animals. It is an irritant, causing redness and necrosis of the skin. It is extensively metabolized, with the mercapturic acid conjugate being the major urinary metabolite. Data on genotoxicity and carcinogenity are equivocal. Because it lacks herbicidal properties, dichloropropene is often formulated with chloropicrin.

Metam Sodium

Metam sodium ($C_2H_4NNaS_2$) is hydrolyzed to methyl isothiocyanate (MITC), which is toxic toward soil nematodes, fungi, and weed seeds. In mammals, metam sodium is metabolized in vivo to carbon disulfide and MITC. Upon chronic exposure in various species, toxic effects in the bladder, kidney, and liver have been reported. Metam sodium is not genotoxic but is classified as a probable human carcinogen (B2). In humans, metam sodium can act as a contact sensitizer, inducing allergic dermatitis. Acute exposure of vaporized MITC are irritated or burning eyes, nasal and throat irritation, nausea, coughing, and shortness of breath.

Sulfur Compounds

Elemental sulfur is effective for the control of fungal plant diseases. In agriculture, sulfur finds its major use in grapes and tomatoes, and it can be used in organic farming. The primary health effect in humans is dermatitis. In ruminants, excessive sulfur ingestion can cause cerebrocortical necrosis (polioencephalomalacia), possibly due to conversion by microorganisms in the rumen to hydrogen sulfide.

Sulfuryl fluoride (SO_2F_2) is also used as a fumigant and for postharvest fumigation of stored commodities. There is no evidence of developmental toxicity, mutagenicity, or carcinogenicity. Upon chronic exposure, the primary effect in multiple species is neurotoxicity, evidenced by microvacuolation in various brain areas; the mechanism is unknown.

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QUESTIONS

1. Which of the following does NOT contribute to the environmental presence of organochlorine insecticides?
   - **a.** high water solubility.
   - **b.** low volatility.
   - **c.** chemical stability.
   - **d.** low cost.
   - **e.** slow rate of degradation.

2. All of the following are characteristics of DDT poisoning EXCEPT:
   - **a.** paresthesia.
   - **b.** hypertrophy of hepatocytes.
   - **c.** increased potassium transport across the membrane.
   - **d.** slow closing of sodium ion channels.
   - **e.** dizziness.

3. Anticholinesterase agents:
   - **a.** enhance the activity of AChE.
   - **b.** increase ACh concentration in the synaptic cleft.
   - **c.** only target the neuromuscular junction.
   - **d.** antagonize ACh receptors.
   - **e.** cause decreased autonomic nervous system stimulation.

4. All of the following symptoms would be expected following anticholinesterase insecticide poisoning EXCEPT:
   - **a.** bronchodilation.
   - **b.** tachycardia.
   - **c.** diarrhea.
   - **d.** increased blood pressure.
   - **e.** dyspnea.

5. Which of the following insecticides blocks the electron transport chain at NADH–ubiquinone reductase?

a. nicotine.
b. carbamate esters.
c. nitromethylenes.
d. pyrethroid esters.
e. rotenoids.

6. What is the main mechanism of pyrethroid ester toxicity?
a. blockage of neurotransmitter release.
b. inhibition of neurotransmitter reuptake.
c. acting as a receptor agonist.
d. causing hyperexcitability of the membrane by interfering with sodium transport.
e. interfering with Cl^- transport across the axonal membrane.

7. Which of the following herbicides is NOT correctly paired with its mechanism of action?
a. glufosinate—inhibition of glutamine synthetase.
b. paraquat—interference with protein synthesis.
c. glyphosate—inhibition of amino acid synthesis.
d. chlorophenoxy compounds—growth stimulants.
e. diquat—production of superoxide anion through redox cycling.

8. Captan:
a. is an herbicide that inhibits root growth.
b. is an insecticide that targets the reproductive organs.
c. is a fungicide that could cause duodenal tumors.
d. is an herbicide that stimulates growth.
e. is a fungicide that is a known teratogen.

9. What is a mechanism of action of nicotine?
a. Nicotine antagonizes ACh at the neuromuscular junction.
b. Nicotine decreases the rate of repolarization of the axonal membrane.
c. Nicotine interferes with sodium permeability.
d. Nicotine acts as an ACh agonist in the synapse.
e. Nicotine inhibits the release of neurotransmitter.

10. Which of the following is the most characteristic of warfarin poisoning?
a. diarrhea.
b. cyanosis.
c. decreased glucose metabolism.
d. hematomas.
e. seizures.

CHAPTER 23

Toxic Effects of Metals

Alexander C. Ufelle and Aaron Barchowsky

KEY POINTS

- Persons at either end of the life span, young children or elderly people, are more susceptible to toxicity from exposure to a particular level of metal than most adults.
- Metals that provoke immune reactions include mercury, gold, platinum, beryllium, chromium, and nickel.
- *Complexation* is the formation of a metal ion complex in which the metal ion is associated with a charged or uncharged electron donor, referred to as a *ligand.*
- *Chelation* occurs when bidentate ligands form ring structures that include the metal ion and the two ligand atoms attached to the metal.
- Metal–protein interactions include binding to numerous enzymes, the metallothioneins, nonspecific binding to proteins such as serum albumin or hemoglobin, and specific metal carrier proteins involved in the membrane transport of metals.

INTRODUCTION

What Is a Metal?

Metals are typically defined by physical properties of the element in the solid state. General metal properties include high reflectivity (luster), high electrical conductivity, high thermal conductivity, and mechanical ductility and strength. A characteristic of toxicological importance is that metals exhibit variable oxidation states and may react in biological systems by losing one or more electrons to form cations. An overview of *toxic effects of metals* is shown in Fig. 23–1.

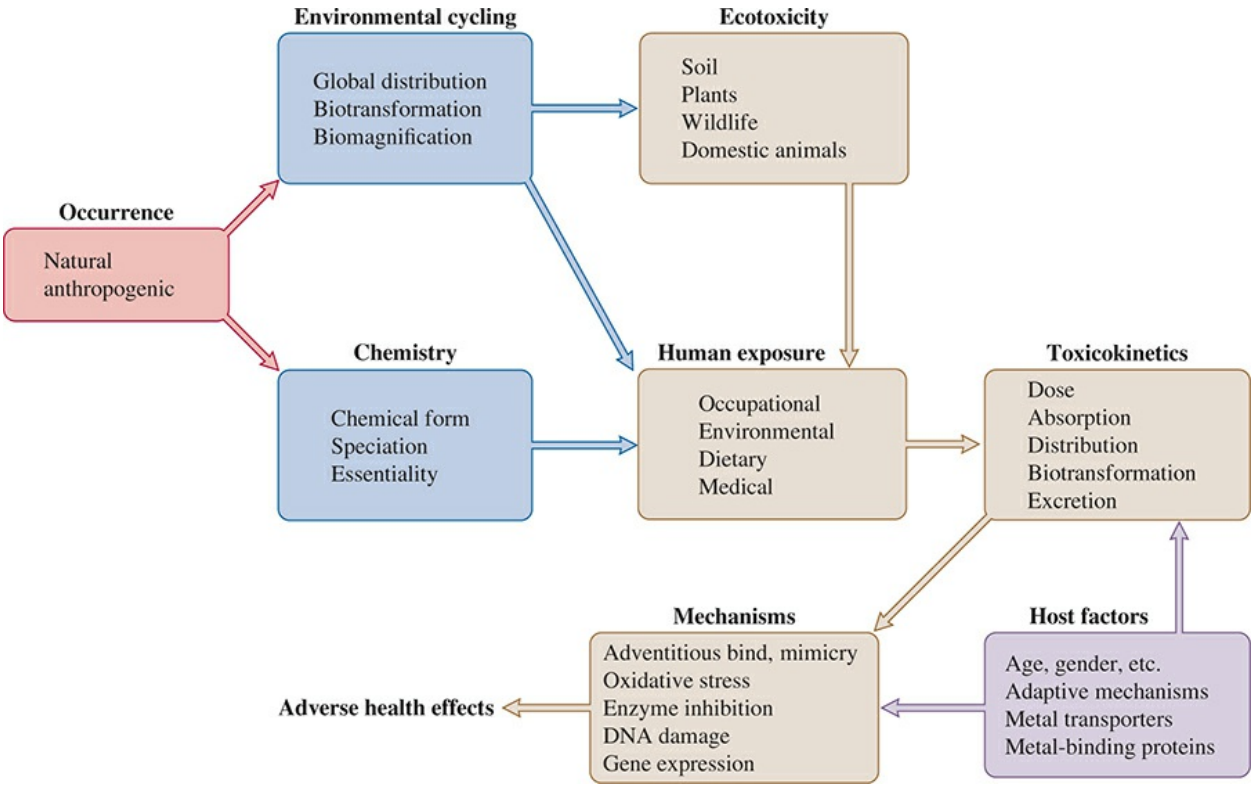

FIGURE 23–1 *Overview of metal toxicology.*

Metals as Toxicants

Unique among pollutant toxicants, metals are all naturally occurring and are ubiquitous to some level within the human environment. Regardless of how safely metals are used in industrial processes or consumer products, some level of human exposure is inevitable. Metals are elements that are neither created nor destroyed by human endeavors. Human use of metals can alter its chemical form or speciation and thereby impact toxic potential. Production of metal nanoparticles has created many novel metal properties including unique toxicities. Metals are persistent and potentially more toxic in the human environment. Metal exposures contribute to the etiology of diseases of essentially all organ systems (Table 23–1). Several metals are known or probable carcinogens promoting a range of cancer types.

TABLE 23–1 Toxicity of several metals or metalloids.

Metal	CNS	GI Tract	Lung	Kidney	Liver	Heart	Blood	Skin
Aluminum	*		*					
Antimony			*			*		*
Arsenic	*	*	*	*	*		*	
Beryllium			*					*
Bismuth				*	*			*
Cadmium	*	*	*	*	*	*		
Chromium	*		*	*	*			*
Cobalt	*	*	*			*		*
Copper		*					*	
Iron	*	*	*		*		*	
Lead	*	*		*			*	*
Lithium	*	*		*		*		
Manganese	*		*					
Mercury	*	*	*	*				
Nickel	*		*					*
Selenium		*		*				*
Silver			*					*
Zinc		*					*	

Movement of Metals in the Environment

Metals are redistributed naturally in the environment by both geological and biological cycles. Rainwater entering surface and groundwater aquifers releases soluble metals from rocks and ores. Surface water runoff and exchange of groundwater with surface waters, as well as human pumping of water from aquifers, release metals into the biosphere and rivers that eventually transport these substances to the ocean to be precipitated as sediment or taken up into forming rainwater, reinitiating the natural water cycle. Biological cycles moving metals include biomagnification by microorganisms, plants, and animals resulting in incorporation in food cycles. Human activities shorten the residence time of metals in ore deposits and greatly enhance metal distribution in the global environment by discharge to soil, water, and air.

Routes of Exposure and Absorption of Metals

The main routes of human exposure to metals include oral ingestion, inhalation, and dermal contact. Oral ingestion of naturally occurring metals comes primarily from drinking water drawn from contaminated groundwater sources (e.g., arsenic, fluoride, manganese, and iron) and food. Metals accumulate in various foods, especially those grown in soils naturally containing or anthropogenically contaminated with metals. The absorption of metals through the gastrointestinal tract depends on the metal solubility, pH of the environment, secreted ligands, rate of transport through the mucous layer, and the presence and density of specific transport proteins in the gut wall.

Occupational exposures to metals and metal nanoparticles (e.g., cobalt ferrite, cesium, and cadmium core) primarily enter the human body through inhalation and secondarily via oral ingestion. Inhalation of metal-containing fumes generated by welding and related processes may result in “metal fume fever” that presents with nonspecific complaints including influenza-like symptoms, fever, shaking chills, arthralgias, myalgias, headache, and malaise. In addition to specific metal transporters that move the metals from apical to basolateral surfaces for absorption into the blood stream, soluble metals are absorbed through the alveoli and follow water between epithelial cells. Insoluble forms are coughed out or moved by the mucocilliary tree into the gastrointestinal tract for excretion.

Dermal exposures are usually limited due to poor uptake of the small, charged metal ions across the impervious lipophilic epidermis. Corrosive forms of metals such as strong acids (e.g., chromic acid) or bases (e.g., metal hydroxides) cause dermal and mucus membrane corrosion and chemical burns. Exogenous factors that increase dermal exposure include dose, vehicle, molecular volume, counter ion, valence, protein reactivity, tissue deposition, solubility, and pH. Endogenous factors include age of the skin, anatomical site, homeostatic control, skin layer, and the importance of oxidation and reduction of xenobiotics on the skin.

Metals introduced therapeutically either by injection/infusion or by implantation pose toxic risk with either overdose or, in the case of orthopedic prostheses, immune or foreign body reactions. Degradation products of cobalt-, titanium-, and chrome/nickel-based implants evoke pathologic foreign body reactions, lymphocyte proliferation, and allergic rejection reactions.

Distribution of Metals in the Body

Organ/tissue distribution of metals depends on organ blood flow and organ-specific properties. Many nonessential, toxic metals distribute and accumulate in the body by mimicking and competing with essential metals of similar physical character. Metals disrupt metabolic functions by accumulating in organs or by displacing vital nutrients or metals.

Metal Transporters and Metal-Binding Proteins

Ionic metals must be absorbed into the body and in cells by transport through selective and relatively nonselective ion channels. All cells possess mechanisms for metal ion homeostasis that involve a balance between uptake and efflux systems. The 30 different transporters and channels for zinc (the third most abundant transition element in the body) exemplify the breadth of metal transporters and their complex regulation of metal ion concentrations in the circulation, in cells, and within cellular compartments. Magnesium (the fourth most abundant metal in the body) has seven transporters and channels that are distributed differentially throughout the body. The TRPM7 channel is a signal-generating receptor, as well as a transcellular membrane transporter. The extracellular and intracellular concentrations of all of the essential metals are tightly controlled by their transporters and chaperone proteins such that these proteins become rate-limiting regulators of metal action and toxicity.

Many transporters are promiscuous in conveying a number of similarly sized and valenced cations. This provides an axis for nonessential, toxic metals to have cellular access and opportunity to cause toxicity. Toxicity also occurs when toxic metals or excessively high concentrations of essential metals outcompete an essential metal for transport and action.

Metal chaperones are proteins or small molecules that prevent metal ions from roaming freely within the circulation and in cells. Transferrin is a glycoprotein that binds most of the ferric iron in plasma and helps transport iron across cell membranes where it is delivered to ferritin, the primary cellular iron storage protein. Transferrin also transports aluminum and manganese and may serve as a general metal detoxicant protein, because it binds many toxic metals including cadmium, zinc, beryllium, and aluminum. Ceruloplasmin is a ferroxidase enzyme that is the major copper-carrying protein in blood and helps convert ferrous iron to ferric iron, which then binds to transferrin. Chaperones are also responsible for delivering metals into metalloproteins, such as the approximately 1000 Zn and many Cu-containing enzymes in humans. The 30 zinc channels and transporters chaperone zinc across membranes and through specific cellular compartments. These chaperones may be a target of toxicity if they are overwhelmed by a competing metal or bind an incorrect metal. Examples include systems to keep redox active iron, manganese, and nickel from damaging organelles and cells.

Metallothioneins are metal chaperones that function in essential metal homeostasis and metal detoxification. Twenty of the 60 amino acids in the metallothioneins are highly conserved cysteines essential for binding and coordinating Zn and Cu, Cd, Mg, and other d10 electron configuration metals. Expression of metallothioneins is induced by various metals acting on metal transcription factor-1. The high induction of metallothioneins in the kidney by Cd creates a sink for sequestering Cd. However, this is also the source of renal toxicity when Cd overwhelms the sequestering chaperone.

Excretion of Metals

Metals are excreted through fecal, biliary, and urinary elimination, as well as through sweat and storage in hair and nails. Several metals including lead, cadmium, arsenic, inorganic mercury, organic mercury, iron, manganese, magnesium, chromium, zinc, copper, nickel, cobalt, tin, and aluminum are excreted through bile and urine. Factors that affect urinary excretion of metals include induction of metallothionein and chaperone proteins and degree of protein affinity. Arsenic is somewhat unique in being metabolized by a highly conserved As(III) *S*-adenosylmethionine methyltransferase (AS3MT) to monomethylated and dimethylated metabolites that are excreted in the urine more readily than the parent compound. Cadmium, lead, and mercury are also excreted through the skin. Hair and nails sequester metals in keratin matrices to reduce toxicity.

Biomarkers of Metal Exposure

Exposure biomarkers, such as concentrations in blood, urine, nails, and hair, have long been used for metals. Techniques in molecular toxicology have greatly expanded the possibilities for biomarkers. Thus, in the case of chromium, DNA–protein complexes may serve as a biomarker of both exposure and carcinogenic potential. The capacity for expression of genes for metallothioneins and hemeoxygenase may also be used.

A critical indicator of retention of a metal is its biological half-life, which varies according to the metal as well as the organ or tissue. Hair and nail content should be considered excreted metal as it is irreversibly sequestered away from biological action. Results from single measurements may reflect recent, long-term, or past exposure depending on retention time in the

particular tissue. Blood and urine concentrations usually reflect recent exposures and correlate with acute adverse effects, unless metals are released owing to acute kidney injury. The hair can be useful in assessing variations in exposure to metals over the period of its growth. Analyses can be performed on segments of the hair, so that metal content of the newest growth can be compared with past exposures. Fingernails and toenails have been reliably used for measuring chronic exposures and provide ample material for quantifying multiple metals. However, often only total metal levels can be quantified in nails and it is not possible to identify different metal and metalloid species.

Chemical Mechanisms of Metal Toxicology

Ionic metals can be very reactive, interacting with biological systems in many ways. Cells present numerous biologically active metal-binding sites in proteins and nucleic acids. Such adventitious binding is an important mechanism by which exogenous metals exert toxic effects. Binding of inappropriate toxic metals in enzymes is a major mechanism for disrupting normal function. Binding of genotoxic metals (e.g., chromium and methylmercury) disrupts DNA structure, promotes ineffective DNA repair, and is mutagenic. Toxic metals may bind physiological sites normally reserved for essential elements. Through mimicry, the toxic metals gain access to and disrupt important actions of the essential metal and critical metal-mediated cellular functions. Indeed, molecular or ionic mimicry at the level of transport is often a key event in metal toxicity. Metals in their ionic form can form DNA and protein adducts in biological systems, inducing aberrant gene expression, which, in turn, produces adverse effects.

Free ionic transition metals (e.g., iron, copper, manganese, and molybdenum) produce significant toxicity through one electron reduction generating free electrons and reactive oxygen species (ROS). Tight coordination of these metals in enzymes catalyzes efficient electron flow and transfer. However, when in excess or unbound, ionic metals catalyze formation of cell membrane damaging oxygen-centered lipid radicals and peroxides, as well as incomplete reduction of molecular oxygen through Haber–Weiss and Fenton reactions with superoxide and H_2O_2.

$$Fe^{2+} + O_2 \longrightarrow (FeO_2)^{2+}(\text{oxidant}) \longrightarrow \text{Lipid}$$

$$\text{Lipid}\bullet + O_2 \rightarrow \text{LipidOO}\bullet$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + OH^-$$

The resultant lipid or hydroxyl radicals react indiscriminately with macromolecules damaging proteins and DNA. In addition to oxygen-based radicals, damaging carbon- and sulfur-based radicals also occur. In contrast, lower levels of generated lipid and hydrogen peroxides are signal generating and affecting gene regulation through protein interactions. Signal-generated low levels of ROS, such as H_2O_2, are second messengers for many receptor-mediated vasoactive and mitogen responses, and high ROS levels from respiratory bursts or mitochondrial injury overwhelm cellular antioxidant defenses, promote mitochondrial calcium leak, and damage or kill cells.

Molecular Responses to Metal Exposure

Exposure to essential metals produces homeostatic signaling for normal tissue maintenance. Nonessential metals signal for homeostatic disruption or directly cause inappropriate activation of growth or metabolic signaling, loss of essential enzymatic activity, direct structural damage, and if severe, cellular apoptosis and/or necrosis. Disruption of normal cell signaling through inappropriate growth factor signaling, promotion of maladaptive stress responses, and cell senescence can be as detrimental in disease promotion as cell death.

The ability of metals to affect gene expression and generate pathogenic cell phenotypes is well-documented. Modern genomic technologies have identified hundreds to thousands of genes whose levels of expression are affected after exposure to excess metals. The intended consequence of metal activation of gene expression is often to protect the organism from metal-induced damage as metal exposure is associated with increased expression of genes encoding proteins that remove the metal from the cell via chelation or increased export, reduce the level of oxidative stress, and repair the metal-induced intracellular damage.

Metals initiate cell signaling by activating cell receptors. Targeted receptors include both tyrosine kinase receptors (e.g., epidermal growth factor receptors) and G protein–coupled receptors. However, the activation differs from tightly regulated activation by normal endogenous ligands. Signal amplification allows for a relatively small amount of toxic metal in chronic exposures to affect broad programs of genetic and epigenetic change that ultimately generate pathogenic cell phenotypes. These broad signaling programs include activation of the pleiotropic mitogen-activated protein kinase (MAPK) cascades that can lead to enhanced cell proliferation at low metal concentrations or cell death at higher concentrations depending on the MAPK family member (e.g., Erk, JNK, or p38). Similarly, metal signaling activates the Src family kinases that regulate programs of cell proliferation. The kinases are intermediates in amplifying signals for metal-induced transcriptional programmatic change that include the stress-responsive activation of activator protein-1 (AP-1), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), metal transcription factor-1 (MTF-1), nuclear factor (erythroid-derived 2)-like 2 (Nrf2), hypoxia inducible factors (HIF-1α and HIF-2α), and the signal transducer and the signal activator of transcription (STAT) family. The ability of nickel, cadmium, arsenic, and chromium to induce cancer has been linked to metal-inducible epigenetic changes.

Factors Impacting Metal Toxicity

Exposure-related factors include dose, route of exposure, duration, and frequency of exposure. Because metals can be highly reactive, the portal of entry is often initially the organ most affected. Host-based factors that can impact metal toxicity include age at exposure, gender, and capacity for biotransformation. In addition to overt teratogenicity, fetal and perinatal exposures can result in epigenetic reprograming of development that can lead to enhanced risk of adult disease. Developing stem cells, as well as rapidly proliferating and differentiating cells, are important pathogenic targets in the fetus.

The oxidation state and valence of the metal greatly affect its competition for uptake and mechanism of interaction with cellular macromolecules. Metabolism by microbes or mammals affects distribution, site of action, and excretion.

Lifestyle factors such as smoking or alcohol ingestion may have direct or indirect impacts on

the level of metal intoxication. For instance, tobacco smoke contains many toxic metals, such as cadmium and arsenic. Alcohol ingestion may influence toxicity by altering diet, reducing essential mineral intake, and altering hepatic iron deposition. Alcohol can also synergize with metals, such as arsenic, through amplification of interacting signal transduction pathways that may promote cancers. The composition of the diet can significantly alter gastrointestinal absorption of various dietary metals.

Adaptive mechanisms are critical to susceptibility to the toxic effects of metals. Typically, adaptation is acquired after the first few exposures and can be long-lasting or transient after exposure ceases. Adaptation can be at the levels of uptake and excretion or, with some metals, through long-term storage in a toxicologically inert form (e.g., hair and nails). Metal exposure can also induce a cascade of molecular/genetic responses that may reduce toxicity, such as with metal-induced oxidative stress responses.

Therapeutic Use and Toxicity of Metals

Metals and metal compounds have been used in chemotherapeutic settings for millennia. Other examples of medicinal metals used today include aluminum (antacids and buffered analgesics), bismuth (peptic ulcer), lithium (mania and bipolar disorders), and gold (arthritis). Metallic compounds find their way into pharmacological preparations as active or inactive ingredients.

Treatment of metal poisoning is sometimes used to prevent, or even attempt to reverse, toxicity. The therapeutic strategy is to give metal chelators that complex the metal and enhance its excretion. Most chelators are not specific and will interact with several metals, eliminating more than just the metal of concern. Chelator therapy can be used for many different metals including lead, mercury, iron, and arsenic.

MAJOR TOXIC METALS

Arsenic

Arsenic (As) is a toxic and carcinogenic metalloid. The most common inorganic trivalent arsenic compounds are arsenic trioxide and sodium arsenite, while common pentavalent inorganic compounds are sodium arsenate, arsenic pentoxide, and arsenic acid. Important organoarsenicals include arsanilic acid, arsenosugars, and several methylated forms biotransformed by various organisms, including humans. Arsine (AsH_3) is an important gaseous arsenical. Industrial and military toxic arsenicals include phenylarsine oxide, roxarsone, and lewisite.

Occupational exposure to arsenic occurs in the manufacture of pesticides, herbicides, and other agricultural products, and from arsenic fumes and dusts owing to smelting industries. Environmental arsenic exposure mainly occurs from arsenic-contaminated drinking water, which is generally from natural sources. Food, especially seafood and rice, may contribute significantly to daily arsenic intake.

Toxicokinetics—Inorganic arsenic is well absorbed (80% to 90%) from the gastrointestinal tract, distributed throughout the body, often metabolized by methylation, and then excreted primarily in urine. Trivalent inorganic arsenic and trivalent methylated metabolite absorption from the

gastrointestinal track and movement into cells throughout the body occur through aquaglyceroporins 7 and 9. Trivalent arsenicals freely distribute throughout the body with the volume of distribution of water. Pentavalent inorganic and methylated arsenic species resemble phosphate and must compete with phosphate for cell entry. Skin is a potential route of exposure to industrial levels of arsenic.

Arsenic has a predilection for skin and is excreted by desquamation of skin and in sweat. It also concentrates in forming fingernails and hair bound to keratins. Arsenic exposure produces characteristic transverse white bands across fingernails (Mees' line), which appear about 6 weeks after the onset of symptoms of arsenic toxicity. Arsenic in the fingernails and hair has been used as a biomarker for exposure, including both current and past exposures, while blood and urinary arsenic is a good indicator for current exposure.

Toxicity

***Acute Poisoning*—**Ingestion of 70 to 180 mg doses of inorganic arsenic can be fatal. Symptoms of acute intoxication include fever, anorexia, hepatomegaly, melanosis, cardiac arrhythmia, and terminal cardiac failure. Acute arsenic ingestion can damage mucous membranes of the gastrointestinal tract, causing irritation, vesicle formation, and even sloughing. Sensory loss in the peripheral nervous system is the most common neurological effect, appearing at 1 to 2 weeks after large doses and consisting of Wallerian degeneration of axons. Anemia and leucopenia, particularly granulocytopenia, occur a few days after high-dose arsenic exposure.

Arsine gas is a potent hemolytic agent, producing acute symptoms of nausea, vomiting, shortness of breath, and headache accompanying the hemolytic reaction. Exposure to arsine is fatal in up to 25% of the reported human cases. Persistent exposure may be accompanied by hemoglobinuria, renal failure, jaundice, and anemia in nonfatal cases.

***Chronic Toxicity*—**The skin is a major target organ in chronic inorganic arsenic exposure. In humans, chronic exposure to arsenic induces characteristic changes in skin epithelium of diffuse or spotted hyperpigmentation. Palmar-plantar hyperkeratosis usually follows the initial appearance of arsenic-induced pigmentation changes within a period of years. Skin cancer that presents as in situ squamous carcinoma is common with protracted high-level arsenical exposure.

Cardiovascular disease and coronary artery disease in particular are strongly associated with environmental arsenic exposures. The dominant form of disease stems from arsenic's propensity to enhance both vessel disease and prolongation of the cardiac Q-T interval.

Chronic exposure also produces respiratory symptoms of chronic obstructive pulmonary disease (COPD) and increased respiratory disease mortality. In addition, arsenic exposure is associated with increased respiratory tract infections and chronic lung infections. Liver injury, characteristic of long-term arsenic exposure, manifests initially as jaundice, abdominal pain, and hepatomegaly, but may progress to cirrhosis and ascites, even to hepatocellular carcinoma.

Arsenic produces a peripheral polyneuropathy after severe poisoning. This neuropathy usually begins with sensory changes, such as numbness in the hands and feet, but later may develop into a painful "pins and needles" sensation. Both sensory and motor nerves can be affected, and muscle tenderness often develops, followed by weakness, progressing from proximal to distal muscle groups. Histological examination reveals a dying-back axonopathy with demyelination, and effects are dose-related.

Arsenic, especially with chronic early-life exposure, is immunotoxic and potentially increases risk of infections and inflammatory-like diseases during childhood and in adulthood. This

immunosuppressive potential is prevalent in respiratory tract infections, and enhancement of a global inflammatory state may underlie the etiology of neural, cardiovascular, metabolic, and cancer disease promotion.

***Mechanisms of Toxicity*—**The trivalent compounds of arsenic are thiol-reactive, and thereby activate or inhibit enzymes or alter protein structure by reacting with proteinaceous thiol groups. Pentavalent arsenate mimics phosphate and can inhibit phosphotransfer reactions, such as mitochondrial oxidative phosphorylation, when present in concentrations that are stoichiometrically competitive. Arsenic and its metabolites stimulate oxidant production through activation of NADPH oxidases and inference with mitochondrial respiration. Transition between trivalent and pentavalent oxidation states occurs through two electron transfer and thus arsenic is not capable of directly generating reactive oxygen species. Instead, reaction with critical cysteines in receptors and signaling enzymes that stimulate NADPH oxidase generate ROS.

Carcinogenicity—Arsenic is a known human carcinogen, mostly associated with tumors of the skin, lung, and urinary bladder, and possibly the kidney, liver, and prostate. Arsenic is not mutagenic in bacteria and acts weakly in mammalian cells, but can induce chromosomal abnormalities, aneuploidy, and micronuclei formation. Arsenic can also act as a co-mutagen and/or co-carcinogen. Reaction with thiols in critical proteins produces transcriptional and epigenetic changes that promote cancers and tumor growth. Arsenic inhibits a number of DNA repair mechanisms and downregulates expression of some DNA repair genes. Inhibition of the tumor suppressor p53 and promotion of maladaptive antioxidant and antiapoptotic responses may underlie the co-carcinogenic properties of arsenicals. Arsenic signaling for increased ROS generation may promote cell proliferation at lower concentrations and DNA damage at higher concentrations that interfere with mitochondrial respiration.

Arsenic-induced skin cancers include basal cell carcinomas and squamous cell carcinomas, both arising in areas of arsenic-induced hyperkeratosis. In humans, the skin cancers often occur on areas of the body not exposed to sunlight (e.g., on palms of hands and soles of feet) and often occur as multiple primary malignant lesions.

The majority of arsenic-related cancer deaths result from lung cancer with in utero exposures increasing the risk of lung cancer later in life. It is likely that arsenic is not the primary carcinogen, but synergizes with the carcinogenic potential of tobacco smoke constituents. Arsenic-promoted lung cancer is independent of route of exposure with oral ingestion and inhalation giving equivalent increases in risk of disease.

The unique kinetics of arsenic and its methylated trivalent metabolites greatly increase risk for transitional cell carcinoma of the bladder. Bladder cancer risk is up to sixfold higher in arsenic-exposed females relative to males, and the association of arsenic exposure with bladder cancer risk is greatly enhanced in smokers or ever-smokers.

Treatment—For acute arsenic poisoning, treatment is symptomatic, with particular attention to fluid volume replacement and support of blood pressure. The chelators, penicillamine, succimer, and dimercaptopropanesulfonic acid (DMPS) have been used for acute arsenic poisoning. For chronic poisoning, chelator therapy has not proved effective in relieving symptoms.

Cadmium

About 75% of cadmium produced is used in batteries, especially nickel–cadmium batteries. Cadmium has been used in electroplating or galvanizing alloys for corrosion resistance. It is also used as a color pigment for paints and plastics, in solders, as a barrier to control nuclear fission, as a plastic stabilizer, and in some special application alloys.

Exposure—Ingestion of plant-derived foods and certain types of seafood, as well as inhalation of tobacco smoke and industrial emissions are the major routes of exposure. Both natural and anthropogenic sources of cadmium contaminate soil, including fallout of industrial emissions, some fertilizers, soil amendments, and use of cadmium-containing water for irrigation, resulting in a slow but steady increase in the cadmium content in vegetables over the years. Inhalation is the dominant route of exposure in occupational settings. Occupations potentially at risk from cadmium exposure include those involved with refining zinc and lead ores, iron production, cement manufacture, and industries involving fossil fuel combustion, the manufacture of paint pigments, cadmium–nickel batteries, and electroplating.

Toxicokinetics—Gastrointestinal absorption of cadmium is limited to 5% to 10% of a given dose. Cadmium absorption can be increased by dietary deficiencies of calcium or iron and by diets low in protein. Absorption of cadmium after inhalation ranges from 10% to 60%.

The relationship of cadmium metabolism and toxicity is shown in Fig. 23–2. Once absorbed, cadmium is very poorly excreted through the urine and feces. The half-life of cadmium in humans is estimated to be more than 26 years. Cadmium binds to albumin and other higher-molecular-weight proteins. Transport into cells is mediated through calcium channels and through molecular mimicry. Cadmium induces the metallothioneins (MT) that are responsible for cellular retention. Cadmium–MT may be released from the liver and transported via blood to the kidney, where it is reabsorbed and degraded in the lysosomes of the renal tubules. This releases cadmium to induce more cadmium–MT complex or cause renal toxicity.

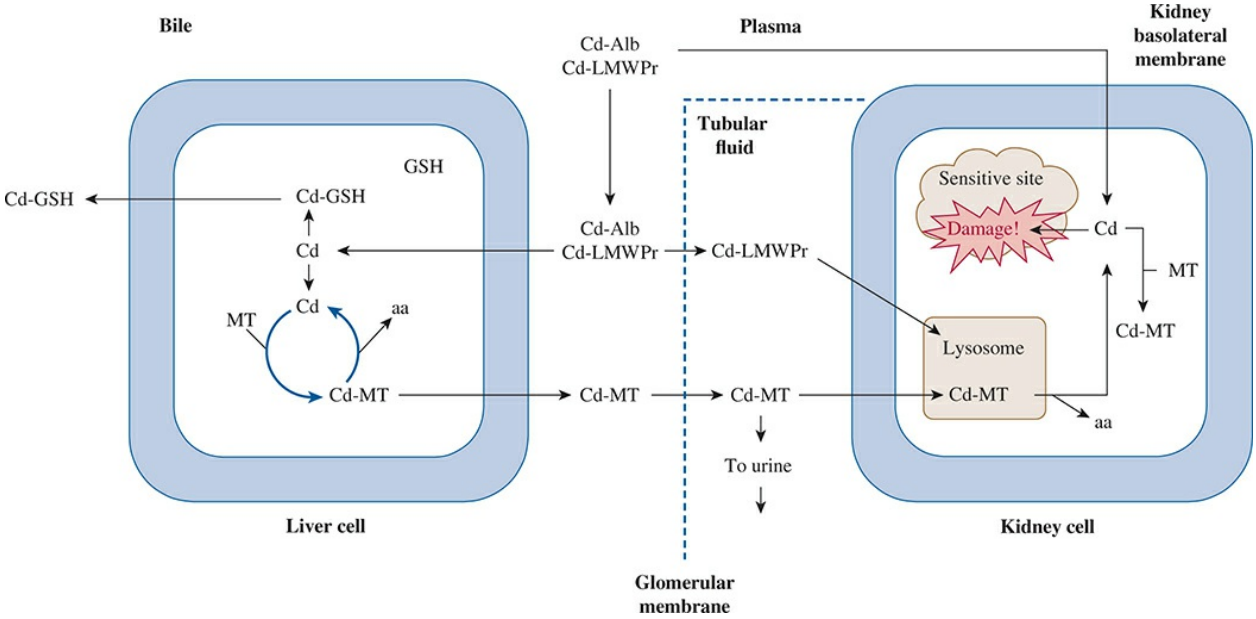

FIGURE 23–2 *Cadmium transport, protein binding, and toxicity.* aa, amino acids; Cd-Alb, Cd–albumin; Cd-LMWPr, Cd associated with low-molecular-weight proteins; GSH, glutathione; MT, metallothionein.

Toxicity—Acute cadmium toxicity from ingestion of high concentrations of cadmium-contaminated beverages or food causes nausea, vomiting, and abdominal pain. Inhalation of cadmium fumes or other heated cadmium-containing materials may produce acute pneumonitis with pulmonary edema. The major long-term toxic effects of low-level cadmium exposure are renal injury, obstructive pulmonary disease, osteoporosis, and cardiovascular disease.

***Nephrotoxicity*—**Cadmium markedly impairs renal function by causing an initial tubular cell necrosis and degeneration, progressing to an interstitial inflammation and fibrosis. The proteinuria includes β_2-microglobulin, *N*-acetyl-β-d-glucosaminidase (NAG), and MT, as well as retinol-binding protein, lysozyme, ribonuclease, α_1-microglobulin, and immunoglobulin light chains. The presence of larger proteins, such as albumin and transferrin, in the urine after occupational cadmium exposure suggests a glomerular effect as well.

***Chronic Pulmonary Disease*—**Cadmium-induced obstructive lung disease in humans can be slow in onset, and results from chronic bronchitis, progressive fibrosis of the lower airways, and accompanying alveolar damage leading to emphysema. Pulmonary function is reduced with dyspnea, reduced vital capacity, and increased residual volume.

***Skeletal Effects*—**Occupational cadmium exposure can cause hypercalciuria, renal stone formation, osteomalacia, and osteoporosis. The skeletal changes are possibly related to a loss or decrease of calcium absorption, and interference with the actions of parathyroid hormone, and disruption of collagen metabolism. Cadmium may also stimulate osteoclast activity, resulting in the breakdown of bone matrix, and interfere with calcification and bone remodeling.

***Cardiovascular Effects*—**There is strong association between cadmium exposure and peripheral vascular disease and cadmium may partially mediate the negative effect of smoking on peripheral artery disease. Low-level cadmium induces endothelial cell angiogenesis, but that higher, environmentally relevant levels may directly damage the vascular endothelium.

***Neurotoxicity*—**The blood–brain barrier severely limits cadmium access to the central nervous system, and a direct toxic effect appears to occur only with cadmium exposure prior to blood–brain barrier formation (young children), or with blood–brain barrier dysfunction under certain pathological conditions.

***Reproductive Health Effects*—**Cadmium exposure may be associated with multiple adverse women's reproductive health outcomes. Pregnant women appear to accumulate more cadmium than nonpregnant women. Prenatal cadmium exposure is associated with adverse effects on birth outcomes, child health, and development.

Carcinogenicity—In humans, occupational respiratory exposure to cadmium is associated with lung cancer. Both the kidney and pancreas accumulate high concentrations of cadmium, and exposure to cadmium may be associated with human renal and pancreatic cancer. Tumors of the testes, pancreas, adrenals, liver, kidney, pituitary, and hematopoietic system have been noted in mice, rats, or hamsters. Cadmium can be carcinogenic in animals.

Treatment—At the present time, there is no effective clinical treatment for cadmium intoxication. Chelation therapy for cadmium generally results in significant adverse effects.

Chromium

Most naturally occurring chromium (Cr) is found in the trivalent state in chromite ores. Sodium dichromate, a hexavalent chromium compound, is used as an oxidizing agent in stainless steel production and welding, chromium plating, ferrochrome alloys and chrome pigment production, and tanning industries. Hexavalent chromium is a human carcinogen. Chromium in ambient air originates primarily from ferrochrome production, ore refining, and chemical processing. Chromium fallout is deposited on land and water. Widespread industrial uses have increased chromium levels in the environment.

Toxicokinetics—Absorption of hexavalent chromium compounds is 2% to 10% and that of trivalent chromium compounds is 0.5% to 2%. Inhaled chromium compounds are absorbed in the lung via transfer across alveolar cell membranes. Dermal absorption depends on the chemical form, vehicle, and integrity of the skin. Concentrated potassium chromate may cause chemical burns to the skin and facilitate absorption. Hexavalent chromium readily crosses cell membranes via sulfate and phosphate transporters. In the blood, hexavalent chromium is taken up by erythrocytes; trivalent chromium is only loosely associated with erythrocytes. Chromium compounds distribute to all organs, with highest levels in the liver, spleen, and kidney. Chromium can remain in the lungs for years. Absorbed chromium is excreted primarily in urine.

Toxicity—Hexavalent chromium is corrosive and may cause chronic ulceration and perforation of the nasal septum, as well as chronic ulceration of other skin surfaces. It elicits allergic contact dermatitis among previously sensitized individuals, which is a type IV allergic reaction. Accidental ingestion of high doses of hexavalent chromium compounds may cause acute renal failure characterized by proteinuria, hematuria, and anuria.

Carcinogenicity—Hexavalent chromium compounds in the chrome production and pigment industries are associated with increased risk of lung cancer. Hexavalent chromium compounds are genotoxic, and can generate reactive oxygen radicals, inhibit protein synthesis, and arrest DNA replication. Hexavalent chromium can disrupt the p53 signaling pathway, alter the ATM/ATR cell cycle checkpoints, induce apoptosis, and interfere with DNA damage repair. Hexavalent chromium can also activate cell signaling enzymes, such as Src family kinases, to stimulate signal cascades leading to transcriptional repression of cellular protectant proteins.

Lead

Lead (Pb) in the environment comes mainly from human activity. Lead is not biodegradable and ecotoxicity of lead remains a worry.

Exposure—Occupational exposure to lead occurs for workers in the lead smelting and refining industries, battery manufacturing plants, glass manufacturing, steel welding or cutting operations, construction, rubber products and plastics industries, printing industries, firing ranges, radiator repair shops, and other industries requiring flame soldering of lead solder. In these occupations, the major routes of lead exposure are inhalation and ingestion of lead-bearing dusts and fumes.

Lead-containing paint in older housing and aging water infrastructure are primary sources of lead exposure in the general population and in children. Major environmental sources of lead for

infants and toddlers up to 4 years of age is hand-to-mouth transfer of lead-containing paint chips or dust from floors of older housing. Lead in household dust can also come from outside of the home in neighborhood soil. Food and water are also a major route of exposure.

Toxicokinetics—Adults absorb 5% to 15% of ingested lead and usually retain less than 5% of what is absorbed. Children absorb 40% to 50% of ingested lead with 32% retention. Lead absorption can be enhanced by low dietary zinc, manganese, iron, and calcium, especially in children. Airborne lead is a minor component of exposure. Lead in blood is primarily (about 99%) in erythrocytes bound to hemoglobin; only 1% of circulating lead in serum is available for tissue distribution. Lead is initially distributed to soft tissues such as the kidney and liver, and then redistributed to the skeleton and hair. The half-life of lead in the blood is about 30 days and is about 20 years in bone. Lead crosses the placenta, so that accumulation in fetal tissues, including brain, is proportional to maternal blood levels. Renal excretion of lead is usually through glomerular filtrate with some renal tubular resorption. Fecal excretion via biliary tract accounts for one-third of total excretion of absorbed lead.

Toxicity

Neurological, Neurobehavioral, and Developmental Effects in Children—Clinically overt lead encephalopathy may occur in children with high exposure to lead. Symptoms begin with lethargy, vomiting, irritability, loss of appetite, and dizziness, progressing to ataxia, a reduced level of consciousness, coma, and death. Recovery is often accompanied by sequelae including epilepsy, mental retardation, and, in some cases, optic neuropathy and blindness.

Lead can affect the brain by multiple mechanisms. It may act as a surrogate for calcium and/or disrupt calcium homeostasis. Lead affects virtually every neurotransmitter system in the brain, including glutamatergic, dopaminergic, and cholinergic systems. The stimulation of protein kinase C through mimicking calcium may alter the blood–brain barrier.

Neurotoxic Effects in Adults—Adults with occupational exposure may demonstrate abnormalities in neurobehavior. Peripheral neuropathy including foot drop and wrist drop are seen rarely in workers with excessive occupational exposure to lead. Peripheral neuropathy is characterized by segmental demyelination and possibly axonal degeneration.

Hematologic Effects—Lead has multiple hematologic effects, ranging from increased urinary porphyrins, coproporphyrins, δ-aminolevulinic acid (ALA), and zinc protoporphyrin to anemia. The heme biosynthesis pathway and the sites of lead interference are shown in Fig. 23–3. The most sensitive effects of lead are the inhibition of δ-aminolevulinic acid dehydratase (ALAD) and ferrochelatase. ALAD catalyzes the condensation of two units of ALA to form porphobilinogen (PBG). Inhibition of ALAD results in accumulation of ALA. Ferrochelatase catalyzes insertion of iron into the protoporphyrin ring to form heme. Inhibition of ferrochelatase results in accumulation of protoporphyrin IX, which takes the place of heme in the hemoglobin molecule and, as the erythrocytes containing protoporphyrin IX circulate, zinc is chelated at the site usually occupied by iron. Anemia only occurs in very marked cases of lead toxicity, and is microcytic and hypochromic, as in iron deficiency.

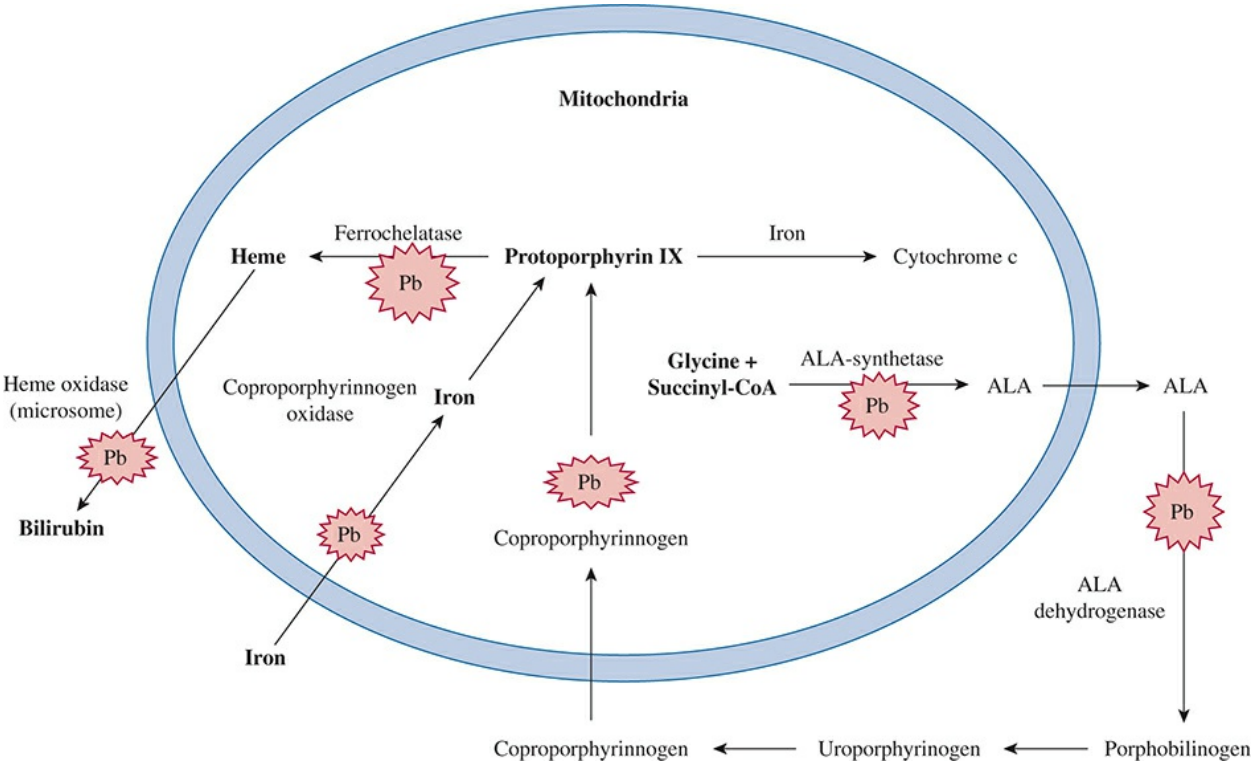

FIGURE 23–3 *Lead interruption of heme biosynthesis.* ALA, δ-aminolevulinate; Pb, sites for lead effects. The major lead inhibition sites are ALA dehydrogenase and ferrochelatase.

***Renal Toxicity*—**Acute lead nephrotoxicity consists of proximal tubular dysfunction and can be reversed by treatment with chelating agents. Chronic lead nephrotoxicity consists of interstitial fibrosis and progressive nephron loss, azotemia, and renal failure. A characteristic microscopic change is the presence of intranuclear inclusion bodies, which are composed of a lead–protein complex. The inclusion bodies are a form of aggresome accumulating large amounts of lead in a relatively inert, nontoxic state. MT on the outer surface of lead inclusion bodies may transport the metal to the forming inclusion. Lead nephrotoxicity impairs the renal synthesis of the heme-containing hydroxylase involved in vitamin D metabolism causing bone effects.

***Effects on Cardiovascular System*—**The pathogenesis of lead-induced hypertension is multifactorial including (1) interferening with endogenous nitric oxide generation and subsequent cGMP-dependent vasorelaxation, (2) activating renin secretion and the renin–angiotensin–aldosterone system, (3) altering calcium-activated contraction and functioning of vascular smooth muscle cells by decreasing Na^+/K^+-ATPase activity and stimulating the Na^+/Ca^{2+} exchange pump, and (4) increasing secretion of vasoconstrictive ligands, such as endothelin and thromboxane.

***Immunotoxicity*—**The developing immune system is sensitive to toxic effects of lead. A hallmark of lead-induced immunotoxicity is a pronounced shift in the balance in T-helper cell function toward Th2 responses at the expense of Th1 functions, resulting in elevated IgE levels. In occupational exposure, lead-associated immunologic changes include altered T-cell subpopulations, reduced immunoglobulin levels, and reduced polymorphonuclear leukocyte chemotactic activity.

***Bone Effects*—**Lead can affect bone by interfering with metabolic and homeostatic mechanisms including parathyroid hormone, calcitonin, vitamin D, and other hormones that influence calcium metabolism, as lead substitutes for calcium in bone. It is known to affect osteoblasts, osteoclasts, and chondrocytes and has been associated with osteoporosis and delays in fracture repair. Lead is deposited in teeth, inhibits mineralization of enamel and dentine, and affects metabolism of the cells in the dental pulp.

***Other Effects*—**Lead colic is a rare gastrointestinal symptom of severe lead poisoning, and is characterized by abdominal pain, nausea, vomiting, constipation, and cramps.

Carcinogenicity—Lead and lead compounds are reasonably anticipated to be human carcinogens. Lead does not appear to be directly genotoxic in vivo or in vitro, and lead may interact with other toxicants to facilitate chemical carcinogenesis. Mechanisms proposed for lead-induced carcinogenesis include inhibition of DNA synthesis or repair, generation of ROS with oxidative damage to DNA, substitution of lead for zinc in transcriptional regulators, interaction with DNA-binding proteins, and aberrant gene expression.

Treatment—The oral chelating agent succimer has advantages over EDTA in that it can be given orally and is effective in temporarily reducing blood lead levels.

Mercury

Also called quicksilver, metallic mercury is in liquid state at room temperature. Mercury vapor (Hg^0) is much more hazardous than the liquid form. Mercury binds to other elements (such as chlorine, sulfur, or oxygen) to form inorganic mercurous (Hg^{1+}) or mercuric (Hg^{2+}) salts. This metal can form stable organometallic compounds by attaching to one or two carbon atoms.

Global Cycling and Ecotoxicology—Mercury exemplifies movement of metals in the environment (Fig. 23–4). Atmospheric mercury, in the form of mercury vapor (Hg^0), is derived from natural degassing of the earth's crust, through volcanic eruptions and evaporation from oceans and soils. Anthropogenic sources include emissions from metal mining and smelting (mercury, gold, copper, and zinc), coal combustion, municipal incinerators, and chloralkali industries. Mercury vapor is a chemically stable monatomic gas and its residence time in atmosphere is about 1 year. Eventually oxidized to a water-soluble inorganic form (Hg^{2+}), it returns to the earth's surface in rainwater. The metal may then be reduced back to mercury vapor and returned to the atmosphere, or it may be methylated by microorganisms present in sediments of bodies of fresh and ocean water. Methylmercury (MeHg) enters the aquatic food chain starting with plankton, then herbivorous fish, and finally ascending to carnivorous fish and sea mammals. Organomercurial compounds are generally more toxic than inorganic mercury to aquatic organisms, plants, and birds.

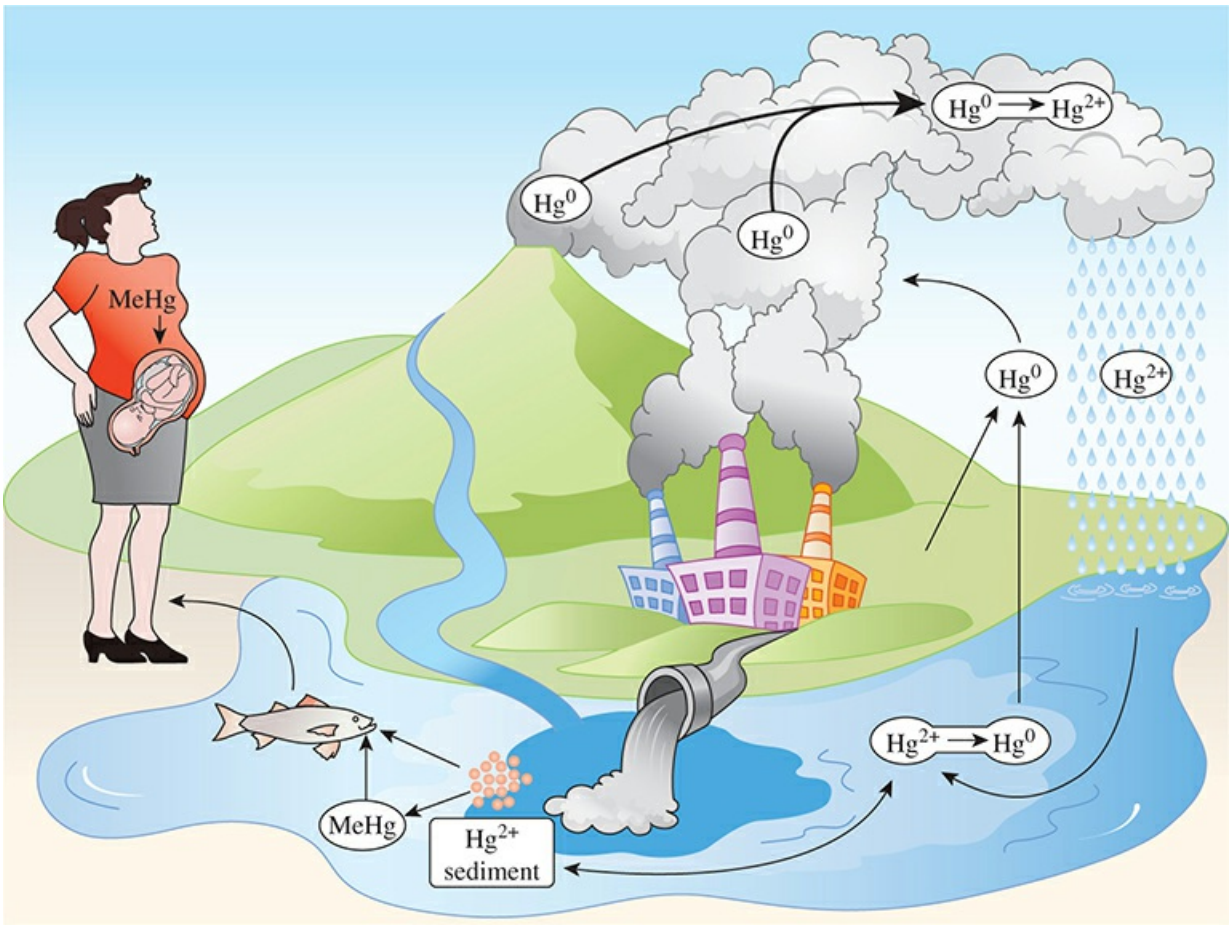

FIGURE 23–4 *The movement of mercury in the environment.* In nature, mercury vapor (Hg^0), a stable monoatomic gas, evaporates from the Earth's surface (both soil and water) and is emitted by volcanoes. Anthropogenic sources include emissions from coal-burning power stations and municipal incinerators. After approximately 1 year, mercury vapor is converted to soluble form (Hg^{2+}) and returned to the Earth by rainwater. It may be converted back to the vapor by microorganisms and reemitted into the atmosphere. Thus, mercury may recirculate for long periods. Mercury attached to aquatic sediments is subjected to microbial conversion to methylmercury, starting with plankton, then herbivorous fish, and finally ascending to carnivorous fish and sea mammals. This biomethylation and biomagnification result in human exposure to methylmercury through consumption of fish, and pose the health risk to humans, especially the developing fetus.

Exposure

***Dietary Exposure*—**Consumption of fish is the major route of exposure to methylmercury. Cooking the fish does not lower the methylmercury content. Inorganic mercury compounds are also found in other foods, but the amounts ingested are typically below toxic levels. Mercury in the atmosphere and in drinking water is generally low.

***Occupational Exposure*—**Inhalation of mercury vapor can occur from working in the chloralkali industry, where mercury is used as a cathode in the electrolysis of brine. Occupational exposure may also occur during manufacture of a variety of scientific instruments and electrical control

devices, in dentistry where mercury amalgams are used in tooth restoration, and in extraction of gold.

***Medicinal Exposure*—**The use of mercury amalgam in dental restoration releases mercury vapor in the oral cavity and can result in increased mercury body burden. Although the potential health effects of amalgams have been fiercely debated, the amounts are low compared with occupational exposure.

***Accidental Exposure*—**Elemental mercury exposure can occur in many ways, such as from broken elemental mercury containers, medicinal devices, barometers, and melting tooth amalgam fillings to recover silver.

Toxicokinetics

***Mercury Vapor*—**Mercury vapor is readily absorbed (about 80%) in the lungs, rapidly diffuses across alveolar membranes into the blood, and distributes to all tissues in the body due to its high lipid solubility. Once the vapor has entered cells, it is oxidized to divalent inorganic mercury by tissue and erythrocyte catalase. A significant portion of mercury vapor crosses the blood–brain barrier and placenta before it is oxidized by erythrocytes, and thus shows more neurotoxicity and developmental toxicity compared with administration of inorganic mercury salts that cross membranes less rapidly. Approximately 10% of mercury vapor is exhaled within a week of exposure, and that converted to inorganic mercury is excreted mainly in urine and feces, with a half-life of 1 to 2 months. Liquid metallic mercury is poorly absorbed by the gastrointestinal tract (0.01%), and not biologically reactive.

***Inorganic Mercury*—**Inorganic mercury is poorly absorbed from the gastrointestinal tract (7% to 15% of ingested dose). Renal uptake of mercury salts occurs through two routes: either from luminal membranes in proximal tubule in the form of cysteine *S*-conjugates (Cys-*S*-Hg-*S*-Cys) or from the basolateral membrane through organic anion transporters. Inorganic mercury salts do not readily pass the blood–brain barrier or placenta, are mainly excreted in urine and feces, and have a half-life of about 2 months.

***Methylmercury*—**Methylmercury is well absorbed from the gastrointestinal tract, and is distributed to all tissues in about 30 hours. About 10% of absorbed methylmercury is distributed to the brain and 5% remains in the blood. The concentration in erythrocytes is 20 times that in plasma. Methylmercury is bound to thiol-containing molecules such as cysteine, which mimic methionine to cross the blood–brain barrier and placenta through the neutral amino acid carrier. About 90% of the methylmercury is eliminated in the feces, with a half-life of 45 to 70 days.

Toxicity

***Mercury Vapor*—**Inhalation of mercury vapor at extremely high concentrations may produce an acute, corrosive bronchitis and interstitial pneumonitis and, if not fatal, may be associated with central nervous system effects such as tremor or increased excitability. With chronic exposure to mercury vapor, the major effects are on the central nervous system. The *asthenic-vegetative syndrome* or *micromercurialism* requires neurasthenic symptoms and three or more of the following clinical findings: tremor, enlargement of the thyroid, increased uptake of radioiodine in the thyroid, labile pulse, tachycardia, dermographism, gingivitis, hematologic changes, or increased excretion of mercury in urine. The triad of tremors, gingivitis, and erethism (memory

loss, increased excitability, insomnia, depression, and shyness) has been recognized historically as the major manifestation of mercury vapor poisoning.

***Inorganic Mercury*—**Although a high dose of mercuric chloride is directly toxic to renal tubular cells, chronic low-dose exposure to mercury salts may induce rare immunologic glomerular disease.

***Methylmercury*—**The major human health effect from exposure to methylmercury is neurotoxicity. Clinical manifestations of neurotoxicity include paresthesia, ataxia, and difficulty in swallowing and articulating words. Other signs include neurasthenia, vision and hearing loss, and spasticity and tremor. These may finally progress to coma and death. The overall acute effect is cerebral edema, but with prolonged destruction of gray matter and subsequent gliosis, cerebral atrophy results.

***Mechanism of Toxicity*—**High-affinity binding of divalent mercury to sulfhydryl groups of cellular proteins is an important mechanism for producing nonspecific cell injury or even cell death. Other general mechanisms of toxicity include upregulation of genes of oxidative stress, affects on the MAPK signaling pathway, and impaired neurodevelopment by affecting neural progenitor cell proliferation, differentiation, and apoptosis, as well as impairing key signaling molecules (Rac1, Cdc42, and RhoA) critical to migration of cerebrocortical neurons. Both inorganic and methylmercury damage mitochondria and disrupt intracellular calcium homeostasis.

Treatment—For the most severe cases with acute renal failure, hemodialysis may be the first measure, along with administration of chelating agents for mercury, such as cysteine, EDTA, BAL, or penicillamine. Oral administration of a nonabsorbable thiol resin can bind alkyl mercury interrupting its enterohepatic circulation and facilitating its fecal excretion.

Nickel

Nickel (Ni) is used in various metal alloys, including stainless steels, and in electroplating, batteries, pigments, catalysts, and ceramics. Occupational exposure to nickel occurs by inhalation of nickel-containing aerosols, dusts, or fumes, as well as dermal contact in workers engaged in nickel mining, milling, and refining. The general population is exposed to low levels of nickel in air pollution, cigarette smoke, water, and food.

Toxicokinetics—About 25% to 35% of the inhaled nickel that is retained in the lungs is absorbed into the blood. The insoluble nickel particles can be taken up into cells by phagocytosis. Intestinal nickel absorption occurs through calcium or iron channels, or by the divalent metal transport protein-1. The main plasma transport proteins for nickel are albumin and α_2-microglobulin. Nickeloplasmin and MT also can bind and transport nickel. Absorbed nickel is excreted into urine.

Toxicity

***Contact Dermatitis*—**Nickel-induced contact dermatitis is found in 10% to 20% of the general population. It can result from exposure to airborne nickel, liquid nickel solutions, or prolonged skin contact with metal items containing nickel, such as coins and jewelry. The resulting

dermatitis is an inflammatory reaction mediated by type IV delayed hypersensitivity.

***Nickel Carbonyl Poisoning*—**Metallic nickel combines with carbon monoxide to form extremely toxic nickel carbonyl ($Ni[CO]_4$). Intoxication begins with headache, nausea, vomiting, and epigastric or chest pain, followed by cough, hyperpnea, cyanosis, gastrointestinal symptoms, and weakness. The symptoms may be accompanied by fever and leukocytosis. The more severe cases can progress to pneumonia, respiratory failure, and eventually to cerebral edema and death.

Carcinogenicity—Risks are highest for lung and nasal cancers among workers heavily exposed to nickel compounds.

***Mechanism for Nickel Carcinogenesis*—**The carcinogenicity of nickel is thought to be due to the generation of ionic nickel in target cells. Carcinogenic nickel particles that are phagocytized and delivered to the nucleus can produce specific chromosomal damage; the heterochromatic long arm of the X chromosome suffers regional decondensation, frequent deletions, and other aberrations. Many soluble and insoluble forms of nickel cause genetic damage, including DNA damage, cell transformation, and DNA repair disruption. The redox activity of nickel may produce ROS that could attack DNA directly.

***Epigenetic Effects*—**Nickel-induced epigenetic effects include perturbed DNA methylation and posttranslational histone modification. Nickel produces low but measurable ROS in cells and depletes cellular glutathione. Oxidative DNA damage, oxidative protein damage, lipid peroxidation, and inhibition of DNA repair enzymes can be observed after nickel exposure.

Treatment of Nickel Toxicity—Sodium diethylcarbodithioate (DDTC) is the preferred drug for nickel treatment. Disulfiram has been used in nickel dermatitis and in nickel carbonyl poisoning.

ESSENTIAL METALS WITH POTENTIAL FOR TOXICITY

Metals generally accepted as essential include cobalt, copper, iron, magnesium, manganese, molybdenum, selenium, and zinc. All can produce some target organ toxicity as shown in Table 23–1.

Cobalt

Cobalt (Co) is a ferromagnetic transition metal used in various alloys, in permanent magnets, as a paint or varnish dryer, in catalysts, and in production of pigments. Cobalt is essential as a metal cofactor coordinated in vitamin B_{12}.

Toxicokinetics—Oral absorption in humans varies between 5% and 45%. Absorption of inhaled cobalt compounds appears to be relatively effective in humans and animals. About 80% of absorbed cobalt is excreted in urine, and about 15% is excreted in feces.

Essentiality—Cobalamin, a cobalt-containing tetrapyrrolic ring is a critical component of vitamin B_{12}, which is required for red blood cell production and the prevention of pernicious anemia.

Toxicity—Occupational inhalation of cobalt-containing dust may cause respiratory irritation at air concentrations between 0.002 and 0.01 mg/m^3. High concentrations of cobalt–tungsten carbide may cause of "hard metal" pneumoconiosis, a progressive form of pulmonary interstitial fibrosis. Occupational dermal exposure is sometimes associated with an allergic dermatitis.

Intravenous exposure to cobalt can cause increased blood pressure, slowed respiration, tinnitus, and deafness due to nerve damage. The clinical emergence of cobalt cardiomyopathy seems to require the coexistence of one or more cofactors, particularly a low-protein diet, thiamine deficiency, alcoholism, and hypothyroidism. Chronic cobalt exposure targets mitochondrial enzymes in complex II and complex III, with modest effects on respiration and a minimal lowering of ATP levels. Cobalt-stabilized HIF-1α increases expression of endothelin-1, which would signal for cardiac hypertrophy and remodeling.

Inhalation of cobalt sulfate induces lung tumors, including carcinomas in rats and mice. The mechanisms by which cobalt may produce cancer include inhibition of DNA repair, formation of DNA-damaging ROS, and alterations of important cellular functions by replacing other essential metal ions. Cobalt–tungsten carbide powders and hard metals are *reasonably anticipated to be human carcinogens* based on limited evidence from human studies. Cobalt may cause cytotoxicity, genotoxicity, inflammation, and apoptosis.

Copper

Food, beverages, and drinking water are major sources of exposure in the general population. Copper exposure in industry is primarily from inhaled particulates in mining or metal fumes in smelting operations, welding, or related activities.

Toxicokinetics—Approximately 55% to 75% of an oral dose of copper is absorbed from the gastrointestinal tract, primarily in the duodenum. Copper is bound to ceruloplasmin, which releases Cu^+ ions to the membrane-bound Cu importer CTR1 for transfer to the chaperone ATOX1 and delivery to cytoplasmic metal-binding domains of ATP7A and two homologous ATPases located in the trans-Golgi network. The copper is then available for coordination in proteins synthesized by the Golgi. In mammals, the major route of excretion for excess copper is via the feces. Biliary secretion, enterohepatic recirculation, and intestinal reabsorption all help to maintain copper homeostasis.

Essentiality—Copper is an essential component of cytochrome *c* oxidase (COX), lysyl oxidase (LOX), and copper/zinc superoxide dismutase (SOD). Disruption of copper binding in any of these proteins has profound consequences on enzyme production (LOX), cytoplasmic antioxidant activity (SOD1), and energy metabolism (COX).

Toxicity—Commonly reported adverse health effects of excess oral copper intake are gastrointestinal distress (nausea, vomiting, and abdominal pain). Ingestion of large amounts of copper salts, most frequently copper sulfate, may produce hepatic necrosis and death.

Hereditary Disease of Copper Metabolism

***Menkes Disease*—**This rare sex-linked genetic defect in copper metabolism results in copper deficiency in male infants. It is characterized by failure to thrive, severe mental retardation, neurological impairment, connective tissue dysfunction, and death usually by 3 to 5 years of age. Deficiency in *ATP7A* gene results in reduced copper transporter and reduced copper transport across the basolateral membrane of intestinal cells into the portal circulation. Copper accumulates in enterocytes and is deficient systemically. The transport of copper to the brain is also blocked, causing severe neurological abnormalities.

***Wilson Disease*—**This autosomal recessive genetic disorder is characterized by the excessive accumulation of copper in the liver, brain, kidneys, and cornea. Patients with Wilson disease have impaired biliary excretion of copper resulting in copper overload in hepatocytes, mild hepatitis, acute liver failure and an increased incidence of hepatocarcinoma. Neurological symptoms include dystonia, tremor, dysarthria, and psychiatric disturbances. The defect in copper transport results from mutations of the Wilson disease locus (WND) on chromosome 13, encoding P-type ATPase (*ATP7B*).

***Hereditary Aceruloplasminemia*—**This is the autosomal recessive genetic disorder of copper-binding protein ceruloplasmin, associated with the iron overload syndrome. Iron overload contributes to mental confusion, memory loss, dementia, cerebellar ataxia, altered motor function, retinal degeneration, and diabetes.

***Indian Childhood Cirrhosis (ICC)*—**This disorder in young children is characterized by jaundice due to an insidious and progressive liver disease. Two distinguishing features are a widespread brown orcein staining (indicating copper) and intralobular hepatic fibrosis progressing to portal cirrhosis and chronic inflammation. The hepatotoxic injury occurs in genetically susceptible infants from ingested copper.

Treatment—Treatment for diseases of copper overload includes chelation with d-penicillamine, Trien (triethylenetetramine 2HCl) and supplementation with zinc acetate and tetrathiomolybdate.

Iron

Iron (Fe) is essential for erythropoiesis and a key component of hemoglobin, myoglobin, heme enzymes, metalloflavoprotein enzymes, and mitochondrial enzymes. In physiological systems, iron mainly exists as the ferrous (2^+) and ferric (3^+) forms. Toxicological considerations are important in terms of iron deficiency, accidental acute exposures, and chronic iron overload.

Toxicokinetics—Soluble inorganic iron is taken up in the proximal duodenum by divalent metal transporter protein 1 (DMT1), which couples the transport of Fe^{2+} to a proton gradient. Iron taken up at the apical membrane of enterocytes is either sequestered within the iron storage protein ferritin or exported into the portal circulation through ferroportin—a receptor for hepcidin, the master regulator of systemic iron homeostasis. Ferroportin exports Fe^{2+} that must be oxidized by copper-containing ferroxidases, such as ceruloplasmin and hephaestin, before being loaded onto transferrin for systemic delivery to cells. Transferrin delivers iron to tissues by binding the transferrin receptor-1 on the cell membrane, followed by endocytosis. Intracellular

iron homeostasis is regulated by an iron response element/iron regulatory protein system and antioxidant response elements. Iron elimination occurs through release of heme-containing proteins into the gastrointestinal tract and eventually the feces, as well as elimination of porphyrins in the urine.

Essentiality and Deficiency—The major manifestation of iron deficiency is anemia with microcytic hypochromic red blood cells, with impaired psychomotor development and intellectual performance, decreased resistance to infection, adverse pregnancy outcomes, and possibly increased susceptibility to lead and cadmium toxicity. Oral ferrous sulfate is the treatment of choice for iron deficiency.

Toxicity—Acute iron poisoning from accidental ingestion of iron-containing dietary supplements is the most common cause of acute toxicity. After ingestion of more than 0.5 g of iron or 2.5 g of ferrous sulfate, symptoms of toxicity ensue, including abdominal pain, diarrhea, and vomiting. Of particular concern are metabolic acidosis, liver damage, and cardiac collapse. Supportive therapy and iron chelation with deferoxamine should be used as soon as possible.

Chronic iron toxicity from iron overload in adults is a relatively common problem. Hereditary hemochromatosis is an insufficiency of hepcidin caused by mutations in genes regulating the protein's expression allowing excessive iron absorption, excessive mobilization of macrophage iron stores, saturation of transferrin with iron, and increased serum non-transferrin bound iron. *Hemochromatosis* refers to excessive iron deposition in organs. *Hemosiderosis* refers to increased iron stores in the form of hemosiderin.

Tissue injury from excessive iron may cause cirrhosis, diabetes, cardiomyopathy, atherosclerosis, and endocrine failure. Neurodegenerative disorders associated in the brain include neuroferritinopathy, aceruloplasminemia, and manganism. Much of the tissue injury results from ROS generation. Injuries are worsened when damaged tissue becomes hypoxic and necrotic, and additional iron is released from intracellular stores.

Treatment—Desferrioxamine is the chelator of choice for the treatment of acute iron intoxication and chronic iron overload. Deferoxamine therapies are effective in reducing iron overloads caused by thalassemias. Proposed alternatives to chelation include using transferrin infusions.

Magnesium

Magnesium (Mg) is a nutritionally essential metal that plays a key role in a wide range of important fundamental cellular reactions.

Toxicokinetics—Ingested magnesium is absorbed mainly in the small intestine. Calcium and magnesium are competitive with respect to absorption, and excess calcium will partially inhibit magnesium absorption. Of the magnesium filtered by the glomeruli, about 95% is reabsorbed.

Essentiality and Deficiency—Magnesium is a cofactor of many enzymes. In the glycolytic cycle, there are seven key enzymes that require divalent magnesium. Magnesium-containing enzymes are involved in the citric acid cycle and in β-oxidation of fatty acids and MgATP is essential for most energy and phosphotransfer reactions. Deficiency may occur as a complication of various disease states such as malabsorption syndromes, renal dysfunction, and endocrine

disorders. Magnesium deficiency in humans causes neurological depression, neuromuscular irritability, frank tetany, and even convulsions. Magnesium deficiency induces an inflammatory syndrome, and is a risk factor for diabetes mellitus, hypertension, hyperlipidemia, and ischemic heart diseases. Supplementation of magnesium is beneficial.

Toxicity—Toxic effects of hypomagnesemia are more common than hypermagnesemia. In industrial exposures, no ill effects are produced with a twofold increase in serum magnesium, although concurrent increases occur in serum calcium. Inhaled freshly generated magnesium oxide can cause metal fume fever, like that caused by zinc oxide. In nonoccupationally exposed individuals, toxicity may progress from nausea and vomiting to hypotension, electrocardiograph abnormalities, central nervous system effects, coma, and systolic cardiac arrest.

Molybdenum

Molybdenum (Mo) is a cofactor for four enzymes in humans: sulfite oxidase, xanthine oxidase, aldehyde oxidase, and mitochondrial amidoxime reductase. From molybdenite (MoS_2), molybdem is used in the manufacture of high-temperature-resistant steel alloys for gas turbines and jet aircraft engines and in the production of catalysts, lubricants, and dyes. Ammonium tetrathiomolybdate is a molybdenum-donating copper chelator used to treat Wilson disease.

Toxicokinetics—Water-soluble molybdenum compounds are readily absorbed when ingested. The highest molybdenum concentrations are found in the kidneys, liver, and bones. Very little molybdenum appears to cross the placenta. Excretion, primarily via the urine, is rapid.

Essentiality and Deficiency—Molybdenum cofactor (Moco) deficiency is a pleiotropic genetic disorder characterized by the loss of the molybdenum-dependent enzymes sulfite oxidase, xanthine oxidoreductase, and aldehyde oxidase, due to mutations in the genes involved with Moco biosynthesis. This rare human disorder causes severe neurodegeneration and childhood death.

Toxicity—Molybdenum is of low toxicity. Chronic exposure to excess molybdenum in humans is characterized by high uric acid levels in serum and urine. Molybdenum toxicity resembles copper deficiency. Treatment with supplemental copper can often reverse the adverse effects of excess molybdenum, and treatment of Wilson disease with molybdenum compounds reduces copper burden.

Zinc

Zinc (Zn) toxicity is relatively uncommon and occurs only at very high exposure levels. The major route of zinc intake is through the diet. Occupational exposure to dusts and fumes of metallic zinc occurs in zinc mining and smelting.

Toxicokinetics—About 20% to 30% of ingested zinc is absorbed from the intestinal lumen by passive diffusion and by zinc-specific transmembrane transporters such as ZnT-1. It is involved in over 300 enzymatic reactions essential for health and is responsible for tertiary and quaternary protein structure required for protein and protein/DNA interactions. In plasma, zinc is bound to

albumin (60% to 80%) or α_2-macroglobulin and transferrin. Zinc is excreted in both urine and feces. Zinc is an effective inducer of metallothionein synthesis. Metallothionein is also an important storage depot for cellular zinc.

Essentiality and Deficiency—There are more than 300 catalytically active zinc metalloenzymes and 2000 zinc-dependent transcription factors. Zinc supports a healthy immune system, and is essential for normal growth and development during pregnancy, childhood, and adolescence.

Toxicity—Acute zinc toxicity from excessive ingestion is uncommon, but gastrointestinal distress and diarrhea have been reported after ingestion of beverages standing in galvanized cans. After inhalation, the most common effect is “metal fume fever” characterized by fever, chest pain, chills, cough, dyspnea, nausea, muscle soreness, fatigue, and leukocytosis. Acute inhalation of high levels of zinc chloride in “smoke bombs” results in more pronounced damage including interstitial edema, fibrosis, pneumonitis, bronchial mucosal edema, and ulceration.

***Neuronal Toxicity*—**Zinc deficiency may alter activity of the antioxidant enzyme Cu–Zn SOD, resulting in excess ROS. A genetic abnormality of Cu–Zn SOD may be the basis of a familial form of amyotrophic lateral sclerosis. Zinc modulates the solubility of β-amyloid in the brain and protects against β-amyloid toxicity, but excess zinc may trigger neuronal death. Synaptically released zinc might contribute to excitotoxic brain injury, which could set the stage for the later development of Alzheimer disease.

***Pancreatic Toxicity*—** Zinc accumulating in secretory granules of pancreatic islet β-cells can affect the function or survival of islet cells and cause β-cell death. Excess dietary zinc is associated with damage to exocrine pancreas. A single, high-dose injection of zinc increases plasma α-amylase activity and can produce fibrosis and necrosis of pancreatic exocrine cells.

METALS RELATED TO MEDICAL THERAPY

Aluminum

Aluminum compounds occur typically in the trivalent state (Al^{3+}). Aluminum binds strongly to oxygen donor ligands such as citrate and phosphate. Human exposure to aluminum comes primarily from food and secondarily from drinking water. Occupational exposures to aluminum occur during mining and processing, as well as in aluminum welding.

Toxicokinetics—Aluminum is poorly absorbed after either oral or inhalation exposure and is not absorbed dermally. Inhalation of particulate aluminum may result in direct transfer to brain tissue via the olfactory system. Less than 1% of aluminum in the diet is absorbed. In plasma, 80% to 90% of aluminum binds to transferrin. The transferrin pathway is also considered a mechanism for aluminum transport across the blood–brain barrier. Aluminum is excreted in urine.

Toxicity—Acute aluminum toxicity is rare. Most cases of aluminum toxicity are observed in patients with chronic renal failure or persons exposed in the workplace, with the lung, bone, and

central nervous system as major target organs. Aluminum can produce developmental effects.

***Lung and Bone Toxicity*—**Aluminum dust can produce lung fibrosis in humans. Osteomalacia has been associated with excessive intake of aluminum-containing antacids, presumably due to interference with intestinal phosphate absorption. Osteomalacia also can occur in uremic patients exposed to aluminum in dialysis fluid. In these patients, osteomalacia may be a direct effect of aluminum on bone mineralization as bone levels are high.

***Neurotoxicity*—**Aluminum is neurotoxic to experimental animals, with wide species and age variations. Susceptible rabbits and cats show progressive neurological impairment resulting in death associated with status epilepticus. The most prominent early pathological change is the accumulation of neurofibrillary tangles (NFTs) in large neurons, proximal axons, and dendrites of neurons of many brain regions. Rats fail to develop NFTs or encephalopathy and monkeys develop NFTs only after more than a year of aluminum infusion. Impairment of cognitive and motor function and behavioral abnormalities are often observed.

***Dialysis Dementia*—**Patients on long-term intermittent hemodialysis for chronic renal failure develop a speech disorder followed by dementia, convulsions, and myoclonus typically after 3 to 7 years of dialysis treatment. The aluminum content of brain, muscle, and bone increases in these patients. Sources of the excess aluminum may be from oral aluminum hydroxide commonly given to these patients or from aluminum in dialysis fluid derived from the tap water used to prepare the dialysate fluid. The syndrome may be prevented by avoiding the use of aluminum-containing oral phosphate binders and by monitoring of aluminum in the dialysate.

***Alzheimer Disease*—**Aluminum is associated with various components of the pathological lesions in Alzheimer disease brain tissue. Elevated aluminum levels in Alzheimer disease brains may be a consequence and not a cause of the disease. The reduced effectiveness of the blood–brain barrier in Alzheimer disease might allow more aluminum into the brain. Also, recent studies have raised the possibility that the staining methods in earlier studies may have led to aluminum contamination. Furthermore, the NFTs seen in aluminum encephalopathy differ structurally and chemically from those in Alzheimer disease. There are conflicting conclusions regarding the role of aluminum exposure in Alzheimer disease. However, there is increasing evidence suggesting a link between aluminum in the brain and other neurodegenerative diseases.

Treatment—Chelation therapy for aluminum, mostly in dialyzed and/or uremic patients, resembles that for iron overload, with deferoxamine and deferiprone.

Lithium

Lithium (Li) is used in batteries, alloys, catalysts, photographic materials, and the aerospace industry. Lithium hydride is used in ceramics and in chemical analysis. Lithium carbonate and lithium citrate are widely used for mania and bipolar disorders. In this regard, lithium is active possibly through its effects on signal transduction, such as phosphoinositide hydrolysis, glycogen synthase kinase-3, and neurotropic cascades. Topical applications of lithium succinate are still used in the treatment of seborrheic dermatitis.

Toxicokinetics—Lithium is readily absorbed from the gastrointestinal tract, and distributes to

total body water. Excretion is chiefly through the kidneys with 80% of the filtered lithium reabsorbed. It enters cells via the amiloride-sensitive sodium channel or the Na/H^+ exchanger. The greater part of lithium is retained in the cells, perhaps at the expense of potassium. It may be competing with sodium at certain sites, such as in renal tubular reabsorption.

Toxicity—Except for lithium hydride, lithium salts are not considered to be industrial hazards. nor toxic. Lithium hydride is intensely corrosive, may produce burns on the skin because of the formation of hydroxides, and is explosive. Intoxications related to lithium exposure are mainly related to its medicinal use, as the therapeutic index of lithium is very narrow. The toxic responses to lithium include neuromuscular changes (tremor, muscle hyperirritability, and ataxia), central nervous system disorders (blackout spells, epileptic seizures, slurred speech, coma, psychosomatic retardation, and increased thirst), cardiovascular disturbances (cardiac arrhythmia, hypertension, and circulatory collapse), gastrointestinal symptoms (anorexia, nausea, and vomiting), and renal damage (albuminuria and glycosuria).

Interstitial nephritis may occur with long-term exposure even when lithium levels remain within the therapeutic range. Lithium nephrotoxicity primarily targets distal and collecting tubes, with a higher incidence of proteinuria and associated glomerular pathology. Chronic lithium-induced neurotoxicity, nephritis, and thyroid dysfunction may occur. Acute lithium overdose produces neurological sequelae and cardiac toxicity, which can be fatal. The toxicity may be treated by the administration of diuretics (amiloride), accompanied by replacement of water and electrolytes, and by hemodialysis.

Platinum

Platinum (Pt) compounds are used as automobile catalysts, in jewelry, in electronics, and in dental alloys. Platinum coordination complexes are important antitumor agents.

Toxicokinetics—After a single exposure, most of the inhaled platinum is rapidly cleared from the lungs by mucociliary action, swallowed, and excreted in the feces, with half-life of about 24 hours. After intravenous administration of clinical doses, the drug has an initial elimination half-life in plasma of 25 to 50 minutes. More than 90% of the platinum in the blood is bound to plasma proteins. After administration of the main metallochemotherapeutic form, *cis*-dichlorodiammine platinum(II) (cisplatin), high concentrations are found in the kidney, liver, intestine, spleen, and testes, but there is poor penetration into the brain.

Toxicity—Platinum can produce profound hypersensitivity reactions in susceptible individuals, who show urticaria, contact dermatitis of skin, and respiratory distress, ranging from irritation to an asthmatic syndrome, after exposure to platinum dust. The skin and respiratory changes are termed *platinosis*. They are mainly confined to persons with a history of industrial exposure to soluble compounds such as sodium chloroplatinate.

***Antitumor Effects of Platinum Complexes*—**The platinum-coordinated complexes cisplatin, carboplatin, and oxaloplatin are routinely administered, often in combination with other anticancer drugs, in the treatment of a wide spectrum of malignancies, especially epithelial cancers. Platinum complexes are neutral and have a pair of *cis*-leaving groups. Hydrolysis of chloride-leaving groups in cisplatin yields a positively charged molecule that reacts with DNA

and proteins, forming both intrastrand and interstrand DNA cross-links with guanine and/or adenine. In tumor cells, the replication of DNA is impaired due to cisplatin-induced DNA cross-links, while in normal cells, guanine is repaired before replication.

Carcinogenic Effects of Platinum Complexes—Although cisplatin has antitumor activity in humans, it is *reasonably anticipated to be a human carcinogen* and clearly carcinogenic in rodents. Cisplatin is a strong mutagen in bacterial systems and causes chromosomal aberrations in cultured hamster cells and a dose-dependent increase in sister chromatid exchanges.

Toxicities of Platinum Antitumor Complexes—Cisplatin produces proximal and distal tubular cell injury, mainly in the corticomedullary region, where the concentration of platinum is highest. Cisplatin is also associated with cumulative peripheral sensory neuropathy, ototoxicity due to irreversible damage of the hair cells in Corti organ, and significant nausea and vomiting. Bone marrow suppression, manifested as anemia, neutropenia, and thrombocytopenia, is relatively common during treatment. Second- and third-generation platinum compounds, such as carboplatinum and oxaliplatinum, have lower toxicity profiles.

MINOR TOXIC METALS

Additional metals for which toxicity has been described include antimony (Sb), barium (Ba), cesium (Cs), palladium (Pd), silver (Ag), tellurium (Te), thallium (Tl), tin (Sn), titanium (Ti), uranium (U), and vanadium (V). Table 23–1 lists toxic effects of some of these elements. Additional details may be found in the parent textbook.

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QUESTIONS

1. Which of the following is NOT a major excretory pathway of metals?
   - **a.** sweat.
   - **b.** urine.
   - **c.** respiration.
   - **d.** feces.
   - **e.** hair.

2. Metallothioneins:
   - **a.** are responsible for metal transport in the bloodstream.

b. are involved in the biotransformation of metals.
c. invoke hypersensitivity reactions.
d. provide high-affinity binding of copper and mercury.
e. are involved in extracellular transport of metals.

3. Which of the following metal-binding proteins is NOT correctly paired with the metal it binds?
a. transferrin—iron.
b. ceruloplasmin—copper.
c. metallothioneins—zinc.
d. ferritin—lead.
e. albumin—nonspecific metal binding.

4. Which of the following groups is LEAST likely to chelate metals?
a. —COOH.
b. —Cl.
c. —NH.
d. —OH.
e. —SH.

5. What is the mechanism of toxicity of arsenic (As)?
a. inhibition of mitochondrial respiration.
b. impairment of calcium uptake by membrane transporters.
c. accumulation in renal corpuscle.
d. abolition of sodium–potassium gradient.
e. destruction of surfactant in the lungs.

6. Lead's toxicity is largely due to its ability to mimic and interfere with normal functioning of which of the following ions?
a. Na^+.
b. K^+.
c. Cl^-.
d. Fe^{2+}.
e. Ca^{2+}.

7. Which of the following statements regarding mercury (Hg) toxicity is FALSE?
a. A major source of environmental mercury is rain-water.
b. Mercury vapor is much more dangerous than liquid mercury.
c. Mercury vapor inhalation is characterized by fatigue and bradycardia.
d. Microorganisms in bodies of water can convert mercury vapor to methylmercury.
e. Methylmercury is the most important source of human mercury toxicity.

8. Which of the following is a common symptom of nickel exposure?

a. renal failure.
b. diarrhea.
c. hepatic cirrhosis.
d. contact dermatitis.
e. tachycardia.

9. Which of the following statements regarding Wilson disease is FALSE?
a. Serum ceruloplasmin is high.
b. Urinary excretion of copper is high.
c. There is impaired biliary excretion of copper.
d. The disease can be treated with liver transplantation.
e. This is an autosomal recessive disorder.

10. Which of the following statements regarding metals and medical therapy is FALSE?
a. There are elevated levels of aluminum in the brains of Alzheimer's patients.
b. Lithium is used to treat depression.
c. Chronic nephrotoxicity is a common result of excess aluminum exposure.
d. Platinum is used as cancer treatment.
e. Platinum salts can cause an allergic dermatitis.

CHAPTER 24

Toxic Effects of Solvents and Vapors

James V. Bruckner, S. Satheesh Anand, and D. Alan Warren

CHLORINATED HYDROCARBONS

Trichloroethylene

- Metabolism
- Modes of Carcinogenic Action in Target Tissues
- Liver Cancer
- Lung Cancer
- Kidney Cancer
- Risk Assessment

Tetrachloroethylene

- Metabolism
- Modes of Cytotoxicity/Carcinogenicity
- Hepatorenal Toxicity
- Cancer Bioassays in Rodents
- Cancer Epidemiology Studies
- Risk Assessment

1,1,1-Trichloroethane

- Toxicokinetics (TK) and Metabolism
- Toxicity
- Potential Carcinogenicity

Methylene Chloride

- Metabolism
- Modes of Toxicity/Carcinogenicity
- Cancer Bioassays in Rodents
- Cancer Epidemiology Studies
- Risk Assessment

Carbon Tetrachloride

Chloroform

AROMATIC HYDROCARBONS

Benzene

Toluene

KEY POINTS

- The term *solvent* refers to a class of liquid organic chemicals of variable lipophilicity and volatility, small molecular size, and lack of charge.
- Absorption of inhaled volatile organic compounds occurs in the alveoli, with almost instantaneous equilibration with blood in the pulmonary capillaries.
- Solvents are readily absorbed from the gastrointestinal tract and across the skin.
- Most solvents produce some degree of CNS depression.

INTRODUCTION

The term *solvent* refers to a class of organic chemicals of variable lipophilicity and volatility with small molecular size and lack of charge. Solvents undergo ready absorption across membranes of the lung, gastrointestinal (GI) tract, and skin. Lipophilicity generally increases with increasing numbers of carbon and/or halogen atoms, while volatility decreases. Organic solvents are frequently used to dissolve, dilute, or disperse materials that are insoluble in water. Naphthas and gasoline are complex mixtures, often consisting of hundreds of compounds.

According to molecular structure or functional group, classes of solvents include aliphatic hydrocarbons, many of which are halogenated (i.e., halocarbons), aromatic hydrocarbons, alcohols, ethers, esters/acetates, amides/amines, aldehydes, ketones, and complex mixtures that defy classification. The main determinants of a solvent's inherent toxicity are: (1) its number of carbon atoms, (2) whether it is saturated or has double or triple bonds between adjacent carbon atoms, (3) its configuration (i.e., straight chain, branched chain, or cyclic), (4) whether it is halogenated, and (5) the presence of functional groups. Subtle differences in chemical structure can translate into dramatic differences in toxicity.

Nearly everyone is exposed to solvents during normal activities. Environmental exposures to solvents in air and groundwater use multiple exposure pathways (Fig. 24–1). Even household use of solvent-contaminated water may result in solvent intake from inhalation and dermal absorption as well as ingestion. In many cases, risk assessment guidelines stipulate that risks be determined for physiologically diverse individuals who are exposed to several solvents by multiple exposure pathways. For many common/toxic solvents, toxicity factors, such as reference concentrations (RfCs), reference doses (RfDs), and cancer slope factors (CSFs) are available.

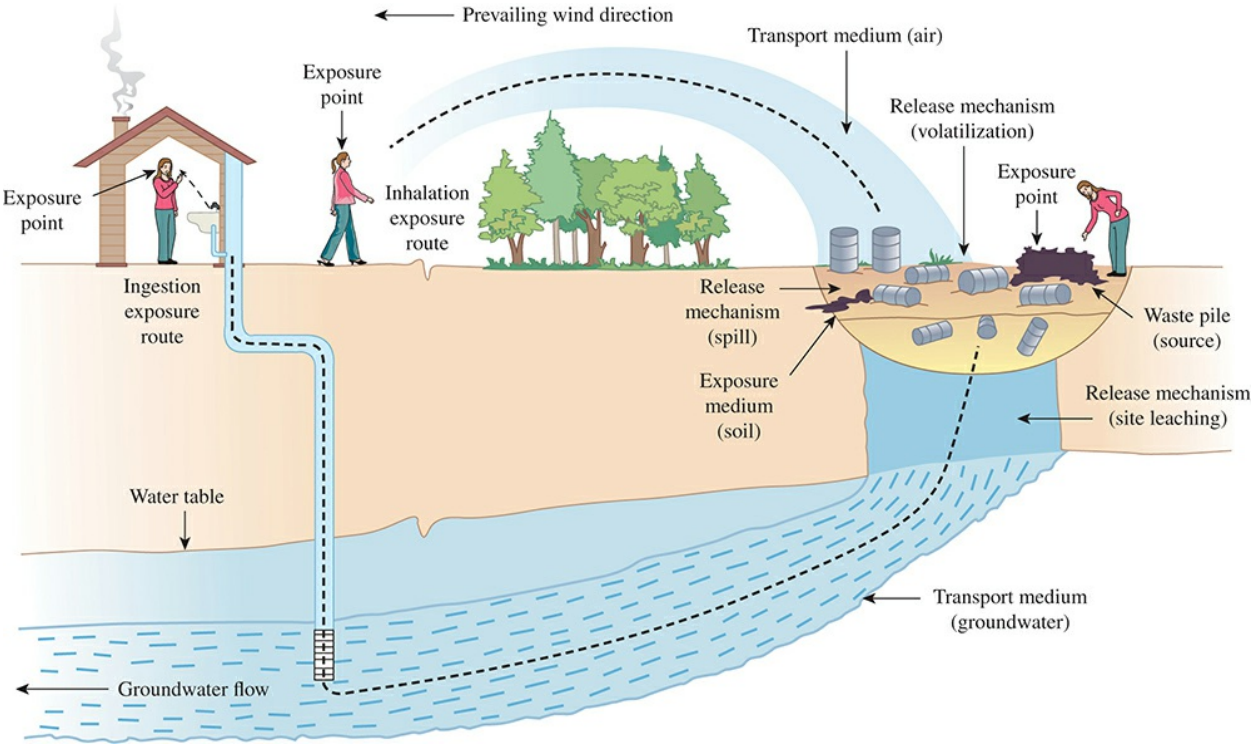

FIGURE 24–1 *Solvent exposure pathways and media*. (Adapted from EPA Risk Assessment Guidance for Superfund. *Human Health Evaluation Manual Part A, Interim Final*. Washington, DC: Office of Emergency and Remedial Response, 1989.)

The U.S. Occupational Safety and Health Administration (OSHA) has established legally enforceable permissible exposure limits (PELs) for over 100 solvents. The majority of existing PELs were adopted from a list of threshold limit values (TLVs) previously published by the American Conference of Governmental Industrial Hygienists (ACGIH). Whereas the ACGIH's TLVs for an 8-hour workday, 40-hour workweek are designed to be protective for a working lifetime, its short-term exposure limits (STELs) and ceiling values are designed to protect against the acute effects of high-level, short-term solvent exposure. If warranted, ACGIH will assign a skin notation to a solvent, indicating that a significant contribution to overall exposure is possible by the dermal route.

Most solvent exposures involve a mixture of chemicals, rather than a single compound. Our knowledge of the toxicity of solvent mixtures is rudimentary relative to the toxicology of individual solvents. While the assumption is frequently made that the toxic effects of solvents are additive, the chemicals may also interact synergistically or antagonistically. Significant data gaps exist in the toxicology of mixtures, and these can be significant sources of uncertainty in risk assessments.

Although degree of hazard varies, virtually all solvents can cause adverse effects. Most have the potential to induce some level of narcosis and cause respiratory and mucous membrane irritation. As with other chemicals, whether adverse health effects occur from solvent exposure is dependent on: (1) toxicity/carcinogenicity of the solvent, (2) exposure route, (3) amount or rate of exposure, (4) duration of exposure, (5) individual susceptibility, and (6) interactions with other chemicals.

IS THERE A SOLVENT-INDUCED CHRONIC ENCEPHALOPATHY?

The CNS-depressant effects of acute, high-level exposures and the potential for permanent neurologic damage in chronic solvent abusers are not a matter of debate. Far less clear is whether chronic, low-level exposure to virtually any solvent or solvent mixture can produce a pattern of neurologic dysfunction referred to as *painters' syndrome, organic solvent syndrome, psychoorganic syndrome,* and *chronic solvent encephalopathy* (CSE). CSE is characterized by nonspecific symptoms (e.g., headache, fatigue, mood disturbances, and sleep disorders) with or without changes in neuropsychological function. A reversible form of CSE referred to as *neurasthenic syndrome* consists of symptoms only. The "mild" and "severe" forms are accompanied by objective signs of neuropsychological dysfunction that may or may not be fully reversible. There is universal agreement that CSE is a nonprogressive disease in which no severe deterioration of functioning occurs after diagnosis, provided exposure ceases.

SOLVENT ABUSE

Inhalants are volatile substances that can be inhaled to induce a psychoactive or mind-altering effect. Solvents are among the most popular classes of drugs of abuse, given their presence in a multitude of inexpensive, readily available products that are legal to buy and possess. Solvent

abuse is a unique exposure situation, in that participants repeatedly subject themselves to vapor concentrations high enough to produce intoxication. Solvents can be breathed in through the nose or the mouth by “sniffing” or “snorting” vapors from containers, spraying aerosols directly into the nose or mouth, “bagging” by inhaling vapors from a plastic or paper bag, or “huffing” from a solvent-soaked rag stuffed into the mouth. The abuser can begin to experience effects after a matter of seconds. While intoxication may last only a few minutes, abusers frequently seek to prolong the “high” by inhaling repeatedly over the course of several hours. Death may be a consequence of cardiac arrhythmias, asphyxiation, and/or cachexia.

ENVIRONMENTAL CONTAMINATION

Widespread use of solvents has resulted in their dissemination throughout the environment (Fig. 24–1). The majority of the more volatile organic chemicals (VOCs) volatilize when products containing them (e.g., aerosol propellants, paint thinners, cleaners, and soil fumigants) are used as intended. Solvent loss into the atmosphere also occurs during production, processing, storage, and transport activities, resulting in elevated concentrations in air in the proximity of point sources. Winds dilute and disperse solvent vapors across the world. Atmospheric concentrations of most VOCs are usually extremely low (i.e., nondetectable to nanograms or a few micrograms per cubic meter (m^3) of air). Higher atmospheric concentrations of certain solvents have been measured in urban areas, around petrochemical plants, and near hazardous waste sites.

Solvent contamination of drinking water supplies is a major health concern. Solvents spilled onto the ground may permeate the soil and migrate through it until reaching groundwater or an impermeable material. All solvents are soluble in water to some extent. Some (e.g., alcohols, ketones, glycols, and glycol ethers) are freely water soluble. Concentrations diminish rapidly after VOCs enter bodies of water, primarily due to dilution and evaporation. VOCs in waters rise to the surface or sink to the bottom, according to their density. VOCs on the surface will largely evaporate. VOCs on the bottom must depend on solubilization in water or mixing by current or wave action to reach the surface.

TOXICOKINETICS

Toxicity is a dynamic process, in which the degree and duration of injury of a target tissue depend on the net effect of toxicodynamic (TD) and toxicokinetic (TK) processes including systemic absorption, tissue deposition, metabolism, interaction with cellular components, elimination, and tissue repair. Volatility and lipophilicity are two properties that govern solvent absorption and disposition in the body. Lipophilicity can vary from quite water soluble (e.g., glycols, esters, and alcohols) to quite lipid soluble (e.g., halocarbons and aromatic hydrocarbons). With relatively low-molecular-weight and no charge, many solvents passively diffuse through membranes.

Absorption

The majority of systemic absorption of inhaled VOCs occurs in the alveoli, with limited

absorption occurring in the upper respiratory tract. Gases in the alveoli equilibrate almost instantaneously with blood in the pulmonary capillaries. Blood:air partition coefficients (PCs) are the ratio of concentration of VOCs achieved between two different media at equilibrium. More hydrophilic solvents have relatively high blood:air PCs, which favor extensive uptake. Increases in respiratory rate (to maintain a high alveolar concentration) and in cardiac output/pulmonary blood flow (to maintain a large concentration gradient by removing capillary blood containing the VOC) enhance absorption.

Solvents are well absorbed from the GI tract. Peak blood levels are observed within minutes of dosing fasted subjects. The presence of fatty food in the GI tract can significantly delay absorption of lipophilic solvents. It is frequently assumed that 100% of an oral dose of most solvents is absorbed systemically. The vehicle or diluent in which a solvent is ingested can affect its absorption and TK.

Dermal absorption of solvents can result in both local and systemic effects. Lipophilic solvents penetrate the stratum corneum, the skin's barrier to absorption, by passive diffusion. Important determinants of the rate of dermal absorption include solvent concentration, surface area exposed, exposure duration, integrity and thickness of the stratum corneum, and lipophilicity and molecular weight of the solvent.

Transport and Distribution

Solvents absorbed into portal venous blood from the GI tract can undergo presystemic elimination. Solvents are also subject to exhalation during their first pass through the pulmonary circulation. Therefore, solvents that are well metabolized and quite volatile are most efficiently eliminated before they enter the arterial blood. Hepatic first-pass elimination depends on the chemical, as well as the rate at which it arrives in the liver. Pulmonary first-pass elimination, in contrast, is believed to be a zero-order process, as a fixed percentage of the chemical is thought to exit the pulmonary blood at each pass through the pulmonary circulation.

Solvents transported by arterial blood are taken up by tissues according to their blood flow and mass and tissue:blood PCs. Relatively hydrophilic solvents solubilize in plasma. Lipophilic solvents do not bind appreciably to plasma proteins or hemoglobin, but partition into hydrophobic sites in these molecules as well as into phospholipids, lipoproteins, and cholesterol present in the blood.

Elimination

Systemic elimination of different solvents varies considerably. Exhalation is determined largely by the rate of pulmonary blood flow, the chemical's blood:air PC, and the alveolar ventilation rate. The more volatile, lipophilic VOCs are exhaled readily. Blood levels of such solvents drop very rapidly during the initial elimination phase following cessation of exposure. This redistribution phase is characterized by rapid diffusion of solvents from the blood into tissues. Equilibration of blood and adipose tissue is prolonged due to the small fraction of cardiac output (about 3%) supplying fat depots. Body fat increases the volume of distribution and total body burden of lipophilic solvents.

Metabolism

Biotransformation can modulate the toxicities of many solvents. Organic solvents are poorly soluble in water and must be converted to relatively water-soluble derivatives, which may be more readily eliminated in the largely aqueous urine and/or bile. Toluene, benzene, 1,1,1-trichloroethylene, hexane, and carbon tetrachloride may be bioactivated primarily by CYP2E1. Other solvents undergo detoxication.

Physiological Modeling

Physiologically based toxicokinetic (PBTK) models are used to relate the administered dose to the blood and/or tissue dose of parent compound and/or bioactive moiety. With knowledge of the physiology of the test animal/tissue and interactions of the bioactive moiety with cellular components and ensuing responses, physiologically based toxicodynamic (PBTD) models can be developed. Biologically based dose–response (BBDR) models consist of a chemical-specific PBPK model for each chemical linked to a PBPD model of cytotoxicity and cellular proliferation, linked in turn to a clonal growth model to predict tumor incidence. The models are well suited for use with species-to-species extrapolations, because human physiological and metabolic parameter values can be inputted into animal models, and simulations of target tissue doses and effects in humans generated. Thus, solvent exposures necessary to produce the same target organ dose in humans, as those found experimentally to cause unacceptable cancer or noncancer risks in test animals, can be determined for some chemicals with reasonable certainty.

POTENTIALLY SENSITIVE SUBPOPULATIONS

Endogenous Factors

Children—Very little information is available on the toxic potential of solvents in infants and children. GI absorption of solvents varies little with age. Systemic absorption of inhaled solvents may be greater in infants and children. Their cardiac output and respiratory rates are relatively high, although their alveolar surface area is lower than that of adults. Reduced plasma protein binding in neonates and infants may be of consequence. Extracellular water, expressed as percent of body weight, is highest in newborns and diminishes through childhood. The larger volume of distribution for water-soluble solvents results in slower clearance and longer duration of action. As lipophilic solvents accumulate in adipose tissue, more body fat would result in higher body burdens, slower clearance, and longer duration of action.

Changes in xenobiotic biotransformation during maturation may impact susceptibility to chemical toxicity and carcinogenicity. CYP isoforms are expressed asynchronously. Levels of CYP2E1, the primary catalyst of oxidation of a variety of solvents, are very low in fetal liver, increase steadily for the first year, and remain relatively constant through adolescence. Glomerular filtration and renal tubular secretion are quite low at birth, but increase substantially during the first 6 months to 1 year. The half-life of creatinine is about half the adult value at 1 year.

Elderly—The aging CNS undergoes changes (e.g., neuronal loss, altered neurotransmitter and receptor levels, and reduced adaptability to effects of toxicants) that may predispose to more pronounced neurologic effects by solvents. Memory, attention, visual perception, and motor skills diminish with aging. With aging, body fat content usually increases substantially at the expense of the lean mass (i.e., skeletal muscle) and body water. Thus, polar solvents tend to reach higher blood levels during exposures. Relatively lipid-soluble solvents accumulate in adipose tissue and are released slowly. Cardiac output diminishes with aging, as do renal and hepatic blood flows.

Gender—Physiological and biochemical differences between men and women have the potential to alter tissue dosimetry and health effects of certain chemicals. There are few data on gender-dependent dermal or pulmonary absorption of solvents. Most men have leaner body mass and larger body size. Women typically have smaller volumes of distribution for polar solvents, but larger volumes of distribution for lipophilic solvents.

Genetics—Polymorphisms for xenobiotic-metabolizing enzymes may affect the quantity and quality of enzymes and the outcomes of exposures to solvents. It is often difficult to disentangle the influences of genetic traits from those of lifestyle, socioeconomic status, and geographics.

Exogenous Factors

P450 Inducers and P450 Inhibitors—Preexposure to compounds that induce CYP isoforms can potentiate or reduce toxicity/carcinogenicity of high doses of solvents that undergo metabolism. An increasing number of drugs and other chemicals, dietary supplements, fruit juices, and vegetable constituents are being identified as inhibitors and/or inducers of CYPs, phase II enzymes, and efflux transporters.

Lifestyle—Exercise increases alveolar ventilation rate and cardiac output/pulmonary blood flow. Polar solvents with relatively high blood:air PCs (e.g., acetone, ethanol, and ethylene glycol) are very rapidly absorbed into the pulmonary circulation. Alveolar ventilation is rate limiting for these chemicals. In contrast, pulmonary blood flow and metabolism are rate limiting for uptake of more lipophilic solvents. Light to heavy exercise can increase pulmonary uptake of solvents. Blood flow to the liver and kidneys diminishes with exercise, so biotransformation solvents and urinary elimination of polar metabolites may be diminished.

Dietary habits can influence the absorption, metabolism, and toxicity of solvents in several ways. The mere bulk of food in the GI lumen can inhibit absorption by preventing contact of the chemical with the GI epithelium. VOCs in the GI tract partition into dietary lipids, largely remaining there until the lipids are emulsified and absorbed. Food intake increases splanchnic blood flow, which favors GI absorption, liver blood flow, and biotransformation.

Fasting for 1 to 3 days results in decreased hepatic concentrations of reduced glutathione, detoxication of electrophilic metabolites of several solvents, and formation of cytotoxic, mutagenic metabolites.

Chronic ethanol ingestion induces CYP2E1, which causes marked potentiation of hepatic or renal damage in persons occupationally exposed to potent hepatorenal toxicants, such as CCl_4, 1,1,1-trichlorethane, 1,1,2-trichloroethylene, or tetrachloroethylene.

Medications and drugs of abuse that induce or inhibit CYP2E1 and other enzymes involved in

the metabolism of VOCs can potentially alter the chemicals' toxicity or carcinogenicity. Tobacco smoke contains a number of compounds that are strong CYP inducers.

Solvent Mixtures—Many occupational and environmental exposures to VOCs involve multiple chemicals. The most common four-component VOC mixture is trichloroethylene, perchloroethylene, 1,1,1-trichloroethane, and 1,1-dichloroethane. Many U.S. cities' drinking-water supplies contain complex mixtures of VOCs. Trace amounts of numerous VOCs are present in the blood of many nonoccupationally exposed members of the general population. Knowledge of mechanisms of VOC interactions involves largely the influence of one VOC on the metabolic activation or inactivation of another.

Diseases—Illness can cause variability in response to solvents. Impaired drug metabolism and clearance are commonly seen in patients with cirrhosis and hepatitis. Reduced metabolism of solvents may result from decrease in hepatic parenchymal mass, diminished enzymatic activity, and/or decreased portal blood flow. Progressive loss of kidney function leads to impaired renal excretion of numerous chemicals and metabolites that may be toxic. Kidney disease can affect uptake and efflux transporters and metabolic enzymes in the liver and the GI tract. Diabetes mellitus resulting from either insulin deficiency or insulin resistance may impact VOC metabolism. Persons with gram-negative infections may render cells more susceptible to damage by solvents and other chemicals.

CHLORINATED HYDROCARBONS

Trichloroethylene

TCE was widely used as a solvent and metal degreasing agent.Moderate-to-high doses of TCE, as with other halocarbons, are associated with a number of noncancer toxicities, including autoimmune disorders, immune system dysfunction, male reproductive toxicity, and cancer.

Metabolism—CNS depression is due to the parent compound and a major metabolite, trichloroethanol. TCE is rapidly and extensively absorbed into the systemic circulation via the oral and inhalation routes. Dermal absorption is considerably slower and less extensive. Most of the TCE is oxidized in the liver, with a small amount conjugated with glutathione (GSH).

Modes of Carcinogenic Action in Target Tissues—Both metabolic pathways are implicated in the carcinogenicity of TCE: reactive metabolite(s) of the GSH pathway in kidney tumors in rats, and oxidative metabolites in liver and lung tumors in mice. Tumor formation in many cases is species-, strain-, sex-, and route of exposure–dependent.

Liver Cancer—TCE can produce hepatocellular carcinoma in B6C3F1 mice, but not in other strains of mice or in rats. This differential susceptibility may be owing to the greater capacity of that mouse to bioactivate large quantities of TCE via the oxidative pathway.

Trichloroacetate (TCA) is a species-specific carcinogen that induces peroxisome proliferation and hepatocellular carcinoma in male and female B6C3F1 mice when administered in very high doses in drinking water or by gavage. TCA does not produce liver tumors in any strain of rats tested under these conditions. Very high doses of dichloroacetate (DCA) produce hepatic tumors

in both B6C3F1 mice and F344 rats. Hypomethylation due to TCA and DCA induces DNA replication and prevents methylation of newly synthesized strands of DNA.

Lung Cancer—Inhaled TCE is carcinogenic to the mouse lung but not to that of the rat. Oral TCE is not carcinogenic to the lung, probably due to first-pass hepatic metabolism that limits the amount of TCE reaching the lungs. The primary target of TCE in the mouse lung is the nonciliated Clara cell. Cytotoxicity is characterized by vacuolization and increased replication of these cells in the bronchiolar epithelium. A dose-dependent reduction in CYP activity in Clara cells is observed as well. Clara cells of the mouse efficiently metabolize TCE to the putative toxicant, chloral, which accumulates due to its efficient production and low activity of ADH, the enzyme responsible for its reduction to TCOH. The Clara cells' lack of glucuronosyltransferase reduces the formation of TCOH-GLU.

Kidney Cancer—TCE can cause cytomegaly and karyomegaly of tubular cells in the renal corticomedullary region. Frank toxic nephropathy was observed with higher frequency in male rats beginning at 52 weeks of exposure. Renal adenomas or adenocarcinomas were occasionally seen in male rats of different strains after 2 years of high-dose oral exposure. Adverse effects of TCE on the kidneys are due largely to metabolites formed via the GSH conjugation pathway.

TCE induces renal cell carcinoma (RCC) in humans. Somatic mutations of the von-Hippel Lindau (VHL) tumor suppressor gene are found in workers exposed to high levels of TCE in metal-processing factories. The VHL gene may be a specific and susceptible target of reactive GSH pathway metabolites.

Risk Assessment—TCE was utilized as a pilot chemical for evaluation and implementation of a "weight of evidence" approach that includes characterization of dose–response relationships, modes of action, and metabolic/TK processes. Where adequate data are available to support reversible binding of the carcinogenic moiety to biological molecules as the initiating event, a nonlinear (i.e., threshold) risk assessment/approach is to be used. Otherwise, the default assumption of a linear (i.e., no-threshold) model/approach is used to estimate cancer risk. In contrast to liver cancer, kidney cancer is widely accepted to be qualitatively similar in rats and humans, though rats form greater quantities of reactive metabolites via the GSH pathway. Despite genotoxic events, kidney tumor formation in humans may require promotion resulting from frank cytotoxicity. But other modes of action of multiple metabolites may operate at low doses. TCE is now classified as a known human carcinogen.

Tetrachloroethylene

Tetrachloroethylene (perchloroethylene, PERC) is commonly used as a dry cleaner, fabric finisher, degreaser, rug and upholstery cleaner, paint and stain remover, solvent, and chemical intermediate. The highest exposures usually occur in occupational settings via inhalation. Although releases are primarily atmospheric, PERC enters surface water and groundwater by accidental and intentional discharges.

Metabolism—The systemic disposition and metabolism of PERC and TCE are similar in many respects, though PERC is much less extensively metabolized. Both chemicals are well absorbed from the lungs and GI tract, distributed to tissues according to their blood flow and lipid content,

partially exhaled unchanged, and metabolized. PERC, like TCE, is metabolized by cytochrome P450-catalyzed oxidation and glutathione conjugation.

Modes of Cytotoxicity/Carcinogenicity—Two additional intermediate metabolites of PERC may contribute to its hepatocytotoxicity: PERC oxide and trichloroacetyl chloride, which is primarily responsible for activation of the nuclear receptor PPARα, which stimulates peroxisomal enzymes and selected CYPs involved in lipid metabolism. The resulting peroxisome proliferation generates reactive oxygen moieties that can cause lipid peroxidation, cellular injury, and altered expression of cell-signaling proteins.

The metabolism and mode of nephrotoxicity of PERC and TCE appear to be quite similar. Renal effects of both halocarbons are due primarily to metabolites formed via the GSH pathway. The sites, enzymes, and products associated are almost identical for both PERC and TCE.

Hepatorenal Toxicity—PERC, like TCE, has quite limited ability to adversely affect the liver or kidneys of rodents. Humans are less susceptible to hepatorenal injury by PERC than rodents, due to lower target-organ doses of the parent compound and its bioactive metabolites. Rats achieve a substantially higher internal dose of VOCs than do humans upon inhalation exposures. In occupational settings, humans may develop mild but reversible liver injury and mild renal tubular damage upon chronic exposure to PERC.

Cancer Bioassays in Rodents—High, chronic doses of PERC have been demonstrated to produce species- and in certain cases strain- and gender-specific tumors in some organs of mice and rats.

Cancer Epidemiology Studies—Many epidemiology studies of cancer incidence and mortality in groups of dry cleaners and other persons occupationally exposed to PERC have been equivocal. Cigarette smoking and alcohol consumption only partially account for an increased rate of esophageal cancer in dry cleaners. Nevertheless, there was limited/suggestive evidence of an association between chronic PERC exposure and esophageal, kidney, bladder, and lung cancers, but insufficient/inadequate evidence to determine whether an association exists between chronic PERC exposure and hepatobiliary cancer.

Risk Assessment—Much research has been conducted to characterize PERC's dose–response relationships, modes of action, and metabolism/toxicokinetics in rodents and humans. Humans appear to be less susceptible than rodents to the toxic or carcinogenic actions of PERC. Humans absorb less inhaled PERC and TCE, attain lower target organ doses of the parent compounds, have lower oxidative and GSH conjugation capacity, and inactivate epoxide intermediates more efficiently. Biotransformation of PERC by the GSH conjugation pathway appears to be qualitatively similar in male rats and humans.

1,1,1-Trichloroethane

1,1,1-Trichloroethane (methyl chloroform, TRI) was a widely used organic solvent, a metal degreaser, general purpose solvent, spot cleaner, and component of aerosols and a variety of household products. Utilization of TRI diminished due to its ozone-depleting properties.

Toxicokinetics (TK) and Metabolism—TRI is rapidly and extensively absorbed from the lungs

and GI tract. Systemic uptake and blood levels are elevated in humans inhaling TRI and other VOCs when they exercise. TRI is distributed throughout the body, with fat achieving the highest concentrations. As biotransformation is limited, TRI is cleared primarily by exhalation.

Toxicity—The primary manifestation of acute or chronic inhalation of TRI is CNS depression, ranging in severity from slight headache or dizziness to anesthesia and death. Very high inhaled concentrations of TRI, particularly when accompanied by hypoxia and stress, can sensitize the myocardium to catecholamines, producing cardiac arrhythmias. TRI has limited cytotoxic potential.

Potential Carcinogenicity—Currently, inadequate information is available for assessment of human carcinogenic potential.

Methylene Chloride

Methylene chloride (dichloromethane, MC) has enjoyed widespread use as a solvent in industrial processes, manufacture of drugs, degreasing agents, aerosol propellants, agriculture, and food preparation. The primary route of exposure to this very volatile solvent is inhalation.

Metabolism—MC metabolism occurs primarily in liver but also in lung. MC is rapidly absorbed and distributed throughout the body. Metabolism of MC in humans and rodents is thought to occur via three pathways: (1) cytochrome P450 2E1-catalyzed oxidation to CO via reactive formyl chloride, (2) theta-class GSH S-transferase (GST), GST-T1, catalyzed formation of a GSH conjugate that is eventually metabolized to carbon dioxide with formation of two reactive intermediates, *S*-(chloromethyl)-GSH and formaldehyde, and (3) oxidative pathway reaction of formyl chloride with a nucleophile such as GSH.

Modes of Toxicity/Carcinogenicity—MC has limited cytotoxicity potential. Manifestations of kidney damage have been rare in laboratory animals, but have occasionally been reported in persons subjected to high vapor levels. There is little information on the identity of MC metabolites that adversely affect the liver or kidney. The CO formed by oxidation of MC binds to hemoglobin to produce dose-dependent increases in carboxyhemoglobin.

Cancer Bioassays in Rodents—High, chronic exposures to MC have been found to produce species- and gender-specific tumors in some organs of mice and rats. Hepatocellular adenomas or carcinomas were noted in male B6C3F1 mice, and bronchoalveolar adenoma and carcinoma were found in male and female B6C3F1 mice.

Cancer Epidemiology Studies—Despite a substantial number of epidemiology studies of MC-exposed workers, evidence of associations between MC and specific tumors is not strong.

Risk Assessment—MC is likely to be carcinogenic in humans and appears to act via a mutagenic mode of action. Bioactivation of MC is qualitatively similar in mice and humans. Based on the mutagenicity of GST pathway metabolites, it is plausible that MC is carcinogenic in humans.

Carbon Tetrachloride

Properties of carbon tetrachloride (CCl_4) include hepatorenal toxicity, carcinogenicity, and contribution to atmospheric ozone depletion.

Early signs of hepatocellular injury in rats include dissociation of polysomes and ribosomes from rough endoplasmic reticulum, disarray of smooth endoplasmic reticulum, inhibition of protein synthesis, and triglyceride accumulation. Hypomethylation of RNA may contribute to inhibition of lipoprotein synthesis, thereby playing a role in steatosis. Ingested CCl_4 undergoes metabolic activation, produces lipid peroxidation, and inhibits microsomal ATPase activity. Single cell necrosis, evident 5 to 6 hours postdosing, progresses to maximal centrilobular necrosis within 24 to 48 hours. Cellular regeneration, manifest by increased DNA synthesis and cell cycle progression, is maximal 36 to 48 hours postdosing.

CCl_4 is bioactivated by cytochromes P450 via reductive dehalogenation to trichloromethyl radicals (CCl_3•), which can react in turn with oxygen to form trichloromethylperoxy free radicals (CCl_3•). Both unstable radicals bind covalently to cellular components including enzymatic and structural proteins and polyunsaturated fatty acids in membranes. This results in lipoperoxidation, loss of intracellular and cellular membrane integrity, and leakage of enzymes. By-products of lipid peroxidation include reactive aldehydes that form adducts with proteins and DNA, thereby contributing to cytotoxicity and carcinogenicity.

Increased cytosolic Ca^{2+} levels may result from influx of extracellular Ca^{2+} due to plasma membrane damage and from decreased intracellular Ca^{2+} sequestration. Ca^{2+} induced activation of phospholipase A_2 exacerbates membrane damage. Elevated Ca^{2+} may also contribute to alterations in calmodulin, phosphorylase, and nuclear protein kinase C activities. High intracellular Ca^{2+} levels activate catabolic proteases, endonucleases, and phospholipases, which kill cells via apoptosis or necrosis. Ca^{2+} may stimulate the release of cytokines and eicosanoids from Kupffer cells.

Development of cellular resistance and tissue repair are important in limiting CCl_4 hepatotoxicity, and in recovery. Hepatocellular regeneration begins within 6 hours of a small dose of CCl_4. This early-phase regeneration (arrested G_2 hepatocytes activated to proceed through mitosis) is followed at about 24 hours by the secondary phase of regeneration (hepatocytes mobilized from G_o/G_1 to proceed through mitosis). Although CCl_4 is a hepatocarcinogen in rodents, there is little experimental evidence of genotoxicity or carcinogenicity in humans.

Chloroform

Chloroform ($CHCl_3$, trichloromethane) is a by-product of drinking water chlorination and has been quantified in municipal drinking water supplies, in swimming pool water and surrounding air. $CHCl_3$ can invoke CNS symptoms similar to those of alcohol intoxication and can sensitize the myocardium to catecholamines, possibly resulting in cardiac arrhythmias. $CHCl_3$ is hepatotoxic and nephrotoxic. $CHCl_3$ is a rodent carcinogen that causes liver and kidney tumors that are species-, strain-, sex-, and route of exposure–dependent.

The metabolite, phosgene, is responsible for $CHCl_3$'s hepatorenal toxicity. The electrophilic

phosgene is initially detoxified by covalent binding to cytosolic GSH. Once GSH is depleted, phosgene is free to bind hepatic and renal proteins and lipids, damaging membranes and other intracellular structures, leading to necrosis and subsequent reparative cellular proliferation. Sustained proliferation with repeated exposures promotes tumor formation in rodents by irreversibly "fixing" spontaneously altered DNA and clonally expanding initiated cells. The expression of certain genes, including *myc* and *fos*, is altered during regenerative cell proliferation in response to $CHCl_3$-induced cytotoxicity. $CHCl_3$ is a probable human carcinogen.

AROMATIC HYDROCARBONS

Benzene

Benzene is used principally in the synthesis of other chemicals and in unleaded gasoline for its antiknock properties. Inhalation is the primary route of exposure in industrial and in everyday settings. Cigarette smoke is the major source of benzene in the home. Smokers have benzene body burdens that are six to ten times greater than those of nonsmokers. Passive smoke can be a significant source of benzene exposure to nonsmokers.

High-level benzene exposures result in an increased risk of acute myelogenous leukemia (AML) in humans. Benzene is also an animal carcinogen, but major species differences exist. The hematopoietic toxicity may manifest initially as anemia, leukopenia, thrombocytopenia, or a combination of these. Bone marrow depression appears to be dose-dependent in both laboratory animals and humans. Continued exposure may result in marrow aplasia and pancytopenia. Survivors of aplastic anemia frequently exhibit a preneoplastic myelodysplasia, which may progress to myelogenous leukemia. Polymorphisms in myeloperoxidase (an enzyme that metabolizes benzene to toxic quinones and free radicals) and NAD(P)H:quinone oxidoreductase (an enzyme that protects against these moieties) conferred increased susceptibility to white cell decreases.

The toxicity of benzene depends upon its conversion to benzene oxide primarily by hepatic CYP2E1. Benzene oxide, which is in equilibrium with its oxepin form, is further metabolized by three pathways: (1) conjugation with GSH to form a premercapturic acid, which is converted to phenyl-mercapturic acid; (2) rearrangement nonenzymatically to form phenol; and (3) hydration by epoxide hydrolase to benzene dihydrodiol, which in turn can be oxidized by dihydrodiol dehydrogenase to catechol. If phenol is hydroxylated in the *ortho* position, more catechol is formed, which can be converted to *o*-benzoquinone. If benzene is hydroxylated in the *para* position, *p*-hydroquinone is formed, which can be oxidized to *p*-benzoquinone. The *o*- and *p*-benzoquinones are believed to be among the ultimate toxic metabolites of benzene.

Investigations of benzene toxicity/leukemogenesis have uncovered several potential mechanisms. (1) A number of benzene metabolites can bind covalently to GSH, proteins, DNA, and RNA, resulting in disruption of the functional hematopoietic microenvironment by inhibition of enzymes, destruction of certain cell populations, and altered growth of other cell types. Covalent binding of hydroquinones to spindle-fiber proteins will inhibit cell replication. Five key events have been proposed for benzene-induced leukemia: (a) benzene metabolism via cytochrome P450; (b) interaction of benzene metabolites with target cells in the bone marrow; (c) formation of initiated, mutated target cells; (d) selective proliferation of the mutated cells; and (e) production of leukemia. (2) Oxidative stress contributes to benzene toxicity. As the bone

marrow is rich in peroxidase activity, phenolic metabolites of benzene can be activated to reactive quinone derivatives. Reactive oxygen moieties (e.g., O_2^- and H_2O_2) are produced via the formation of the semiquinone radicals. These active oxygen species can cause DNA strand breaks or fragmentation, leading to cell mutation or apoptosis, respectively. Modulation of apoptosis may lead to aberrant hematopoiesis and neoplastic progression.

Toluene

Inhalation is the primary route of exposure, though skin contact occurs frequently. Toluene is a favorite of solvent abusers, who intentionally inhale high concentrations to achieve a euphoric effect. Large amounts of toluene enter the environment each year by volatilization. Toluene is well absorbed from the lungs and GI tract. It rapidly accunulates in, and can affect, the brain. Toluene subsequently is deposited in other tissues; adipose tissue attains the highest levels.

The CNS is the primary target organ of toluene and other alkylbenzenes. Cardiac, renal, and hepatic toxicities as well as fetal alcohol-like syndrome have been reported. Increased incidence of spontaneous abortion was linked to toluene exposure at workplaces, but not confirmed by animal testing. Manifestations of acute exposure range from slight dizziness and headache to unconsciousness, respiratory depression, and death. Acute encephalopathic effects are reversible upon cessation of exposure.

Toluene has alcohol-like effects of euphoria and excitation. Motor incoordination, dizziness, relaxation, and lightheadedness are characteristic of toluene intoxication, as well as illusions and hallucinations. Acute inhalation with poor oxygenation can lead to life-threatening cardiac arrhythmias and other complications associated with hypokalemia. Severe neurotoxicity is often diagnosed in persons who have abused toluene chronically. A relatively specific neurobehavioral profile is manifest, including inattention, apathy, memory dysfunction, diminished visuospatial skills, frontal lobe dysfunction, and psychiatric status. Magnetic resonance imaging reveals ventricular enlargement, cerebral atrophy, and white matter hyperintensity, a characteristic profile termed toluene leukoencephalopathy. Such changes represent severe, chronic myelotoxicity. The term “fetal solvent syndrome” was used to describe toluene-abusing women’s children who exhibit microcephaly and cranial facial features similar to those with fetal alcohol syndrome. Existing studies suggest that toluene is not carcinogenic or mutagenic.

Xylenes and Ethylbenzene

Large numbers of people are exposed to xylenes and ethylbenzene occupationally and environmentally. Xylenes and ethylbenzene are major components of gasoline and fuel oil. Xylenes are also used in the production of ethylbenzene. The primary uses of xylenes and ethylbenzene industrially are as solvents and synthetic intermediates. Most of these aromatics that are released into the environment evaporate into the atmosphere.

Xylenes, and other aromatic solvents are well absorbed from the lungs and GI tract, distributed to well-perfused and lipophilic tissues such as liver, fat, brain and skin, exhaled unchanged to some extent, well metabolized by hepatic CYP2E1 primarily, and largely excreted as urinary metabolites. Acute CNS-depressant activity varies directly with lipophilicity. Some alkylbenzenes including xylenes and ethylbenzene are ototoxic in rats. There is limited evidence that chronic occupational exposure to xylenes is associated with residual neurologic effects.

Systemic effects associated with ethylbenzene exposure are respiratory tract and ocular irritation, possible ototoxicity, and hematological alterations.

Xylenes and ethylbenzene have limited capacity to adversely affect organs other than the CNS. Mild, transient liver and/or kidney toxicity have/has occasionally been reported in humans exposed to high vapor concentrations of xylenes.

Levels of xylene and its metabolite, methylhippuric acid, in urine are the primary biomarkers used to detect exposure to xylene. Ethylbenzene levels in blood and mandelic acid and phenylglyoxylic acid in urine are used as biomarkers of ethylbenzene exposure. However, mandelic acid and phenylglyoxylic acid are also metabolites of styrene.

Xylene is not classifiable as to its carcinogenicity to humans. Ethylbenzene caused kidney injury and renal adenoma and carcinoma (combined) in male F344 rats and is classified as a possible human carcinogen.

Styrene

Styrene is primarily used in the manufacture of polystyrene items and in copolymers with acrylonitrile or 1,3-butadiene to produce synthetic rubber, latex, and reinforced plastics. Central nervous system effects and mucous membrane and respiratory irritation are commonly reported effects in humans following styrene exposure, but signs of mild hepatic injury and cholestasis may be exhibited. There is debate as to whether styrene is a human carcinogen. Noncancer effects reported include mucous membrane irritation, changes in color vision, hearing loss, and neurocognitive impairment. Occupational exposure to styrene is a potential risk factor for nonmalignant respiratory disease, and related morbidity and mortality.

ALCOHOLS

Ethanol

Many humans experience greater exposure to ethanol (ethyl alcohol, alcohol) than to any other solvent. Ethyl alcohol is used as an additive in gasoline, as a solvent in industry, in household products and pharmaceuticals, but it is also heavily consumed in intoxicating beverages. Frank toxic effects are less important occupationally than injuries resulting from psychomotor impairment. Driving under the influence of alcohol is the major cause of fatal auto accidents. Ethanol is distributed in body water and to some degree in adipose tissue. Alcohol is eliminated primarily by metabolism (90% to 95%), with the remainder exiting via urinary excretion and exhalation. The blood level in an average adult decreases by approximately 16 mg/dL per hour. Thus, a person with a blood alcohol level of 120 mg/dL would require around 8 hours to reach negligible levels.

Ethanol is metabolized to acetaldehyde by: (1) alcohol dehydrogenase (ADH)-catalyzed oxidation to acetaldehyde, which is rapidly oxidized by acetaldehyde dehydrogenase (ALDH) to acetate; (2) catalase, utilizes H_2O_2 supplied by NADPH oxidase and xanthine oxidase; and (3) CYP2E1 is the principal isoform of the hepatic microsomal ethanol oxidizing system.

Gender differences in responses to ethanol are well recognized. Women are more sensitive to alcohol, exhibit higher mortality at lower levels of consumption than men, and exhibit somewhat

higher blood levels than men following ingestion of equivalent doses of ethanol. Women are more susceptible to alcohol-induced hepatitis and cirrhosis.

Alcohol is the leading preventable cause of birth defects and developmental disorders. The most serious adverse consequence of prenatal alcohol exposure is fetal alcohol syndrome (FAS). Diagnostic criteria for FAS include: (1) heavy maternal alcohol consumption during gestation, (2) pre- and postnatal growth retardation, (3) craniofacial malformations including microcephaly, and (4) mental retardation. However, other alcohol-related brain and behavioral abnormalities that lack abnormal facial features are referred to as fetal alcohol spectrum disorders (FASD) and occur in an estimated 1% of births.

Exposure of embryonic tissue to ethanol adversely affects many cellular functions critical to development, including protein and DNA synthesis, uptake of critical nutrients such as glucose and amino acids, and changes in several kinase-mediated signal transduction pathways. Suggested mechanisms include oxidative stress on fetal tissues; ethanol's effects on neurotransmitter-gated ion channels, particularly the NMDA receptor; its ability to trigger cell death via necrosis and apoptosis; a long-lasting reduction in synaptic efficacy; altered fetal expression of developmentally important genes such as msx2 and insulin-like growth factors; and NMDA and GABA receptor-mediated apoptosis play critical roles in learning impairment. Mechanisms of FASD include: (1) alterations in the regulation of gene expression (e.g., reduced retinoic acid signaling, altered DNA methylation); (2) interference with mitogenic and growth factor responses involved in neural stem cell proliferation, migration, and differentiation; (3) disturbances in molecules that mediate cell-cell interactions (L1, NCAM, loss of trophic support); and (4) derangements of glial proliferation, differentiation, and function.

Alcoholic liver disease (ALD) is one of the most prevalent and fatal conditions of alcohol abuse. ALD follows a characteristic pattern marked by progressive appearance of fatty liver, hepatocyte necrosis, inflammation, regeneration nodules, fibrosis, and cirrhosis. Development of steatosis, characterized by accumulation of triglyceride droplets in the liver, is the initial stage of ALD. This stage is symptomless and reversible, if alcohol consumption is reduced. It is postulated that chronic alcohol consumption increases gut permeability to lipopolysaccharide (LPS), an endotoxin that is a major constituent of the outer membrane of gram negative bacteria. LPS binds to receptors in Kupffer cells to produce reactive oxygen species (ROS), which activate NF-kB, leading to TNF-α and other inflammatory mediators that are cytotoxic to hepatocytes and chemoattractants for neutrophils. Pro-inflammatory cytokines and oxidative stress stimulate collagen synthesis by hepatic stellate cells, leading to alcoholic fibrosis. Chronic alcohol consumption also inhibits components of innate immunity, such as natural killer (NK) cells. The inhibition of NK cells decreases NK cell-mediated killing of stellate cells, contributing to fibrosis. CYP2E1 can generate ROS during its catalytic cycle. Because CYP2E1 metabolizes ethanol and increases during ethanol exposure, it is suggested to play a significant role in ethanol-induced liver toxicity. Necroptosis (necrosis through apoptosis-regulated pathway) is activated by receptor-interacting protein kinase 3, which is dependent on CYP2E1 expression. Necroptosis also exacerbates chronic inflammation.

Prostaglandins released from endotoxin-activated Kupffer cells may be responsible for a hypermetabolic state in the liver. With increased oxygen demand, the viability of centrilobular hepatocytes would be most compromised, due to their relatively poor oxygen supply. Metabolism of ethanol via ADH and ALDH results in a shift in the redox state of the cell. The metabolites and the more reduced state can result in hyperlacticacidemia, hyperlipidemia, hyperuricemia, and hyperglycemia, leading to increased steatosis and collagen synthesis.

Chronic ethanol consumption may promote carcinogenesis by: (1) production of acetaldehyde, a weak mutagen and carcinogen; (2) induction of CYP2E1 and its associated oxidative stressors and conversion of procarcinogens to carcinogens; (3) depletion of SAM with induction of global DNA hypomethylation; (4) increased production of inhibitory guanine nucleotide regulatory proteins and components of extracellular signal-regulated kinase-mitogen-activated protein kinase signaling; (5) accumulation of iron and associated oxidative stress; (6) inactivation of the tumor suppressor gene *BRCA1* and increased estrogen responsiveness (primarily in the breast); and (7) impairment of retinoic acid metabolism.

Methanol

Methanol (methyl alcohol, wood alcohol, and CH_3OH) is primarily used for the synthesis of chemicals, in windshield washer fluid, carburetor cleaners, antifreeze, and copy machine toner, and serves as fuel for Sterno™ heaters, model airplanes, and Indianapolis 500 racecars. It also functions as a denaturant for some ethyl and isopropyl alcohols, rendering them unfit for consumption. It is used to a limited extent as an alternative fuel for fleet vehicles.

Following ingestion, CH_3OH can cause CNS depression, ataxia, difficult breathing, acute gastritis or pancreatitis, anorexia, intense abdominal pain, vomiting, and diarrhea. Left untreated, acute CH_3OH poisoning in humans is characterized by an asymptomatic latent period of 12 to 24 hours followed by formic acidemia, ocular toxicity, coma, and in extreme cases death. These effects are caused by its metabolites. Respiratory failure or sudden respiratory arrest is the most common cause of death in methanol poisoning. Visual disturbances generally develop between 18 and 48 hours after ingestion and range from mild photophobia and misty or blurred vision to markedly reduced visual acuity and complete blindness. Optic disk edema and hyperemia are seen, along with morphological alterations in the optic nerve head and the intraorbital portion of the optic nerve. Rods and cones, the photoreceptors of the retina, are also altered functionally and structurally. Müller cells, neuroglia that function in the maintenance of retinal structure and in intra- and intercellular transport, are early targets of CH_3OH. Formate inhibits the energy-generating mitochondrial cytochrome *c* oxidase, which is critical for the proper functioning of highly oxidative organs like the retina. This mechanism might explain, at least in part, CH_3OH's selective toxicity to photoreceptors and other highly metabolically active cells.

In mammals, HCOH is rapidly converted via formaldehyde dehydrogenase (FLDH) to formate, which is further metabolized to CO_2. The conversion of formate to CO_2 occurs via a two-step, tetrahydrofolate (THF)-dependent pathway. Direct incubation of formate with cultured ocular cells caused ATP depletion and cytotoxicity, indicating that formate acts as a direct ocular toxin and not indirectly through the induction of an acidotic state.

Intraretinal metabolism of CH_3OH initiates retinal toxicity by formate. Not only are the enzymes necessary to produce formate present in the retina, but so too are folate and formyl-THF dehydrogenase. Localized in the mitochondria of Muller cells, formyl-THF dehydrogenase may serve a dual role, one protective of the Müller cell and the other toxic. Protection would come in the form of formate oxidation; toxicity from the overconsumption or depletion of ATP required for formate metabolism via the folate pathway.

GLYCOLS

Ethylene Glycol

Ethylene glycol (1,2-dihydroxyethane, EG) is a constituent of antifreeze, deicers, hydraulic fluids, drying agents, and inks, and is used to make plastics and polyester fibers. Workers may be exposed dermally or by inhalation. The most important exposure route is ingestion, as EG may be accidentally swallowed, taken deliberately in suicide attempts, or used as a cheap substitute for ethanol. EG enters the environment, partitions into surface water and groundwater, does not persist in any environmental medium, and is practically nontoxic to aquatic organisms.

Absorption from the GI tract of rodents and humans is rapid and virtually complete, whereas cutaneous and pulmonary absorption is slower and less extensive. Once absorbed, EG is distributed throughout total body water. As illustrated in Fig. 24–2, EG is metabolized by NAD^+-dependent ADH to glycolaldehyde and on to glycolic acid, which is oxidized to glyoxylic acid. Glyoxylic acid may be converted to formate and CO_2, or oxidized by glyoxylic acid oxidase to oxalic acid (OA). The rate-limiting step in the metabolism of EG is the conversion of GA to glycolic acid (GA). EG has a half-life in humans of 3 to 8.6 hours.

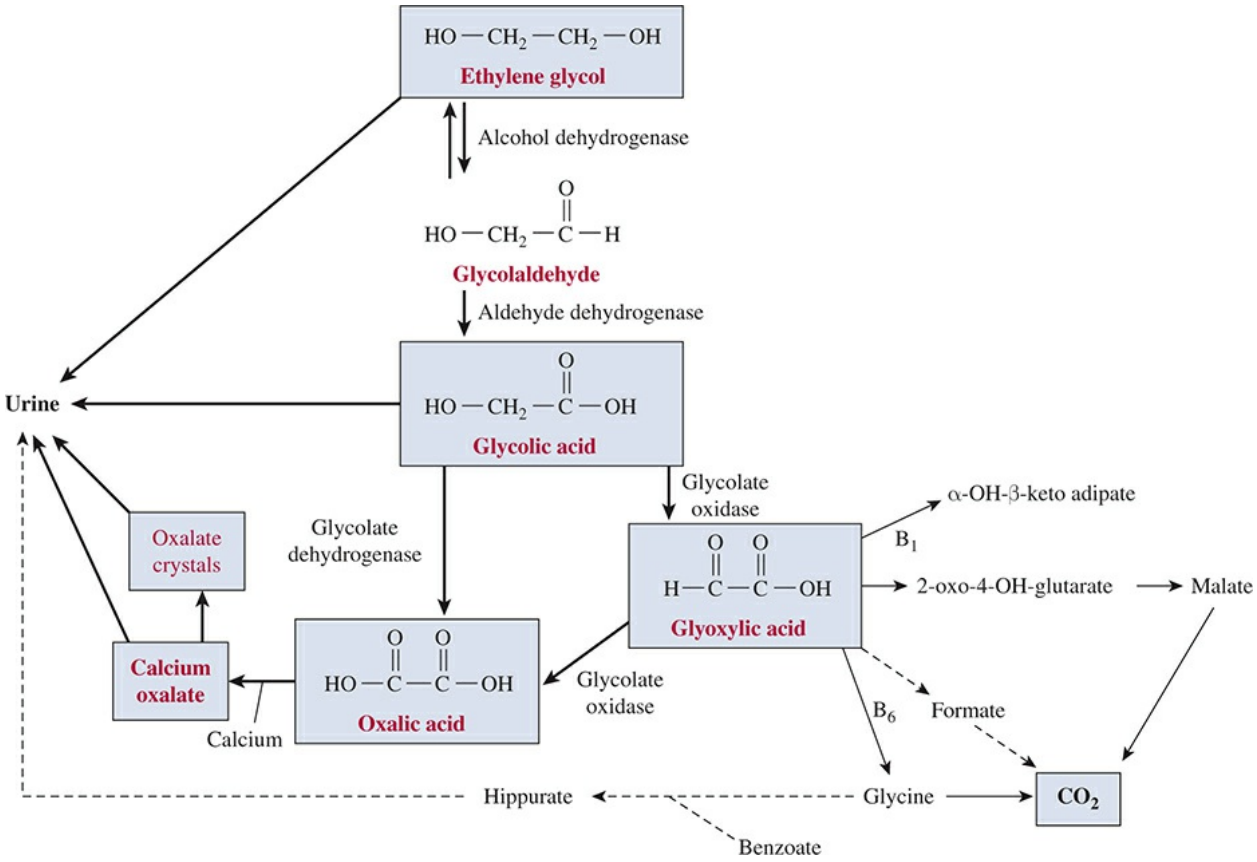

FIGURE 24–2 *Metabolic scheme for ethylene glycol in animals*. Key metabolites that have been observed in vivo are highlighted in boxes. Dashed lines are theoretical pathways that have not been verified in vivo or in vitro. (Reprinted from Corley RA, Bartels MJ, Carney EW, et al. Development of a physiologically based pharmacokinetic model for ethylene glycol

and its metabolite, glycolic acid, in rats and humans. *Toxicol Sci.* 2005;85:476–490.)

Acute poisoning entails three clinical stages after an asymptomatic period, during which EG is metabolized: (1) a period of inebriation, the duration and degree depending on dose; (2) the cardiopulmonary stage 12 to 24 hours after exposure, characterized by tachycardia and tachypnea, which may progress to cardiac failure and pulmonary edema; and (3) the renal toxicity stage 24 to 72 hours postexposure. Metabolic acidosis can progress in severity during stages 2 and 3.

The key mechanistic events in the renal toxicity of EG: (1) metabolism of EG to OA via GA; (2) concentration of OA in tubular urine → precipitation of OA with Ca^{2+} → buildup of COM (calcium oxalate monohydrate) crystals in renal tubular epithelium → adherence of COM crystals to the plasma membrane of proximal tubular cells → subsequent intracellular uptake of COM crystals by endocytosis; and (3) physical trauma by COM crystals and/or production of free radicals and lipid peroxidation leading to cell necrosis, apoptosis, and renal tubular degeneration. Clearly, OA is critical in the induction of renal damage, but the influence of hippuric acid crystals and direct cytotoxicity by other metabolites cannot be ruled out.

Propylene Glycol

Propylene glycol (PG) is used in the synthesis of polyester fibers and resins, as a component of automotive antifreeze/coolants, and as a deicing fluid for aircraft. As PG is “generally recognized as safe” by the FDA, it is a constituent of many cosmetics, processed foods, and tobacco products, and serves as a diluent for oral, dermal, and i.v. drug preparations. Ingestion and dermal contact are important routes of exposure. PG has a high mobility in soil and can leach into groundwater, but is neither persistent nor bioaccumulative. Its soil and water half-lives are a few days under aerobic or anaerobic conditions. PG has very low acute and chronic toxicity, targets no specific organ system, with no accounts of human fatalities. Large i.v. doses given over short periods of time can manifest as hyperosmolality, increased anion gap metabolic acidosis, acute kidney injury, and sepsis-like syndrome. PG is absorbed from the GI tract and distributed throughout total body water. Approximately 55% of PG is metabolized by ADH to lactaldehyde, while about 45% is excreted unchanged by the kidneys. Its serum half-life is 2 to 4 hours in humans.

GLYCOL ETHERS

If one alcohol residue of ethylene glycol ($HO—CH_2—CH_2—OH$) is replaced by an ether, the resulting compound is a monoalkyl glycol ether such as ethylene glycol monomethyl ether (EGME), also called 2-methoxyethanol (2-ME; $CH_3—O—CH_2—CH_2—OH$). If both alcohols are replaced by ethers, the result is a di-alkyl glycol ether such as ethylene glycol dimethyl ether ($CH_3—O—CH_2—CH_2—O—CH_3$). The alkyl group at the end of the ether linkage may be a straight or branched short-chain moiety (e.g., methyl, ethyl, *n*-propyl, isopropyl, or butyl). Acetates of monoalkyl ethers such as 2-ME acetate ($CH_3—CO—O—CH_2—CH_2—O—CH_3$) are common solvents that undergo rapid ester hydrolysis to their parent glycol ethers in vivo, and tend to exhibit the same toxicity profiles as unesterified glycols. Human exposure occurs mainly

via inhalation, but also by dermal absorption.

Glycol ether metabolism varies with chemical structure. For ethylene glycol monoalkyl ethers, the major metabolic pathway is oxidation via ADH and ALDH to alkoxyacetic acids. The propylene series of glycol ethers (e.g., propylene glycol monomethyl ether [PGME]) is predominantly biotransformed to PG via *O*-dealkylation. Glycol ethers may be conjugated with glucuronide or sulfate, but only after saturation of other metabolic pathways.

Reproductive Toxicity

Epidemiological studies have reported associations between glycol ether exposure and increased risk for spontaneous abortion, menstrual disturbances, and subfertility among women employed in the semiconductor industry. For men exposed to glycol ethers, reversible spermatotoxicity including oligospermia and azoospermia, seminiferous tubule atrophy, abnormal sperm head morphology, and infertility have been noted.

Developmental Toxicity

Exposure to certain glycol ethers during organogenesis (e.g., 2-ME and 2-EE) is toxic to the developing embryo. Structural anomalies in rodents have included a variety of minor skeletal variations, hydrocephalus, exencephaly, cardiovascular malformations, dilatation of the renal pelvis, craniofacial anomalies, and digit malformations. Electrocardiograms of fetal rats from dams treated with 2-ME during gestation showed persistent, a berrant QRS waves, suggestive of an intraventricular conduction delay.

Hematotoxicity

Some glycol ethers are hemolytic to red blood cells (RBCs). Typically, the osmotic balance of cells is disrupted, they imbibe water and swell, their ATP concentration decreases, and hemolysis occurs. Subchronic exposure to 2-butoxyethanol (2-BE) causes disseminated thrombosis and bone infarctions in female, but not male rats, likely due to impedance of blood flow by intravascular hemolysis. It is thought that females might be susceptible because they are less efficient in eliminating butoxyacetic acid (BAA), the hemolytic metabolite of 2-BE, and exhibit higher peak blood BAA levels. Humans are less susceptible than rodents.

Immunotoxicity/Carcinogenicity

Based on changes in thymus and splenic weights/cellularities and a variety of in vitro and in vivo immune function assays, the immune system is a potential target for the oxidative metabolites of some glycol ethers.

Only a few chronic bioassays have been conducted with glycol ethers. Two-year inhalation bioassays of 2-BE in F344 rats and B6C3F1 mice revealed some increased incidences of hepatic hemangiosarcoma in male mice and forestomach squamous cell papilloma or carcinoma in female mice. The evidence suggests nonlinear modes of action in both cases and questionable human relevance. Some glycol ethers and their metabolites exhibit a lack of genotoxic potential with others yielding weakly positive responses in certain tests.

FUELS AND FUEL ADDITIVES

Automotive Gasoline

Automotive gasoline is a mixture of hundreds of hydrocarbons predominantly in the C_4 to C_{12} range. Because its composition varies with the crude oil from which it is refined, the refining process, and the use of specific additives, generalizations are difficult to make. Experiments conducted with fully vaporized gasoline may not be predictive of actual risk, because humans are exposed primarily to the more volatile components in the range of C_4 to C_5. These hydrocarbons are generally regarded as less toxic than their higher-molecular-weight counterparts.

Inhalation exposure to gasoline has been measured for service station attendants, self-service customers, truck drivers, distribution workers, and workmen removing leaking underground storage tanks. The most extreme exposures occur with those intentionally sniffing gasoline for its euphoric effects. Acute and chronic encephalopathies have been reported in sniffers. Ingestion of gasoline during siphoning events is typically followed by a burning sensation in the mouth and pharynx, as well as nausea, vomiting, and diarrhea resulting from GI irritation. If aspirated into the lungs, gasoline may produce pulmonary epithelial damage, edema, and pneumonitis.

Gasoline can sensitize the heart to catecholamines, defat the skin upon repeated contact, and induce hepatic P450s and UDP-glucuronyltransferase activities. Chronic inhalation of gasoline at high concentrations has also resulted in increased hepatocellular adenomas and carcinomas in female B6C3F1 mice, possibly due to the promotion of spontaneously initiated cells that occur with unusually high frequency in this mouse strain. However, the evidence for an association between gasoline exposure and cancer in humans is inconclusive.

Oxygenated gasoline contains additives that boost its octane quality, enhancing combustion, and reducing exhaust emissions. Ethanol is a commonly used oxygenate that can increase groundwater plume lengths and persistence of gasoline constituents in groundwater. Another fuel additive, methylcyclopentadienyl manganese tricarbonyl (MMT), could increase manganese inhalation exposures and pose a risk for neurotoxicity. The combustion of MMT gasoline results in the emission of fine Mn particulates mainly as Mn sulfate and Mn phosphate and smaller amounts of oxides.

Methyl Tertiary-Butyl Ether

As an oxygenator, MTBE makes fuel combustion more complete, thereby reducing pollutant emissions from automobile exhaust. Because it is highly water soluble, travels faster and farther in water than other gasoline components, and is resistant to degradation, MTBE is no longer used in gasoline. However, it finds occasional use as an FDA-approved drug for the dissolution of gallstones in the event surgical or endoscopic options are unavailable. It may become an anti-angiogenic treatment for solid tumors.

MTBE is well absorbed following oral, inhalation, and dermal exposure of humans and rats. The majority of absorbed MTBE is exhaled unchanged. Some MTBE is oxidized to *tert*-butyl alcohol (TBA) and HCOH. TBA is relatively water soluble, so it tends to remain in the blood and extracellular fluid, and is slowly exhaled. TBA is further metabolized, first to 2-methyl-1,2-propanediol and then to 2-hydroxyisobutyrate, the major urinary metabolites of MTBE. In addition, glucuronide and sulfate conjugates of TBA are found in trace amounts in urine.

Jet Fuel

Jet A, jet propellant-5 (JP-5), and JP-8 are the predominant jet fuels in use today. All are kerosene-like mixtures of hundreds of aliphatic and aromatic hydrocarbons. These jet fuels differ slightly in hydrocarbon composition and/or additives. Jet fuel exposure is by inhalation and dermal contact. Exposure can occur to liquid, vapor, or aerosol, each phase having a distinct composition and toxicity profile. Jet A, JP-5, and JP-8 have similar toxicity profiles, which suggest their toxicities are largely a function of hydrocarbon content rather than additives.

The pulmonary effects of an aerosol/vapor mixture of JP-8 include increases in pulmonary resistance and alveolar permeability were accompanied by a decrease in the concentration of the tachykinin Substance P (SP) in bronchoalveolar lavage fluid. Pathological changes observed in lower pulmonary structures include inflammation of the terminal bronchioles, degeneration of alveolar type II epithelial (AIIE) cells, and disruption of terminal bronchial airway epithelium.

Mice exposed nose-only to a JP-8 aerosol/vapor mix exhibited decreased spleen and thymus weights and cellularities and an altered number of viable immune cells in lymph nodes, bone marrow, and peripheral blood. Depending on the immune tissue examined, different immune cell sub-populations were lost, including T and B cells and macrophages. Jet fuel–induced immunosuppression in skin can be reversed by inhibiting prostaglandin secretion with a selective COX-2 inhibitor or injecting an anti-IL-10 antibody. The aromatic hydrocarbons in JP-8 are able to produce reactive oxygen species (ROS) in the skin that activate NF-κβ, which in turn activates COX-2, thereby inducing the secretion of PGE2.

Dermal exposure to jet fuel can lead to skin irritation and sensitization and the disruption of skin barrier function. Research implicates cytokine release, oxidative stress, and DNA damage/fragmentation as mechanistic underpinnings. The primary effect of exposure is damage of the stratum corneum barrier and IL-8 release from human epidermal keratinocytes after JP-8 exposure was decreased by SP, which is an agonist for the NK_1 receptor present in keratinocytes and mechanistically linked to IL release. The attenuation of IL release in keratinocytes by SP and the protection SP affords against pulmonary and immunotoxicity suggests that there may be a common mechanistic linkage to these toxicities.

CARBON DISULFIDE

The major uses of CS_2 are in the production of rayon fiber, cellophane, and CCl_4 and as a solubilizer for waxes and oils. Human exposure is predominantly occupational. Two distinct metabolic pathways for CS_2 exist: (1) the direct interaction of CS_2 with free amine and sulfhydryl groups of amino acids and polypeptides to form dithiocarbamates and trithiocarbonates; and (2) microsomal metabolism of CS_2 to reactive sulfur intermediates capable of covalently binding tissue macromolecules. Conjugation of CS_2 with sulfhydryls of cysteine or glutathione (GSH) results in the formation of 2-thiothiazolidine-4-carboxylic acid (TTCA), which is excreted in urine and has been frequently used as a biomarker of CS_2 exposure.

CS_2 is capable of targeting multiple organ systems including the cardiovascular system, central and peripheral nervous systems, male and female reproductive systems, eyes (retinal angiopathy and impairment of color vision), and ears (hearing loss). CS_2 toxicity requires

frequent and prolonged exposures in occupational settings. The most common neurotoxic effect is a distal sensorimotor neuropathy that preferentially affects long axons in the PNS and CNS (particularly the ascending and descending tracks of the spinal cord and the visual pathways). Encephalopathy with motor and cognitive impairment has also been reported following chronic, low-level exposure to CS_2. These clinical syndromes are associated with CS_2: (1) acute and chronic encephalopathy (often with prominent psychiatric manifestations); (2) polyneuropathy (both peripheral and cranial); (3) Parkinsonism; and (4) asymptomatic CNS and PNS dysfunction. CNS pathology consists of neuronal degeneration throughout the cerebral hemispheres, with maximal diffuse involvement in the frontal regions. Cell loss is also noted in the globus pallidus, putamen and cerebellar cortex, with loss of Purkinje cells. Vascular abnormalities with endothelial proliferation of arterioles may be seen, sometimes associated with focal necrosis or demyelination. PNS changes consist primarily of myelin swelling and fragmentation and large focal axonal swellings, characteristic of distal axonopathy.

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QUESTIONS

1. Which of the following statements regarding solvents is FALSE?
   - **a.** Solvents can be absorbed from the GI tract and through the skin.
   - **b.** Equilibration of absorbed solvents/vapors occurs most quickly in the lungs.
   - **c.** Solvents are small molecules that lack charge.
   - **d.** Volatility of solvents increases with molecular weight.
   - **e.** Most solvents are refined from petroleum.

2. What is the route is in which most solvents enter the environment?
   - **a.** chemical spills.
   - **b.** contamination of drinking water.
   - **c.** evaporation.
   - **d.** improper waste disposal.
   - **e.** wind.

3. All of the following statements are true EXCEPT:
   - **a.** Most solvents can pass freely through membranes by diffusion.
   - **b.** A solvent's lipophilicity is important in determining its rate of dermal absorption.
   - **c.** Hydrophilic solvents have a relatively low blood:air partition coefficient.
   - **d.** Biotransformation of a lipophilic solvent can result in the production of a mutagenic compound.

e. Hepatic first-pass metabolism determines the amount of solvent absorbed in the GI tract.

4. Which of the following statements regarding age solvent toxicity is TRUE?
   a. GI absorption is greater in adults than it is in children.
   b. Polar solvents reach higher blood levels in the elderly than they do in children.
   c. Children are always more susceptible to solvent toxicity than are adults.
   d. Increased alveolar ventilation increases uptake of lipid-soluble solvents to a greater extent than water-soluble solvents.
   e. Increased body fat percentage increases clearance of solvent chemicals.

5. Huffing gasoline can result in which of the following serious health problems?
   a. renal failure.
   b. pneumothorax.
   c. Hodgkin's disease.
   d. encephalopathy.
   e. thrombocytopenia.

6. Which of the following statements regarding benzene is FALSE?
   a. High-level exposure to benzene could result in acute myelogenous leukemia (AML).
   b. Gasoline vapor emissions and auto exhaust are the two main contributors to benzene inhalation.
   c. Benzene is used as an ingredient in unleaded gasoline.
   d. Benzene metabolites covalently bind DNA, RNA, and proteins and interfere with their normal functioning within the cell.
   e. Reactive oxygen species can be derived from benzene.

7. Which of the following is NOT a criterion for fetal alcohol syndrome diagnosis?
   a. maternal alcohol consumption during gestation.
   b. pre- and postnatal growth retardation.
   c. microcephaly.
   d. ocular toxicity.
   e. mental retardation.

8. Which of the following is NOT an important enzyme in ethanol metabolism?
   a. alcohol dehydrogenase.
   b. formaldehyde dehydrogenase.
   c. CYP2E1.
   d. catalase.
   e. acetaldehyde dehydrogenase.

9. Which of the following is NOT associated with glycol ether toxicity?
   a. Irreversible spermatotoxicity.
   b. craniofacial malformations.

c. hematotoxicity.
d. seminiferous tubule atrophy.
e. cleft lip.

10. Which of the following statements regarding chlorinated hydrocarbons is FALSE?
a. Toxicities of trichloroethylene (TCE) are mediated mostly by reactive metabolites, not the parent compound.
b. Glutathione conjugation is an important metabolic step of both trichloroethylene (TCE) and perchloroethylene (PERC).
c. Many chlorinated hydrocarbons are used as degreasing agents.
d. Chloroform interferes with intracellular calcium homeostasis.
e. Carbon tetrachloride causes hepatocellular and kidney toxicity.

CHAPTER 25

Toxic Effects of Radiation and Radioactive Materials

David G. Hoel

KEY POINTS

- The four main types of radiation are due to alpha particles, electrons (negatively charged beta particles or positively charged positrons), gamma rays, and x-rays.
- Alpha particles are helium nuclei (consisting of two protons and two neutrons), with a charge of +2, that are ejected from the nucleus of an atom.
- Beta particle decay occurs when a neutron in the nucleus of an element is effectively transformed into a proton and an electron, which is ejected.
- Gamma-ray emission occurs in combination with alpha, beta, or positron emission or electron capture. Whenever the ejected particle does not utilize all the available energy for decay, the excess energy is released by the nucleus as photon or gamma-ray emission coincident with the ejection of the particle.
- The Compton Effect occurs when a photon scatters at a small angle from its original path with reduced energy because part of the photon energy is transferred to an electron.
- Ionizing radiation loses energy when passing through matter by producing ion pairs (an electron and a positively charged atom residue).
- Radiation may deposit energy directly in DNA (direct effect) or may ionize other molecules closely associated with DNA, hydrogen, or oxygen, to form free radicals that can damage DNA (indirect effect).

INTRODUCTION

Gamma-rays and x-rays are ionizing radiations that have sufficient energy to displace electrons from molecules. These freed electrons then have the capability of damaging other molecules including DNA. The amount of radiation that the public receives has greatly increased due to medical applications, especially the higher doses associated with computed tomography (CT) scans, and from environmental exposure to primarily radon. Figure 25–1 summarizes exposure sources.

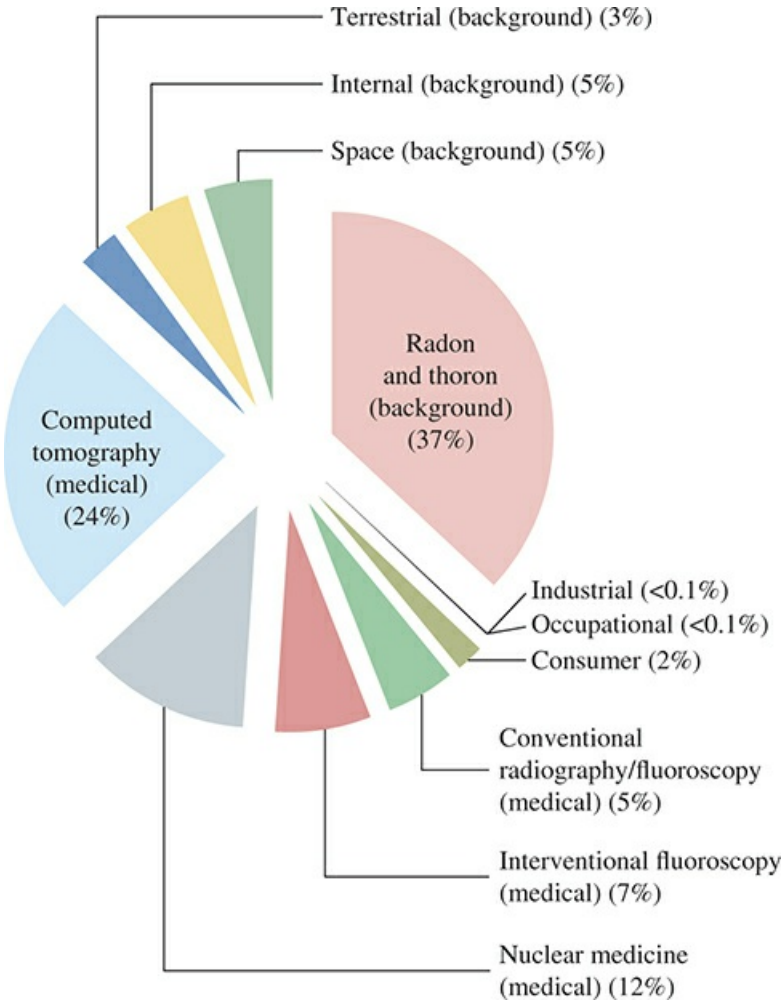

FIGURE 25–1 *Percent contribution of total effective dose to individuals* (*Data from NCRP Report No. 160, Ionizing Radiation Exposure of the Population of the United States*.)

Biological effects of radiation are primarily due to damage to DNA. Atoms of DNA may be directly ionized or indirectly affected by the creation of a free radical that can interact with the DNA molecule. For radiation particles such as neutrons and α particles, the damage is primarily direct, whereas for photons such as x-rays, about two-thirds of the DNA damage in mammalian cells is due to hydroxy radicals. Cancer has been the major adverse health effect of ionizing radiation. More recently, there has been a concern with possible cardiovascular effects,

cataractogenesis, and possibly immunosenescence.

RADIATION BACKGROUND

Types of Ionizing Radiation

When ionizing radiation passes through matter, it has the energy to ionize atoms so that one or more of its electrons can be dislodged and chemical bonds broken. Ionizing radiation is of two types: particulate and electromagnetic waves. Particulate radiation may either be electrically charged (α, β, proton) or have no charge (neutron). Ionizing electromagnetic radiation (photons) in the form of x-rays or gamma-rays has considerably more energy than nonionizing radiation, such as ultraviolet and visible light. Radionuclides (i.e., radioactive atoms), being unstable, release both electromagnetic and particulate radiations during their radioactive decay. The radionuclides decay into either stable elements or through a decay chain of successive radionuclides called decay daughters. The types of radiation emitted, the rate of decay, and the energies of the released radiation are unique to each type of radionuclide. The uranium decay series is illustrated in Fig. 25–2, with specific details in Table 25–1.

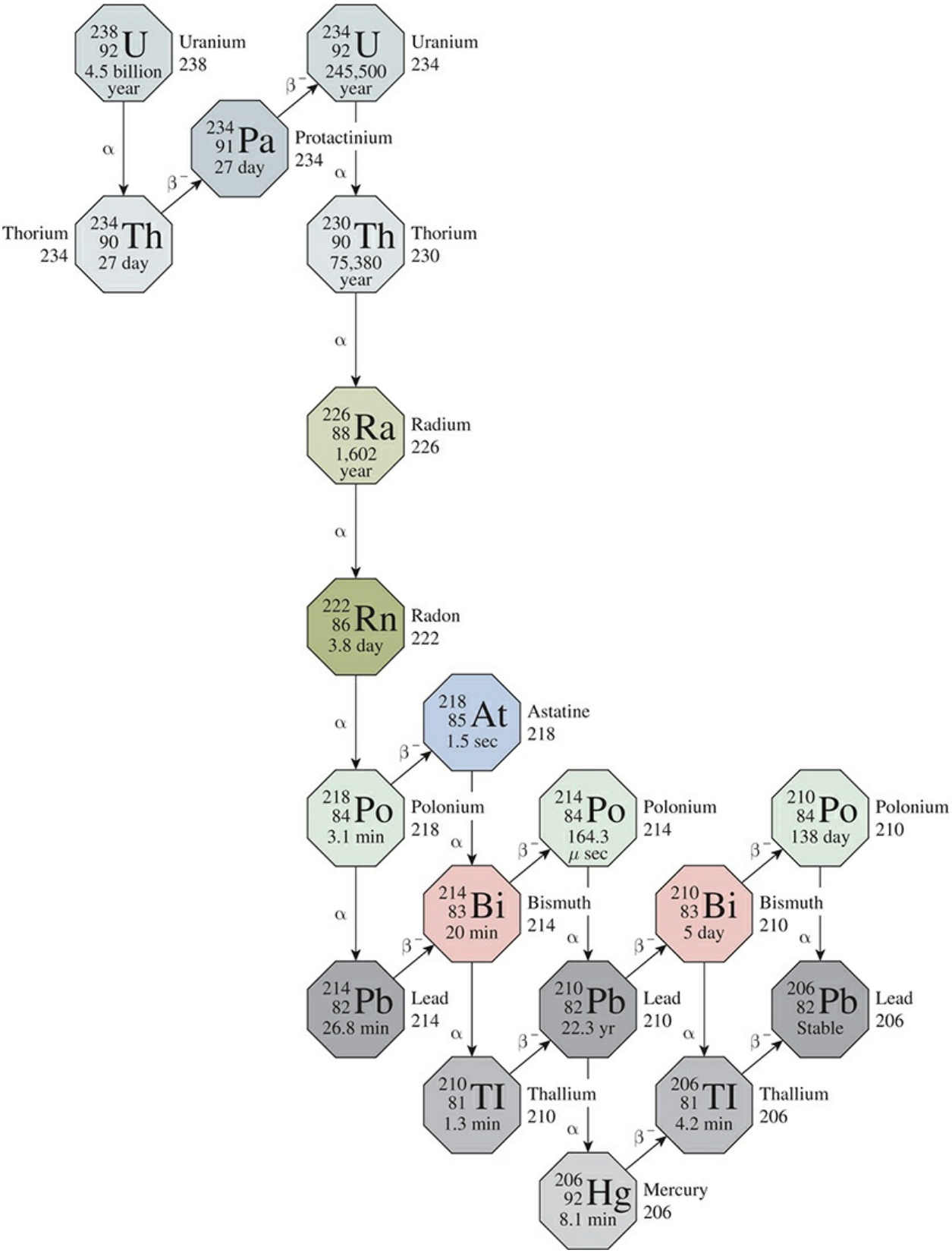

FIGURE 25–2 *Uranium decay chain.*

TABLE 25–1 Radioisotopes in the Uranium Decay Series

Nuclide	Decay Mode	Half-Life (a = Year)	Energy Released (Mev)	Product of Decay
^{238}U	α	4.468×10^9a	4.270	^{234}Th
^{234}Th	β^-	24.10 days	0.273	^{234m}Pa
^{234m}Pa	$\beta-$ 99.84%	1.16 min	2.271	^{234}U
	IT 0.16%		0.074	^{234}Pa
^{234}Pa	β^-	6.70 h	2.197	^{234}U
^{234}U	α	245,500 a	4.859	^{230}Th
^{230}Th	α	75,380 a	4.770	^{226}Ra
^{226}Ra	α	1602 a	4.871	^{222}Rn
^{222}Rn	α	3.8235 days	5.590	^{218}Po
^{218}Po	α 99.98%	3.10 min	6.115	^{214}Pb
	β^- 0.02%		0.265	^{218}At
^{218}At	α 99.90%	1.5 s	6.874	^{214}Bi
	β^- 0.10%		2.883	^{218}Rn
^{218}Rn	α	35 ms	7.263	^{214}Po
^{214}Pb	β^-	26.8 min	1.024	^{214}Bi
^{214}Bi	β^- 99.98%	19.9 min	3.272	^{214}Po
	α 0.02%		5.617	^{210}Tl
^{214}Po	α	0.1643 ms	7.883	^{210}Pb
^{210}Tl	β^-	1.30 min	5.484	^{210}Pb
^{210}Pb	β^-	22.3 a	0.064	^{210}Bi
^{210}Bi	β^-	5.013 days	1.426	^{210}Po
	99.99987%		5.982	^{206}Tl
	α 0.00013%			
^{210}Po	α	138.376 days	5.407	^{206}Pb
^{206}Tl	β^-	4.199 min	1.533	^{206}Pb
^{206}Pb	–	Stable	–	–

The rate of energy dissipation by a single event is referred to as linear energy transfer (LET). The LET of a charged particle is the average energy lost due to interactions per unit length of its trajectory given as kiloelectron volts per micrometer (keV/μm). X-rays, gamma-rays, and β particles of similar energies produce sparse ionization tracks and are classified as low-LET radiation. Particulate radiation (e.g., neutrons and α particles) causes interactions with large amounts of energy being dissipated within short distances. Alpha particles (helium nucleus) released from the nucleus of some radionuclides are slow-moving with a positive charge. Although they cannot penetrate a piece of paper or skin, they are of concern if ingested or inhaled. The most recognized example is the lung cancer risk from the inhalation of radon (Rn 222) and its daughter products.

The rate of decay of a radionuclide is exponential with a decay rate constant λ that is then simply $Ae^{-\lambda t}$ for time *t* and initial quantity of the radionuclide A. The radiological half-life is the time required for the radionuclide to lose 50% of its activity by decay. Each radionuclide has its own unique half-life as illustrated in Table 25–1.

Relative Biological Effectiveness and Quality Factors

Various types of ionizing radiation have similar biological effects because of the ionization of molecules. Without knowing the type of radiation, one cannot specify how much radiation is needed to produce a specific biological effect. This is because a given absorbed dose (energy per unit mass) of x-rays does not have the same biological effect as an identical dose of neutrons. The relative effectiveness of different types of radiation in producing biological changes depends on deposition of energy. The relative biological effectiveness (RBE) is numerically equal to the inverse of the ratio of absorbed doses of the two radiations required to produce equal biological effects. The difficulty is that the RBE may differ depending on the biological endpoint and it may also be dose-dependent.

Units of Radiation Activity and Dose

The basic unit of radiation activity is the Becquerel (Bq), which is nuclear disintegrations per second. The older unit of activity is the Curie (Ci), which corresponds to the number of disintegrations in 1 second from 1 g of radium 226 or 1 Ci = 3.7×10^{10} decays per second; thus, 1 Bq = 2.7×10^{-11}Ci.

The basic unit of dose is the Gray (Gy), which is the amount of energy released in a given mass of tissue. One Gray is defined as 1 joule of energy released in 1 kg of tissue. Another common measure is the Sievert (Sv), which is a dose equivalent; that is, the dose in Gray multiplied by the appropriate quality factor. The roentgen (R) quantifies exposure and is the number of ionizations in a cubic meter of air produced by x-rays or gamma radiation. For cumulative exposure, a working level month (WLM) is used, which is the exposure to 1 WL for 1 working month of 170 hours.

RADIOBIOLOGY

Radiation biology has made significant progress in our understanding of radiation effects at low

doses. Radiation cancer risk extrapolations make two assumptions, namely that the basic mode of action is linearly related to dose and that the individual cell is the unit of risk. However, effects occurring in nontargeted cells, such as with induced genomic instability and bystander effects, suggest that responses can occur nonuniformly over time at the tissue level. Following irradiation, various protective cellular processes occur that depend on the degree of damage and the tissue type. These mechanisms include DNA repair, intracellular metabolic oxidation/reduction reactions, cell cycle checkpoint controls, cellular signaling, senescence, and apoptosis. After an exposure to 5 mGy of low-LET radiation (average background per year), each cell nucleus is on an average hit by one electron, resulting in 5 to 10 damaged bases, 2.5 to 5 single-strand breaks and 0.25 double-strand breaks.

Nontargeted Radiation Effects

Exposure to ionizing radiation can result in direct damage to the irradiated cells as well as produce effects in cells that were not irradiated (bystander effects). These nontargeted effects can occur in the nonirradiated neighbors of irradiated cells and at sites distant from the irradiated cells. Effects can also be observed in the progeny of an irradiated cell (genomic instability). Both targeted and nontargeted effects can result in DNA mutations, gene amplifications, chromosomal rearrangements, carcinogenesis, and cell death.

Bystander Effects—Radiation-induced bystander effects occur when cells that have not been directly exposed to ionizing radiation react as though they have been exposed by receiving a biochemical signal from a radiation-exposed cell. That is, they show chromosomal instability and other abnormalities, or die. For high-LET radiation, a bystander effect has been shown for inducing cell lethality, chromosome aberrations, sister-chromatid exchanges, mutations, genomic instability, altered signal transduction pathways, and in vitro transformations. For low-LET radiation, the bystander effect has been limited to cell lethality and lethal mutations.

These bystander cells can be either adjacent or at some distance from the radiation-exposed cell. Not all cells can produce bystander signals or react to them. The important issue from a risk assessment view is whether bystander effects are beneficial (e.g., adaptive response and apoptosis [removal of damaged cells]) or detrimental to the nonexposed cells, and what impact they may have on the dose response at low doses. It should be noted that most observed effects are detrimental, but beneficial effects are more difficult to measure. However, bystander effects demonstrate that the organism and tissues communicate and are responding as an organized structure to radiation insult. Large DNA deletions are the major type of radiation-induced mutations. In bystander cells, however, mutations are similar to those that occur spontaneously, with the majority being point mutations.

Genomic Instability—Genomic instability has been defined as the increase in rate of acquiring genetic change. Induced genomic instability can be observed in the progeny of irradiated cells and can persist for many generations. When a cell is saturated in repairing radiation damage, it may change its gene-product profile without any specific genetic damage. This has been suggested as a cause of genomic instability, which is an anti-inflammatory response, and is a risk for malignancy. It is hypothetized that genomic instability could be linked to the loss of telomere maintenance and too short telomeres as a mechanism in cancer development.

Adaptive Response—In cells that are exposed to a low priming dose of radiation (e.g., 10 to 20 mGy) followed in a short time interval with a larger challenge dose (e.g., 1 Gy), the frequency of chromosomal aberrations induced by the challenge dose was found to be less than that from the challenge dose given alone. This effect is referred to as "adaptive response." Also, the normal rate of cell transformation and chromosome damage can be decreased to below the normal background level after an initial low-dose radiation exposure. Adaptive responses have been observed both in vitro and in vivo for both cancer and genetic effects, which suggest that low doses may decrease radiation risk. These adaptive responses that enhance normal repair or protective processes may possibly decrease the risk for low-dose radiation-induced cancer.

Hormesis

Hormesis as "the stimulating effect of small doses of substances which in larger doses are inhibitory" probably is not applicable. Available evidence does not support the notion that low doses of ionizing radiation have a beneficial effect, and the possible mechanisms remain obscure.

Gene Expression

Gene expression profiling for monitoring ionizing radiation exposure has shown that dose, dose rate, radiation quality, and time since exposure result in variations in gene responses, allowing gene expression signatures potentially to be markers of radiation exposure. Changes in gene expression in human cell lines occur after as little as 0.02 Gy gamma-rays.

Summary

There is a focus on mechanisms and dose–response as they relate to the induction of chromosomal aberrations and gene mutations because cancer is believed to be associated with these cellular responses. Experimental data indicate that the dose–response relationship over a range of 20 to 100 mGy is most likely to be linear, and not significantly affected by either an adaptive or a bystander effect. The future of understanding low-dose radiation cancer risks will depend on continued advancement of gene expression analysis and computational biology.

CANCER EPIDEMIOLOGY

Epidemiological studies have been extensive and provide the basis for our understanding of radiation-induced cancer effects. Radiation cancer studies are no different from other types of occupational and environmental cancer studies in that radiation-induced cancers are not distinguishable pathologically, and there are the usual issues of exposure confounding, long latencies (e.g., 10 to 20 years for solid tumors), levels of exposure, and study size. For acute exposures, only epidemiological studies with exposures to relatively high doses of radiation (greater than 0.15 Sv) have shown an excess of cancer. Because of these difficulties, the most informative studies involve a large number of individuals with large radiation doses and follow-up of several decades. Table 25–2 lists cancer sites for gamma-ray and x-ray exposures.

TABLE 25–2 IARC: Tumor Sites with Sufficient Evidence of Human Carcinogenicity

Radiation Type	Major Study Populations	Tumor Sites (And Types) On Which Sufficient Evidence Is Based
α-Particle and β-particle emitters		
Radon-222 and decay products	General population (residential exposure), underground miners	Lung
Radium-224 and decay products	Medical patients	Bone
Radium-226, radium-228, and decay products	Radium-dial painters	Bone, paranasal sinus, and mastoid process (radium-226 only)
Thorium-232 and decay products	Medical patients	Liver, extrahepatic bile ducts, gall bladder, and leukemia (excluding CLL)
Plutonium	Plutonium-production workers	Lung, liver, and bone
Phosphorus-32	Medical patients	Acute leukemia
Fission products, including strontium-90	General population, following nuclear reactor accident	Solid cancers and leukemia
Radioiodines, including iodine-131	Children and adolescents, following nuclear reactor accident	Thyroid
X radiation or gamma radiation	Atomic-bomb survivors, medical patients; in utero exposure (offspring of pregnant medical patients and of atomic-bomb survivors)	Salivary gland, esophagus, stomach, colon, lung, bone, skin (BCC), female breast, urinary bladder, brain and CNS, leukemia (excluding CLL), thyroid, kidney (atomic-bomb survivors, medical patients); multiple sites (in utero exposure)
Solar radiation	General population	Skin (BCC, SCC, and melanoma)
UV-emitting tanning devices	General population	Skin (melanoma) and eye (melanoma, particularly choroid and ciliary body)

Abbreviations: BCC, basal cell carcinoma; CLL, chronic lymphocytic leukemia; CNS, central nervous system; SCC, squamous cell carcinoma.

A-bomb Survivor Studies

The Radiation Effects Research Foundation (RERF) has reported a series of mortality studies on a fixed population of A-bomb survivors. Members of the study are given physical examinations every 2 years, and this provides much clinical data as well as biological samples. The problem with the cohort is that the results are extrapolated from a group of Japanese who survived the bombing and have different background cancer rates than other populations. The results of the mortality data show that for total solid cancers, the excess relative risk decreases with attained age and age at exposure. For total solid tumor incidence, the estimated excess relative risk per Gy is 0.47 (0.40 to 0.54).

Occupational Studies

Nuclear Worker Studies—There have been numerous studies over the years among nuclear workers, primarily at governmental facilities. In most of these studies, mortality rates were compared with those in the general population. Interestingly, the cancer mortality rates were less than those for the general public, which may be due to the healthy worker effect and differences between nuclear workers and the public. Workers at the Russian nuclear facility at Mayak generally experienced very high doses from both internal (plutonium, α particles) and external radiation exposures. High levels of body burdens of plutonium greater than 7.4 kBq were found to have a relative risk of liver and bone cancer. To address the problem of varied results, several multinational studies were initiated: a nested case–control study of workers at the Portsmouth Naval Shipyard; an analysis of seven nuclear facilities in Canada, the United Kingdom, and the United States; a 15-country study; and a newer study of 308,297 nuclear workers in France, the United Kingdom, and the United States. Because the results are variable, it is difficult to use epidemiological data to estimate low-dose radiation risks.

Medical Radiation Workers—It is estimated that there are 2.3 million medical radiation workers worldwide. Radiologists and radiological technologists have been studied epidemiologically for many years. These workers were some of the earliest exposed to radiation with the first finding in 1902 that radiation can cause skin cancer. It was recognized later that workers had increased rates of leukemia, breast cancer, and non-chronic lymphocytic leukemias (non-CLLs).

Chernobyl Cleanup Workers—The Chernobyl cleanup workers had higher exposures compared with other nuclear workers. The excess relative risk for solid tumors was significant (ERR = 1.52 per Gy) and the increase was observed in the highest dose interval (mean 200 mSv) with no increase in the lower-dose interval (mean 100 mSv). Besides solid tumors, a significant increase in leukemia incidence was observed for those with exposures greater than 0.15 Gy, compared with workers with lower exposures. Also, increases in the incidence of cardiovascular disease were observed among those at higher exposures. Other than for leukemias, the follow-up period has been relatively short and it is thus too soon to conclude much about solid cancer risks.

Nonoccupationally Exposed Groups

High Natural Background Radiation Areas—Cancer mortality was analyzed in the population

living in the high background radiation areas in Yangjiang, China. Comparing individuals in the high background area to those in a control area, the relative risk was 0.99 (0.87 to 1.14) for total cancer mortality. Dividing the high background area into low, medium, and high radiation exposures, the relative risks for nonleukemia cancer mortality actually decreased with dose (1.07, 0.98 and 0.91, respectively). To evaluate the dose reconstruction, the correlation between estimated radiation exposure and frequency of dicentric and ring chromosomes, which are recognized as a good biomarker of radiation exposure, was determined. For those in the high radiation background area, the incidence of these markers agrees with what has been observed in other studies of radiation exposures and chromosome aberrations. This result provides some evidence in support of the program's exposure estimates. In conclusion, the high background Chinese studies have not shown an increase in cancer incidence at low dose and dose rate exposures.

The coastal area of Kerala, India, is a region of high natural radiation background that has been well studied. A cohort of 70,000 individuals (age 30 to 84 years) were followed for 10.5 years, which resulted in 1379 cancer cases including 30 leukemias. The researchers also obtained individual data on smoking, education, occupation, and other possible cancer risk factors for their analysis. For total cancers and for leukemia, there were no increases observed among any of the dose intervals compared to the lowest dose interval.

A study of childhood cancers in relation to natural background radiation in Great Britain using the National Registry of Childhood Tumours involved over 27,000 cancer cases and matched controls with radiation exposures estimated on the basis of the mother's residence at the time of birth. The mean cumulative dose from birth until diagnosis was 4 mSv. The study found a significant increase in leukemias with an estimated ERR of 12% (95% CI 3 to 22) per mSv of gamma exposure to the red marrow. Other cancers were not significantly increased, and radon was not a significant risk factor.

Semipalatinsk Fallout-Related Exposures—The Semipalatinsk area of Kazakhstan has been studied for possible cancer effects resulting from radiation exposures from local fallout of the Soviet atomic nuclear weapons testing program. Six rural districts that had measurable levels of exposure were compared with six villages that were several hundred kilometers east of the nuclear test site. Follow-up of the populations began 5 to 10 years after the nuclear testing. Using soil samples taken in 1963 of strontium and cesium as well as information on the types of weapons, a dose reconstruction was developed for external and internal exposures.

For the purpose of analysis, the approximate 10,000 individuals in the exposed area were matched with a group of 10,000 in the distant comparison area. Cancer rates were considerably higher in the exposed groups of individuals, which resulted in significant dose–response relationships for most solid tumors. However, there appeared to be an undefined bias in the distant control group since the rate ratios increased relatively little among the exposure categories.

Other Nonoccupational Studies

Buildings with Cobalt 60—A group of 7271 individuals who resided in buildings with high levels of Cobalt 60–contaminated steel in Taiwan had an average cumulative exposure of 50 mSv. After a follow-up period of 16 years, a total of 141 cancer cases were identified. For total solid cancers (82 cases) compared to the national rates, the total cancers were less than expected, but thyroid cancer (7 cases) and non-Hodgkin lymphoma (5 cases) were significantly increased.

This is a small study that shows little effect of continuous exposure. It is also difficult to interpret because the exposed population was likely of a higher social economic status (SES) than the general comparison population.

***Populations Residing Near Nuclear Facilities*—**Ecological cancer studies have been carried out in the vicinity of nuclear power plants in many countries. There has often been reported an unexplained increase in childhood leukemia. A childhood cancer case–control study in the areas in the vicinity of 16 German nuclear power plants noted 1592 cancer cases with 593 cases of leukemia among children under the age of 5 during the period 1983 to 2003. Children within 5 km of a nuclear facility had a significant increase in leukemias, but not for other cancers (37 leukemia cases and 54 controls). Possible confounders were not measured. Radiation exposures near these German nuclear power plants were less than the natural background radiation.

In France a study of childhood leukemia among children living in the vicinity of the 29 French nuclear sites reported 670 cases, with 729 cases expected from national rates. For those within 5 km of a facility, there were 65 leukemia cases, with 75 expected, and for children less than 5 years of age, there were 39 leukemia cases with 40 cases expected.

Radionuclides

Radon—Radon is a natural radioactive gas produced by the decay of uranium and thorium. Originally, exposures to radon and its daughter radionuclides among uranium miners and some other groups of miners established that high exposures were a clear risk for lung cancer. The lung cancer risk was also significantly increased when the cases were restricted to exposures less than 200 Bq/m^3. The lung cancer effects were also consistent, with the risks projected downward from the higher exposed uranium miners.

Radium—There are 25 isotopes of radium of which 4 occur naturally (radium 223, 224, 226, and 228); the others are man-made or decay products of man-made radionuclides. Radium 226 with a half-life of 1601 years is by far the common natural form, followed by 228 with a half-life of 5.75 years. Radium 223 and 224 have half-lives of only a few days. Except for radium 228, which is a β emitter, the other three are all α emitters. The different isotopes have been used both occupationally as luminescent paint on watches and instruments (radium 226 and 228) and in medical applications (radium 223 and 224). These uses, as well as radium found environmentally in drinking water, have provided material for many epidemiological studies. Beginning in the 1920s, young women worked painting the dials of watches with paint containing radium 226 and 228. Many of them “pointed” the tips of their paintbrushes by mouth resulting in ingestion of relatively large amounts of radium for some of the women. Radium as a bone seeker resulted in increases in bone cancer as well as paranasal sinus cancers.

Bone sarcomas were also the major cancer effect among patients with tuberculosis and ankylosing spondylitis who were treated with high doses of radium 224 (mean bone surface dose of 30 Gy) in two cohort studies in Germany. There were increases in bone cancer in both studies, but there were also some increases in other cancer sites.

Plutonium—Plutonium is used for nuclear weapons production, and in the production of mixed oxide fuels. Most of the exposure to plutonium is to workers involved in nuclear weapons (Pu 239) and in nuclear power generation (Pu 238). The major exposure to plutonium is by inhalation

and it is retained primarily in the lung, liver, and bone. Dose–response relationships have been shown for cancers of the lung, liver, and bone over a wide range of doses.

Radioiodine—Releases from nuclear facilities of fission-product radionuclides deposited in the environment as well as internal doses from the ingestion of foods containing fission products have resulted from the Chernobyl and Fukushima accidents. The major observable health effect has been childhood thyroid cancer resulting from the β emitter Iodine 131, which has a half-life of only 8 days. From studies of external radiation exposures in the A-bomb survivors as well as the children who were treated by radiation for tinea capitis (ring worm present on the scalp), it is clear that radiation is a risk for thyroid cancer for exposures to adolescents. The risk of radiation-related thyroid cancer was three times higher in iodine-deficient areas and the use of potassium iodide as a supplement reduced this risk of radiation-related thyroid cancer by a factor of 3.

NONCANCER EPIDEMIOLOGY

Cardiovascular Disease

Cardiovascular disease (CVD) mortality may have a lower relative risk from radiation exposure than solid tumors, but CVD accounts for more total background deaths. Atherosclerosis is an inflammatory disease of the arteries, which can lead to ischemia of the heart. In a study of inflammatory biomarkers (TNF-α, IL-10, IgM, IgA, and IFN-γ) as well as erythrocyte sedimentations rates among A-bomb survivors, it was shown that these markers were associated with radiation exposure.

Cataracts

Cataracts were one of the earliest radiation-associated effects found after the discovery of x-rays. It has long been believed that it results from only high doses of radiation to the lens of the eye. There are basically three types of cataracts: nuclear or nuclear sclerosis, cortical, and posterior-subcapsular (PS) cataracts. Each of these clinical types has its known risk factors such as cigarette smoking for nuclear and possibly PS cataracts, while UV-B is a risk factor for cortical cataracts. Ionizing radiation is a risk factor for both cortical and PS cataracts but not nuclear sclerotic cataracts. Recent studies indicate that radiation exposures to the lens confer the risk of opacities at doses well under 1 Sv. There is limited evidence that those exposed at a younger age are at greater risk.

Mental Effects

In the A-bomb survivor analyses, significant effects on the developing brain were observed among those exposed during the period of the 8th week through 25th week of gestation. During the most sensitive period of 8 to 15 weeks, there was an increased frequency of severe mental retardation, a diminution in IQ scores and school performances, as well as an increase in the occurrence of seizures. During this sensitive period, there is a rapid increase in the number of neurons; they migrate to the cerebral cortex where they lose their capacity to divide, becoming

perennial cells.

DISCUSSION

The major issue in radiation health effects is the causation of cancer. We now see noncancer effects such as CVD at low dose-rate exposures that occur at environmental levels and in diagnostic medical screening. The Linear No Threshold (LNT) model is used to estimate these effects well below what can be observed in epidemiological studies. The simple defense of the LNT model is that the physical energy deposition of ionizing radiation increases cancer risk linearly with increasing dose, and the carcinogenic effectiveness is constant, independent of dose. It is recognized that a cell is not passively affected by the accumulation of lesions induced by ionizing radiation. The cell reacts through at least three main mechanisms: first by reacting against radiation-induced ROS, second by eliminating damaged cells by either apoptosis or through cell death during mitosis of unrepaired cells, and third by immunosurveillance systems that eliminate clones of transformed cells.

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QUESTIONS

1. Which of the following is NOT a main type of radiation?
   - **a.** alpha particles.
   - **b.** microwaves.
   - **c.** beta particles.
   - **d.** gamma rays.
   - **e.** x-rays.

2. Which of the following statements regarding alpha particles is FALSE?
   - **a.** Alpha particles are ejected from the nucleus of an atom.
   - **b.** The atomic number decreases by two after emission of an alpha particle.
   - **c.** The atomic weight decreases by two after emission of an alpha particle.
   - **d.** Energies of most alpha particles range between 4 and 8 MeV.
   - **e.** Alpha particles are helium nuclei.

3. Which of the following types of radiation is likely the MOST energetic?

a. alpha particles.
b. beta particles.
c. positron emission.
d. electron capture.
e. photon emission.

4. Pair production and the Compton effect characterize which type of radiation's interaction with matter?
a. alpha particles.
b. beta particles.
c. positron emission.
d. electron capture.
e. photon emission.

5. Which of the following statements regarding radiation DNA damage is FALSE?
a. Ionizing radiation slows down by forming ion pairs.
b. A main form of radiation DNA damage occurs by the production of free radicals.
c. High-LET radiation causes more ionizations than does low-LET radiation.
d. Most DNA damage caused by radiation happens directly.
e. Direct and indirect ionization cause similar damage to DNA.

6. Low-LET radiation:
a. causes large-scale ionizations throughout the cell.
b. results from alpha particle emission.
c. causes damage that is readily repaired by cellular enzymes.
d. is also known as densely ionizing radiation.
e. usually causes irreparable cell damage.

7. What is the most common type of DNA damage caused by low-LET radiation exposure?
a. base damage.
b. DNA protein cross-links.
c. single-strand breaks.
d. double-strand breaks.
e. thymine-dimer formation.

8. Which of the following statements regarding radon exposure is FALSE?
a. Miners are exposed to increased environmental radon levels.
b. Radon exposure has been linked to the development of lung cancer.
c. Smokers are at a higher risk from radon exposure.
d. Radon levels are relatively higher in urban areas than in rural areas.
e. The use of open flames indoors increases radon exposure.

9. The largest dose of radiation is received from which of the following sources?
a. inhalation.

b. in body.
c. cosmic.
d. cosmogenic.
e. terrestrial.

10. The largest contributor to the effective dose of radiation in the U.S. population is which of the following?
a. nuclear medicine.
b. medical x-rays.
c. terrestrial.
d. internal.
e. radon.

CHAPTER 26

Toxic Effects of Plants and Animals

John B. Watkins, III

CONCLUSION

KEY POINTS

- Different portions of the plant (root, stem, leaves, seeds) often contain different concentrations of a toxic substance.
- The age of a plant contributes to variability. Young plants may contain more or less of some constituents than mature plants.
- Climate and soil influence the synthesis of some toxins.
- Plants contain substances that may exert toxic effects on skin, lung, cardiovascular system, liver, kidney, bladder, blood, nervous system, bone, and the endocrine and reproductive systems.
- Contact dermatitis and photosensitivity are common skin reactions with many plants.
- Gastrointestinal effects range from local irritation to emesis and/or diarrhea.
- Cardiac glycosides in plants may cause nausea, vomiting, and cardiac arrhythmias in animals and humans.
- Venomous animals produce poison in a highly developed secretory gland or group of cells and can deliver their toxin during a biting or stinging act.
- Poisonous animals are those whose tissues, either in part or in their entirety, are toxic. Poisoning usually takes place through ingestion.
- The bioavailability of a venom is determined by its composition, molecular size, amount or concentration gradient, solubility, degree of ionization, and the rate of blood flow into that tissue as well as the properties of the engulfing surface itself.
- The distribution of most venom fractions is rather unequal, being affected by protein binding, variations in pH, and membrane permeability, among other factors.
- A venom may also be metabolized in several or many different tissues.
- Because of their protein composition, many toxins produce an antibody response, which is essential in producing antisera.

INTRODUCTION

The earliest humans used plant extracts and animal venoms for hunting, war, assassination, and political intrigue for millennia. The toxic properties of plants and animals often enhance their ability to survive. Some toxic compounds are used primarily to aid an animal in obtaining food, whereas plants have developed toxic properties to specifically ward off being used as food. Toxins have been utilized as tools to study human biochemistry and physiology in order to pave the way for new pharmaceuticals. In fact, there is considerable effort to harness the natural pharmacopeia for clinical use.

INTRODUCTION TO PLANT TOXICITIES

The plant kingdom contains potentially 300,000 species, and the toxic effects of plants serve primarily as defense mechanisms against natural predators. Toxic effects on humans can range from simple hay fever caused by exposure to plant pollen all the way to serious systemic reactions caused by ingestion of specific plants. Table 26–1 lists some of the poisoning syndromes that plants can produce.

TABLE 26–1 Poisoning Syndromes Caused by Plants

Syndrome	Genera	Mechanism(S)
Antimuscarinic	*Atropa, Datura, Hyoscyamus, Solanum*	Blockade of muscarinic cholinoceptors
Cardiotoxic	*Adenium, Digitalis, Convallaria, Nerium*	Inhibition of cellular Na^+, K^+-ATPase increases contractility, enhanced vagal effect
Convulsants	*Anemone, Conium, Laburnum, Nicotiana, Ranunculus*	Blockade of gamma-aminobutyric acid (GABA) receptor on the neuronal chloride channel, alteration of acetylcholine homeostasis, mimic excitatory amino acids, sodium channel alteration, hypoglycemia
Cyanogenic	*Eriobotrya, Hydrangea, Prunus*	Gastric acid hydrolysis of cyanogenic glycosides releases cyanide
Dysrhythmia	*Aconitum, Rhododendron, Veratrum*	Sodium channel activation
Nicotinic	*Conium, Laburnum, Lobelia, Nicotinia*	Stimulation of nicotinic cholinoceptors
Pyrrolizidine	*Crotalaria, Heliotropium, Senecia*	Pyrroles injure endothelium of hepatic or pulmonary vasculature leading to veno-occlusive disease and hepatic necrosis
Toxalbumin	*Abrus, Ricinus*	Protein synthesis inhibitors leading to multiple organ system failure

Many variables can affect the concentration of a plant's toxin and can be a major factor in the severity of reaction one will experience on exposure. These factors include what part of the plant exposure is from, the age of the plant, amount of sunlight and soil quality that the plant has grown in, and genetic differences within a species. Also, plant toxins have numerous different chemical structures, which is useful in understanding related toxins. Table 26–2 lists some of the common classifications.

TABLE 26–2 Chemical Classification of Plant Toxins

Chemical Category	Genera	Examples
Alkaloids	*Atropa, Senecio, Nicotinia, Coffea, Papaver, Solanum, Acotinum*	Tropines, pyrrolizidines, pyridines, purines, isoquinolines, steroids, diterpines
Glycosides	*Digitalis, Aesculus*	Steroids, coumarins
Proteinaceous compounds	*Abrus, Amanitin, Lathyrus*	Toxalbumins (abrin, ricin), polypeptides (amatoxins, phallotoxins, phalloidin), amines (aminopropionitrile)
Organic acids	*Caladium, Dieffenbachia, Rheum*	Oxalates
Alcohols	*Cicuta, Eupatorium*	Cicutoxin, tremetol
Resins and resinoids	*Cannabis, Rhus*	Tetrahydrocannabinol, urushiol

TOXIC EFFECTS BY ORGAN

Skin

Irritant Contact Dermatitis—Plants causing irritation of the skin on contact are rather common (Table 26–3). The trichomes, or barb-like hairs (Fig. 26–1), found on stinging nettles (*Urtica* species, Urticaceae) puncture skin on contact and release an irritating sap containing a mixture of formic acid, histamine, acetylcholine, and serotonin. *Mucuna pruriens* (cowhage), which also deploys its toxin via barbed trichomes on contact, may cause pain, itching, erythema, and vesication. Mucinain, contained in the toxin, is the proteinase responsible for causing pruritus.

TABLE 26–3 Selective Plants Producing Contact Dermatitis

Botanical Family	Genus Species	Common Name
Amaryllidaceae	*Narcissus*	Narcissus
Apocynaceae	*Nerium oleander*	Oleander
Bromeliaceae	*Ananas comosus*	Pineapple
Asteraceae	*Ambrosia, Aster, Chrysanthemum, Rudbeckia hirta, Tagetesminuta*	Ragweed, aster, chrysanthemum, black-eyed Susan Mexican marigold
Euphorbiaceae	*Ricinus communis*	Castor bean
Fumariaceae	*Dicentra spectabilis*	Bleeding heart
Ginkgoaceae	*Ginkgo biloba*	Ginkgo
Liliaceae	*Allium cepa*	Onion
Myrtaceae	*Eucalyptus globulus*	Eucalyptus
Pinaceae	*Abies balsamea*	Balsam fir
Saxifragaceae	*Hydrangea*	Hydrangea
Solanaceae	*Lycopersicon esculentum, Solanum carolinense, S. turerosum*	Tomato, horse nettle, potato
Umbelliferae	*Daucus carota, Heracleum lanatum*	Carrot, cow parsnip
Urticaceae	*Urtica dioica, U. urens*	Stinging nettle

FIGURE 26–1 *Stinging hairs of Urtica ferox (nettles).*

Allergic Contact Dermatitis—Many people have experienced allergic dermatitis most frequently from contact with poison ivy. Allergic dermatitis is an actual allergic reaction occurring within the skin as opposed to just a response to the presence of an irritant. Due to this immunological component, the severity of the reaction can range widely.

Philodendron scandens (Araceae, arum family) and the toxicodendron group of plants, which contains *Rhus radicans* (poison ivy; Fig. 26–2), *Rhus diversiloba* (poison oak), and *Rhus vernix* (poison sumac), are all known to cause allergic dermatitis. In the *Rhus* species, the allergen is a fat-soluble substance called urushiol that can penetrate the stratum corneum and then bind Langerhans cells in the epidermis. These haptenated cells then migrate to lymph nodes, where T cell activation results in the allergic response.

FIGURE 26–2 *Toxicodendron radicans (poison ivy).*

Photosensitivity—Dermatitis does not necessarily have to be caused by skin contact. Consumption of *Hyperi-cum perforatum* (St. John's wort) by animals can lead to serious dermatitis and even may be life threatening. Once ingested and dispersed systemically, the toxin hypericin (a bianthraquinone) causes photosensitization of the animal's skin. On exposure to sunlight, edematous lesions form on areas of skin that are not protected by hair such as the nose and ears.

Respiratory Tract

Allergic Rhinitis—Rhinitis from inhalation of plant pollens is a seasonal problem for many individuals. Trees, grasses, and weeds are all responsible to contributing to airborne pollen. Grass species *Poa* and *Festuca* are major contributors along with pollen from several weed genera in the Asteraceae (e.g., mugwort, *Artemisia vulgaris*, in Europe, and ragweed, *Ambrosia* sp., in North America).

Cough Reflex—Workers who process peppers have a significantly increased incidence of coughing when specifically handling *Capsicum annuum* (sweet pepper) and *Capsicum frutescens* (red pepper). These two types of peppers produce the major irritants capsaicin (*trans*-8-methyl-*N*-vanillyl-6-nonenamide) and dihydrocapsaicin. Specific nerves in the airway have been found to be capsaicin-sensitive, which leads to the irritation and cough.

Gastrointestinal System

Direct Irritant Effects—Ingestion of a toxic plant can cause irritation of the gastrointestinal tract, often resulting in nausea, vomiting, and diarrhea (Table 26–4).

TABLE 26–4 Selective Plants Causing Gastrointestinal Irritation

Common Name	Scientific Name	Toxic Part	Toxin
Amaryllis	*Hippeastrum equestre*	Bulb	Lycorine
Barberry	*Berberis vulgaris*	Root	Protoberberine and other isoquinoline alkaloids
Boxwood	*Buxus* sp.	Leaves, stems	Steroidal alkaloids
Buttercup	*Ranunculus* sp.	All parts	Ranunculin, protoanemonin
Crown of thorns	*Euphorbia milii*	All parts	Resiniferatoxin
Daffodil	*Narcissus* sp.	All, especially bulb	Lycorine, narcissin, phenanthridine alkaloids
English Ivy	*Hedera helix*	All parts	Hederin from hederagenin
Euonymus	*Euonymus* sp.	All parts	Alkaloids
Hyacinth	*Hyacinthus orientalis*	Bulb	Calcium oxalate, lycorine
Iris	*Iris*	Bulb	Irritant resin
Mayapple	*Podophyllum peltatum*	Green fruit, roots	Podophyllotoxin
Mistletoe	*Phoradendron flavescens*	Berries, other parts	Phoratoxin
Pokeweed	*Phytolacca americana*	All parts	Phytolaccatoxin, related triterpines
Purging nut	*Jatropha curcas*	Seeds	Jatrophin (curcin) (toxalbumin)
Tung nut	*Aleurites fordii*	Nut	Derivative of phorbol, saponins, toxalbumins
Wisteria	*Wisteria sinensis*	Pods	Wistarine (glycoside)

Toxic quinolizidine alkaloids are found in buffalo beans. Ingestion by children causes nausea, vomiting, dizziness, and abdominal discomfort. In addition, consumption by livestock of the mature plant with seeds has been reported to be fatal. Nuts from *Aesculus hippocastanum* (horse chestnut) and *Aesculus glabra* (Ohio buckeye) contain a glucoside called esculin. Ingestion by humans causes gastroenteritis, which increases in severity with the number of nuts consumed.

Antimitotic Effects—The foliage and roots of *Podophyllum peltatum* (May apple, Berberidaceae) contain podophyllotoxin. Low doses induce mild purgation; however, overdose results in nausea and severe paroxysmal vomiting. By binding microtubules, podophyllotoxin blocks mitosis from proceeding, making podophyllotoxin valuable for the treatment of cancer.

Found in the bulbs of *Colchicum autumnale* (autumn crocus, Liliaceae), colchicine blocks the formation of microtubules, ultimately preventing successful mitosis. Ingestion of these bulbs causes severe gastroenteritis (nausea, vomiting, diarrhea, and dehydration). Severe poisoning can result in confusion, hematuria, neuropathy, renal failure, and cardiotoxicity.

Protein Synthesis Inhibition—The castor bean (*Ricinus communis*) is an ornamental plant that produces seeds that, if eaten by children or adults, causes no symptoms of poisoning for several days after ingestion. Gradually, gastroenteritis develops, resulting in some loss of appetite, with nausea, vomiting, and diarrhea. With a fatal dose, the gastroenteritis becomes extremely severe and is marked by persistent vomiting, bloody diarrhea, and icterus followed by death within 6 to 8 days. The beans contain two lectins: ricin I and ricin II of which ricin II is more toxic. Ricin II is made up of an A-chain and a B-chain. The B-chain binds terminal galactose residues on cell surfaces allowing the A-chain to be endocytosed. Once inside, the A-chain inactivates the 60S ribosomal subunit of cells by catalytic depurination of an adenosine residue within the 28S rRNA, thereby blocking protein synthesis.

Cardiovascular System

Cardioactive Glycosides—The best known cardioactive glycoside is *Digitalis purpurea* (foxglove, Scrophulariaceae; Fig. 26–3). However, squill (*Scilla maritima*), which contains scillaren, lily of the valley (*Convallaria majalis*), which contains convallatoxin in the bulbs, milkweeds (*Asclepias* spp., Asclepiadaceae) and other plants contain cardiotoxins (Table 26–5). The cardiac glycosides inhibit Na^+,K^+-ATPase.

FIGURE 26–3 *Digitalis purpurea (common foxglove).*

TABLE 26–5 Selective Plants Causing Cardiotoxicity

Common Name	Scientific Name	Toxic Part	Toxin
Azalea	*Rhodendron* sp.	All	Grayanotoxins
Death camus	*Zigadenus* sp.	All	Zygadenine, veratrine
Foxglove	*Digitalis* sp.	Leaves, seeds	Digitalis glycosides
Larkspur	*Delphenium ambiguum*	All	Delphinine
Lily of the valley	*Convallaria majalis*	All	Convallarin, convallamarin
Milkweed	*Asclepias* sp.		(Hydroxycinnamoyl) desglucouzarin
Monkshood	*Aconitum* sp.	Leaves, roots, seeds	Aconitine, aconine
Oleander	*Nerium oleander*	All	Oleandrin, oleandrosine
Squill	*Scilla maritima*	Bulbs	Scillaren

Actions on Cardiac Nerves—Toxic alkaloids found in *Veratrum viride* (American hellebore, Liliaceae), *V. album* (European hellebore), and *V. californicum* cause nausea, emesis, hypotension, and bradycardia on ingestion. *V. album* has been used for centuries to "slow and soften the pulse." The mixture of alkaloids includes protoveratrine, veratramine, and jervine that affects the heart by causing a repetitive response to a single stimulus resulting from prolongation of the sodium current. *Aconitum* species, which produce the toxic alkaloids aconitine, mesaconitine, and hypoaconitine, cause cardiac arrhythmias and hypotension, gastrointestinal upset and neurological symptoms. The alkaloids work by causing a prolonged sodium current with slowed repolarization in cardiac muscle and in nerve fibers.

Grayanotoxins are produced by *Rhododendron ponticum*, *R. macrophyllum*, and *Kalmia angustifolia* and can cause bradycardia, hypotension, oral parasthesia, and gastrointestinal upset. Severe poisoning can result in respiratory depression and eventually loss of consciousness. Grayanotoxins bind to sodium channels in cardiac and muscle cells resulting in increased sodium conductance.

Vasoactive Chemicals—Mistletoes (*Phoradendron flavescens* and*Viscum album*) produce toxins (phoratoxin and viscotoxin) that cause hypotension, vasoconstriction of the vessels in skin and skeletal muscle, and bradycardia resulting from negative inotropic actions on heart muscle.

Ingestion of the fungus *Claviceps purpurea* (ergot), which grows on grains that are used for food, causes vasoconstriction. In severe cases, the vasoconstriction was intense enough for gangrene to develop in the extremities. Abortion in pregnant women is also common after ingestion of ergot-contaminated grains.

Liver

Hepatocyte Damage—Pyrrolizidine alkaloids found in *Senecio* (groundsel), *Echium* (bugloss), *Cynoglossum* (hound's tongue), *Heliotropium* (heliotrope), and *Symphytum* (comfrey) cause liver damage in the form of hepatic veno-occlusive disease associated with lipid peroxidation. The liver damage caused by ingestion clinically appears similar to cirrhosis and some hepatic tumors that can easily be mistaken to be the source of the disease.

Mushroom Toxins—Most nonedible mushrooms may cause mild discomfort and are not life threatening; however, repeated ingestion of the false morel, *Gyromitra esculenta*, has been found

to cause hepatitis. The toxin gyromitrin is inactivated generally by boiling. Most fatal poisonings related to wild mushrooms are from ingestion of different species within *Amanita*, *Galerina*, and *Lepiota*. *Amanita phalloides* (Fig. 26–4) and *Amanita ocreata* contain two types of toxins, phalloidin and amatoxins. Phalloidin is capable of binding actin in muscle cells; however, it is not readily absorbed during digestion, which limits its harmful effects. Unfortunately, the smaller α-, β-, and γ-amanitins are readily absorbed. Of the amatoxins, α-amanitin is the most toxic as it inhibits protein synthesis in hepatocytes by binding to RNA polymerase II. In addition to the liver, the intestinal mucosa and kidneys are also affected, and serious clinical signs develop about 3 days after ingestion. In cases of severe poisoning, a liver transplant may be required.

FIGURE 26–4 *Amanita phalloides (death cap).*

Mycotoxins—Fumonisin toxins produced by the fungus *Fusarium* are known to grow on corn. The liver is the most affected organ in many species including horses, pigs, chickens, and rats. Ingestion in humans has been suggested to be associated with esophageal cancer. Fumonisins are diesters of propane-1,2,3-tricarboxylic acid and a pentahydroxyicosane containing a primary amino acid that is like sphingosine. This similarity is responsible for their toxicity as they block the enzymes involved in sphingolipid biosynthesis.

Kidney and Bladder

Carcinogens—The bracken fern (*Pteridium aquilinum*) is the only higher plant known to be carcinogenic in animals under natural feeding conditions. Bladder tumors in cattle are usually

epithelial and mesenchymal neoplasms. Ptaquiloside, a norsesquiterpene glucoside, is the known carcinogen present in the fern and it has been found to alkylate adenines and guanines of DNA. Bovine consumption of bracken fern significantly increases chromosomal aberrations.

Kidney Tubular Degeneration—*Xanthium* species (cocklebur, Asteraceae) have been found to contain the toxin carboxyatractyloside, which causes microvascular hemorrhages in multiple organs. Livestock poisoning causes the outward signs of depression and dyspnea; however, internally the toxin causes tubular degeneration and necrosis in the kidney and centrilobular necrosis in the liver. Consumption of the mushroom species *Cortinarius* has been found to cause acute renal failure but different species vary widely in toxicity and, therefore, edibility.

Blood and Bone Marrow

Anticoagulants—Fungal infections in sweet clover (*Melilotus alba*) have been found to produce dicumarol, a potent coumarin-like anticoagulant. Deaths in cattle have been reported and are caused by hemorrhages.

Bone Marrow Genotoxicity—*Argemone* (Papaveraceae), a species of poppy, produces sanguinarine, a benzophenanthridine alkaloid that is known to intercalate DNA and have carcinogenic potential.

Cyanogens—Cyanogens are found in a wide variety of plants including the kernels of apples, cherries, and peaches. The highest concentrations are found in the seeds of the bitter almond, *Prunus amygdalus* var. *amara.* Metabolism of amygdalin releases hydrocyanic acid that binds to the ferric ion in methemoglobin and the cytochrome oxidases, which, if severe enough, results in cyanide poisoning with death from asphyxiation.

Cassava produced from *Manihot esculenta* (Euphorbiaceae) is a major food source for some regions of Africa. The raw root contains a cyanogenic glucoside linamarin that degrades during processing of the root for human consumption. If local processing is inadequate, chronic ingestion of linamarin may cause konzo, a form of tropical myelopathy with sudden onset of spastic paralysis.

Nervous System

Epileptiform Seizures—The common and scientific names for selective plants that produce neurotoxins can be found in Table 26–6. Within the family Apiaceae, which contains carrots, the fleshy tubers of *Cicuta maculata* (water hemlock) produce neurotoxic cicutoxin (a C17-polyacetylene). Consumption of a single tuber can result in fatal poisoning, characterized by tonic–clonic convulsions, owing to the cicutoxin binding to GABA-gated chloride channels.

TABLE 26–6 Selective Plants Producing Neurotoxicity

Common Name	Scientific Name	Part	Toxin	Mechanism
Acacia tree	*Acacia willardiana*	Seeds	Willardiine	Glutamate receptor agonist
Alga	*Digenea simplex*	All	Kainic acid	Depolarization of glutamate-gated channels
	Chondria armata		Domoic acid	
Betel nut	*Areca catechu*	Nut	Guvacine	GABA uptake inhibitor, anticonvulsant
			Arecoline	Stimulates muscarinic cholinoceptors; CNS stimulation
Buckthorn; Coyotillo	*Karwinskia humboldtiana*	Seeds, leaves	Tullidinol	Demyelination of motor neurons leading to paralysis
Chrysanthemum	*Chrysanthemum cinerarifolium*	Seeds	Pyrethrins	Stimulate sodium efflux from neurons in insects
Deadly nightshade	*Atropa belladonna*	Berries	Tropine alkaloid	Blockade of muscarinic cholinoceptors
Fly agaric mushroom	*Amanita muscaria*	All	Muscimol	GABA receptor agonist
Rhododendron	*Rhododendron* sp.	Leaves	Grayanotoxins	Stimulate sodium channel and membrane depolarization
Ryania	*Ryania speciosa*	Stems	Ryanodine	Stimulates calcium channels and muscle contraction
Poison nut tree	*Strychnos nux vomica*	All, especially seeds	Strychnine	Glycine receptor antagonist that produces convulsions
Tobacco	*Nicotiana tabacum*	Leaves	Nicotine	Nicotinic cholinoceptor agonist (low doses) or antagonist (high doses); CNS stimulation

Members of the mint family (Labiatae) such as pennyroyal (*Hedeoma*), sage (*Salvia*), and hyssop (*Hyssopus*) are well known for their essential oils containing monoterpenes. Ingestion of these monoterpenes in concentrations much higher than those used for flavoring can cause tonic–clonic convulsions. Certain species within *Strychnos* (Loganiaceae) produce strychnine and brucine, which stimulate the CNS by blocking glycine-gated chloride channels.

Excitatory Amino Acids—Red algae (*Digenia simplex*) under certain conditions can proliferate rapidly leading to the notorious beach vacating "red tide" and producing kainic acid. Kainic acid may be ingested by humans who eat filter-feeding mussels that have eaten red algae. Acute symptoms are gastrointestinal distress, headache, hemiparesis, confusion, and seizures. Severe exposure can result in significant memory deficits and sensorimotor neuropathy.

The fungi *Amanita muscaria* (fly agaric; Fig. 26–5) and *A. pantherian* (panther agaric) produce the excitatory amino acid ibotenic acid (isoxazole amino acid) and its derivative muscimol. Poisoning produces CNS depression, ataxia, hysteria, and hallucinations. Myoclonic twitching and seizures sometimes develop. Other genera of fungi have been marked for their hallucinogenic actions, notably *Psilocybe*, which contains psilocin and psilocybin.

FIGURE 26–5 *Amanita muscaria (fly agaric).*

Motor Neuron Demyelination—*Karwinskia humboldtiana* produces anthracenones in its seeds. Both human and livestock poisonings have occurred. Several days following ingestion, ascending flaccid paralysis develops with demyelination of large motor neurons in the legs and eventually leads to bulbar paralysis in fatal cases. In addition to neurotoxicity, the anthracenones in *Karwinskia*, especially peroxisomicine A_2, cause lung atelectasis, emphysema, and massive liver necrosis. Inhibition of catalase in peroxisomes may be the mechanism of cell toxicity.

Cerebellar Neurons—The legumes *Swainsonia cansescens*, *Astragalus lentiginosus* (spotted locoweed), and *Oxytropis sericea* (locoweed) produce a toxic indolizidine alkaloid called swainsonine. These weeds can be accidentally consumed by grazing cattle, causing aberrant behavior with hyperexcitability and locomotor difficulty. Swainsonine causes marked inhibition of liver lysosomal and cytosomal α-mannosidase and Golgi mannosidase II, resulting in abnormal accumulation of brain glycoproteins and mannose-rich oligosaccharides that ultimately causes cell death.

Parasympathetic Stimulation—Certain mushrooms of the genera *Inocybe*, *Clitocybe*, and *Omphalotus* contain significant amounts of muscarine, which mimics the action of acetylcholine, the principal neurotransmitter in the parasympathetic nervous system. Consumption of any of these species results in extreme parasympathetic activation resulting in miosis, diarrhea, sweating, salivation, lacrimation, urination, bradycardia, and bronchoconstriction.

Parasympathetic Block—Atropine, l-hyoscyamine, and scopolamine are belladonna alkaloids found in varying concentrations in several genera of Solanaceae such as *Datura stramonium* (jimson weed), *Hyoscyamus niger* (henbane), *Atropa belladonna* (deadly nightshade; Fig. 26–6), and *Duboisia myoporoides* (pituri). These alkaloids effectively block the muscarinic receptor, causing tachycardia, dry mouth, dilated pupils, decreased gastrointestinal motility, and urine

retention.

FIGURE 26–6 *Atropa belladonna (deadly nightshade).*

Sensory Neuron Block—Capsaicin found in *Capsicum annuum* (sweet pepper) and *C. frutescens* (red pepper) causes a burning sensation on vanilloid-type (VR1) sensory receptors. It also desensitizes the transient receptor potential vanilloid 1 (TRPV1) receptor of sensory endings of C-fiber nociceptors to stimuli, a property that has therapeutic use in treating chronic pain. Capsaicin also can relax ileal smooth muscle.

Skeletal Muscle and Neuromuscular Junction

Neuromuscular Junction—Anabasine, an isomer of nicotine, is present in *Nicotiana glauca* (tree tobacco, Solanaceae) and produces prolonged depolarization of the junction after a period of excessive stimulation. Consumption of *N. glauca* leaves can result in flexor muscle spasm and gastrointestinal irritation, followed by generalized weakness, and respiratory compromise. Curare, which is used as a poison placed on the tips of arrows, is also a potent neuromuscular blocking agent and is obtained from tropical species of *Strychnos toxifera* and *Chondrodendron tomentosum*. *Anabaena flosaquae*, a species of alga, can produce under the right conditions a neurotoxin anatoxin A, which, when animals drink pond water with the alga present, depolarizes and blocks the animal's nicotinic and muscarinic acetylcholine receptors, causing death from respiratory arrest within minutes to hours.

Skeletal Muscle Damage—Certain species of *Thermopsis* produce seeds that contain quinolizidine alkaloids. Human poisoning from eating the seeds is rare, but cases have been reported in young children who experienced abdominal cramps, nausea, vomiting, and headache lasting up to 24 hours. Livestock grazing on *Thermopsis montana* (false lupine, mountain goldenbanner) develop locomotor depression and recumbency due to areas of necrosis in skeletal muscle that have been found on autopsy.

Consumption of *Cassia obtusifolia* (sicklepod, Leguminosae) seeds by livestock causes a degenerative myopathy in cardiac and skeletal muscle. Extracts of *C. obtusifolia* have been found to inhibit NADH-oxidoreductase in bovine and swine mitochondria in vitro.

Bone and Tissue Calcification

Bone and Soft Tissue—Consumption of *Solanum malacoxylon* (Solanaceae) by sheep and cows can cause a marked decrease in bone calcium and calcification of the entire vascular system due to the presence of a water-soluble vitamin D–like substance. In severe cases other organs can also be affected such as the lungs, joint cartilage, and kidney. Consumption of *Cestrum diurnum* (day-blooming jasmine, Solanaceae) and *Cestrum laevigatum* has been found to cause hypercalcemia and soft tissue calcification in grazing cattle and chickens.

Reproduction and Teratogenesis

Abortifacients—Besides its actions on the nervous system, swainsonine, the active alkaloid in the legumes *Astragalus* and *Oxytropus*, also causes abortions in pregnant livestock that accidentally ingest locoweeds. Foliage and seeds of *Leucaena leucocephala*, *Leucaena glauca*, and *Mimosa pudica* contain a toxic amino acid, mimosine, which on ingestion by cattle leads to uncoordinated gait, goiter, and reproductive disturbances including infertility and fetal death.

Teratogens—Ingestion of *V. californicum* (California false hellebore, Liliaceae) by pregnant sheep is known to cause malformations in its offspring that can include cyclopia, exencephaly, and microphthalmia. The toxic alkaloid jervine causes teratogenesis by blocking cholesterol synthesis that, among other things, prevents a proper response of fetal target tissue to the sonic hedgehog gene (Shh). Shh has an important role in proper developmental patterning of head and brain and blocking cholesterol synthesis has been shown experimentally to cause a loss of midline facial structures.

CLINICAL STUDY OF PLANT POISONS

Recent research concerning the use of plant toxins in a clinical setting has blossomed. With a growing group of people who wish to return to a more “natural” way of life, a medicine that is derived straight from a plant is much more marketable than one that is completely chemically synthesized. Recent research has shown that Uzara root extract reduced chloride secretion by the gut specifically by inhibiting Na^+,K^+-ATPase. This effect was seen even in the presence of cholera toxin that causes potent diarrhea by increasing chloride secretion in the gut. Interestingly, anemonin, which is the active skin irritant produced by species of *Ranunculus* (buttercup), has been found to show potent anti-inflammation effects under certain conditions. The compound was found to reduce nitric oxide production that resulted in a lessened inflammatory response to inflammatory stimuli.

SUMMARY OF PLANT TOXICITIES

Toxic chemicals produced by plants can cause something as innocuous as a mild rash on the skin all the way to death. It is important to remember that these toxins are primarily defense mechanisms against natural predators. However, we benefit greatly from plants as many have

produced potent pharmacologic therapies for myriad diseases that afflict humans.

INTRODUCTION TO ANIMAL VENOMS

Venomous animals can produce a poison in a highly developed exocrine gland or group of cells and can deliver their toxin during a biting or stinging act. Conversely, poisonous animals have no specific mechanism or structure for the delivery of their poisons and poisoning usually takes place through ingestion. Animal venom may play a role in offense (as in the capture and digestion of food), in the animal's defense (as in protection against predators or aggressors), or in both functions. Venoms used in an offensive posture are generally associated with the oral pole, as in the snakes and spiders, while those used in a defensive function are usually associated with the aboral pole or with spines, as in the stingrays and scorpion fish.

Animal and plant toxins tend to differ significantly in their chemical complexity, yet both can cause massive harm. Plant toxins tend to be smaller compounds or proteins, and often a single offending substance can be pinpointed. Conversely, animal venom typically is very complex and contains many individual toxic compounds and very large proteins that essentially work together to cause their effects.

PROPERTIES OF ANIMAL TOXINS

Venoms contain polypeptides, high- and low-molecular-weight proteins, amines, lipids, steroids, amino-polysaccharides, quinones, glucosides, and free amino acids, as well as serotonin, histamine, and other substances. The venom is a source of peptides and proteins that act on myriad exogenous targets such as ion channels, receptors, and enzymes within cells and on cell membranes.

Studying venom is important for several reasons. First, venoms offer valuable insight into the systems they act on such as the cardiovascular system, nervous system, coagulation, and homeostasis. Second, venoms are useful as a source of potential new drugs. Third, a greater understanding of venoms favors development of improved protection against envenomation.

Novel instrument developments have permitted the greater application of mass spectrometry, coupled with various separation technologies, to tease out the complexity of natural venoms, thereby identifying the peptide and protein components of venoms. The technology allows considerable resolution of extremely small amounts of venom. Figure 26–7 demonstrates the application of gel filtration and high-pressure liquid chromatography (HPLC), as cone snail venom was fractionated into numerous peptides with varying activities. Similar fractionations have been performed on many other types of venom to identify the individual components. Unfortunately, studying the chemistry, pharmacology, and toxicology of venoms requires isolating and dismantling the venoms and losing the synergy among multiple components.

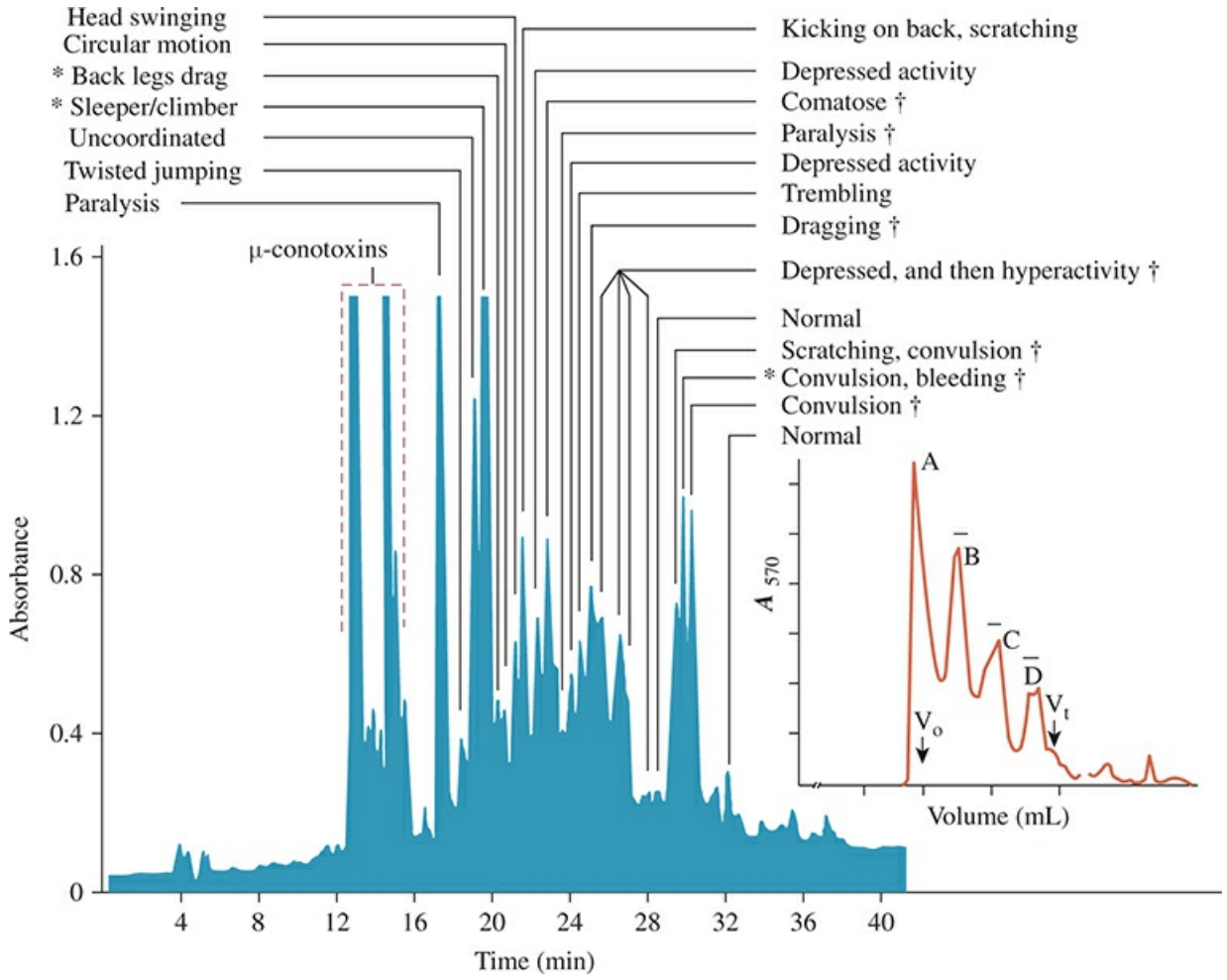

FIGURE 26–7 *Multiple biologically active components were obtained from Conus geographus venom by first subjecting the venom to gel filtration on Sephadex G-25 into four fractions and then separation of fraction B (which contains the α-conotoxins) by high-pressure liquid chromatography on a VYDAC C18 column using a trifluoroacetic acid–acetonitrile gradient.* Various peak fractions were then injected intracerebrally into mice and different responses were noted. (†) The fraction was lethal in at least one injected animal. (Reprinted with permission from Olivera BM, Rivier J, Clark C, et al. Diversity of *Conus* neuropeptides. *Science*. 1990;249:257–263.)

The bioavailability of a venom is determined by its composition, molecular size, amount or concentration gradient, solubility, degree of ionization, and the rate of blood flow into that tissue, as well as the properties of the engulfing surface itself. The venom can be absorbed by active or passive transport, facilitated diffusion, or pinocytosis, among other physiologic mechanisms. Besides the bloodstream, the lymph circulation not only carries surplus interstitial fluid produced by the venom but also transports larger molecular components and other particulates back to the bloodstream. The site of action and metabolism of venom is dependent on its diffusion and partitioning along the gradient between plasma and tissues where the components are deposited. Once the toxin reaches a particular site, its entry to that site is dependent on the rate of blood flow into that tissue, the mass of the structure, and the partition characteristics of the toxin between the blood and that particular tissue.

ARTHROPODS

Arthropods of significant venomous or poisonous importance include the arachnids (scorpions, spiders, whip scorpions, solpugids, mites, and ticks), the myriapods (centipedes and millipedes), the insects (water bugs, assassin bugs, and wheel bugs), beetles (blister beetles), Lepidoptera (butterflies, moths, and caterpillars), and Hymenoptera (ants, bees, and wasps). The number of deaths from arthropod stings and bites is unknown. Among the disease states that are confused with spider or arthropod bites or stings were erythema chronicum migrans, erythema nodosum, periarteritis nodosum, pyroderma gangrenosum, kerion cell-mediated response to a fungus, Stevens–Johnson syndrome, toxic epidermal necrolysis, herpes simplex, and purpura fulminans. Any arthropod may bite or sting and not eject venom.

ARACHNIDA

Scorpions

Of the more than 1000 species of scorpions, the stings of more than 75 can be considered of sufficient importance to warrant medical attention. Some of the more important scorpions are noted along with their location in Table 26–7.

TABLE 26–7 Location of Some Medically Important Scorpions

Genus	Distribution
Androctonus species	North Africa, Middle East, Turkey
Buthus species	France and Spain to Middle East and north Africa, Mongolia, China
Buthotus species	Africa, Middle East, central Asia
Centruroides species	North, Central, South America
Heterometrus species	Central and Southeast Asia
Leiurus species	North Africa, Middle East, Turkey
Mesobuthus species	Turkey, India
Parabuthus species	Southern Africa
Tityus species	Central and South America

Many scorpion venoms contain low-molecular-weight proteins, peptides, amino acids, nucleotides, and salts, among other components. Short-chain toxins with 20 to 40 amino acid residues have three or four disulfide bonds and appear to affect potassium or chloride channels. Long-chain toxins have 58 to 76 amino acid residues with four disulfide bonds and affect mainly

the sodium channels.

The symptoms and signs of scorpion envenomation differ considerably depending on the species. Common offenders are members of the family Vejovidae. Their sting gives rise to localized pain, swelling, tenderness, and mild parasthesia. Systemic reactions are rare, although weakness, fever, and muscle fasciculations have been reported.

Envenomations by some members of the genus *Centruroides* are clinically important. In children, their sting may produce initial pain, although some children do not complain of pain and are unaware of the injury. The area becomes sensitive to touch, and merely pressing lightly over the injury will elicit an immediate retraction. Usually there is little or no local swelling and only mild erythema. The child becomes tense and restless and shows abnormal, random head and neck movements. *Centruroides sculpturatus* stings cause nystagmus, roving eye, and oculogyric movements. Tachycardia, hypertension, and respiratory rates are increased. Fasciculations may be seen and the child may complain of generalized weakness and display some ataxia. The respiratory distress may proceed to respiratory paralysis. If death does not occur, the child usually becomes asymptomatic within 36 to 48 hours. In contrast to children, almost all adults complain of immediate pain after the sting, regardless of the *Centruroides* species involved, becoming tense and anxious. They develop tachycardia and hypertension, and respirations are increased. Most adults are asymptomatic within 12 hours.

Spiders

Of the 30,000 or so species, at least 200 have been implicated in significant bites on humans. Spiders are predaceous, polyphagous arachnids that generally feed on insects or other arthropods. Table 26–8 provides a short list of spiders with associated toxins and their targets.

TABLE 26–8 Some Significant Spiders, Their Toxins, and the Targets of the Toxins

Spider	Peptide	Target
Acanthoscurria gomesiana	Gomesin	PLM
Agelenopsis aperta	ω-Afal-IVA	Ca^{2+}
	μ-Afatoxin 1-6	Na^{+}
Grammostola spatula	HaTx1,2	K^{+}
	GsMTx2,4	MS
	GSTxSIA	Ca^{2+}
Hadronyche versuta	ω-ACTX-Hv1a	Ca^{2+}
	ω-ACTX-Hv2a	Ca^{2+}
	δ-ACTX-Hv1a	Na^{+}
Heteroscodra maculate	HmTx1,2	K^{+}
Ornithoctonus huwena	Huwentoxin I	Ca^{2+}
	Huwentoxin IV	Na^{+}
Psalmopoeus cambridgei	PcTx1	ASIC
Phrixotrichus auratus	PaTx1,2	K^{+}
Thrixopelma pruriens	ProTxI,II	Na^{+}

Abbreviations: ASIC, acid-sensing ion channels; Ca^{2+}, K^{+}, and Na^{+}, calcium, potassium, and sodium ion channels; MS, mechanosensitive ion channels; PLM, phospholipid membranes.

Additional species, their toxins, and their targets may be obtained from the article by Corzo G, Escoubas P. Pharmacologically active spider peptide toxins. *Cell Mol Life Sci.* 2003;60:2409–2426.

All spiders except the Uloboridae family possess a venom apparatus that produces neurotoxins designed to paralyze or kill prey. Spider venoms are complex mixtures of low-molecular-weight components, including inorganic ions and salts, free acids, amino acids, biogenic amines and neurotransmitters, and polypeptide toxins. The acylpolyamines are voltage-dependent open-channel blockers (sodium, calcium, and potassium channels) and/or blockers of the ion channel associated with glutamate receptors. They also act on nicotinic acetylcholine receptors.

***Agelenopsis* Species (American Funnel Web Spiders)—**The American funnel web spider (*Agelenopsis aperta*) contains three classes of agatoxins that target ion channels. The α-agatoxins appear to be use-dependent, noncompetitive antagonists of the glutamate receptor channels. The μ-agatoxins increase spontaneous release of neurotransmitter from presynaptic terminals and repetitive action potentials in motor neurons. In addition, the μ-agatoxins are specific for insect sodium channels. The ω-amatoxins are a structurally diverse group of peptides that are selective for voltage-activated calcium channels. There are four types of ω-amatoxins that can be

distinguished by sequence similarity and their spectrum of action against insect and vertebrate calcium channels. The μ-agatoxins causing channels to open at the normal resting potentials synergize the action of the α-agatoxins. The agatoxins are used as selective pharmacologic probes to characterize ion channels in organs such as brain and heart and have been evaluated as candidate biopesticides.

***Latrodectus* Species (Widow Spiders)—**Found throughout the world in all continents with temperate or tropical climates, these spiders are commonly known as the black widow, brown widow, or red-legged spider (Fig. 26–8).

FIGURE 26–8 *Latrodectus mactans (female black widow spider).*

The latrotoxins, a family of high-molecular-weight proteins that are found in *Latrodectus* venoms, target different classes of animals including vertebrates, insects, and crustaceans. All latrotoxins stimulate massive release of neurotransmitters after binding to specific neuronal receptors.

α-Latrotoxin is the most studied protein that is toxic only to vertebrates and not to insects or crustaceans. The presynaptic toxin is said to exert its toxic effects on the vertebrate central nervous system depolarizing neurons by increasing intracellular [Ca^{2+}] and by stimulating exocytosis of neurotransmitters from nerve terminals. α-Latrotoxin and its mutants are versatile tools for the study of exocytosis. In particular, research with this toxin has helped confirm the vesicular hypothesis of transmitter release, establish the requirement of calcium ion for endocytosis, characterize individual neurotransmitter sites in the central nervous system, and identify two families of important neuronal cell surface receptors.

Bites by the black widow are described as sharp and pinprick-like, followed by a dull, occasionally numbing pain in the affected extremity and by pain and cramps in one or several of the large muscle masses. Muscle fasciculations can be seen, sweating is common, and lymphadenitis is frequently observed. Severe paroxysmal muscle cramps, arthralgia, and hypertension are common findings. Blood studies are usually normal.

***Loxosceles* Species (Brown or Violin Spiders)—**These primitive spiders are variously known in North America as the fiddle-back spider or the brown recluse (Fig. 26–9).

FIGURE 26–9 *Loxosceles reclusa (male brown recluse spider) with the violin pattern on the dorsal cephalothorax.*

The venom of *Loxosceles* spiders appears to contain phospholipase, protease, esterase, collagenase, hyaluronidase, deoxyribonuclease, ribonuclease, dipeptides, dermonecrosis factors, and sphingomyelinase D. The venom has coagulation and vasoconstriction properties and it causes selective vascular endothelial damage. There are adhesions of neutrophils to the capillary wall with sequestration and activation of passing neutrophils by the perturbed endothelial cells.

The bite of this spider produces about the same degree of pain as does the sting of an ant, but sometimes the patient may be unaware of the bite. Pruritus over the area often occurs, and the area becomes red, with a small blanched area surrounding the reddened bite site. With significant envenomations, hemorrhages may develop throughout the area. A small bleb or vesicle may form at the bite site, increase in size, and rupture with subsequent pustule formation. The whole area may become swollen and painful, and lymphadenopathy is common. Systemic symptoms and signs include fever, malaise, stomach cramps, nausea, vomiting, jaundice, spleen enlargement, hemolysis, hematuria, and thrombocytopenia. Fatal cases, while rare, usually are preceded by intravascular hemolysis, hemolytic anemia, thrombocytopenia, hemoglobinuria, and renal failure.

***Steatoda* Species—**These spiders are variously known as the false black widow, combfooted, cobweb, or cupboard spiders. The venom of *Steatoda paykulliana* stimulates the release of transmitter substances like *Latrodectus* venom. The venom is said to form ionic channels permeable for bivalent and monovalent cations, and the duration of time in the open state depends on the membrane potential. Bites by *S. grossa* or *S. fulva* have been followed by local pain, induration, pruritus, and the occasional breakdown of tissue at the bite site.

Cheiracanthium* Species (Running Spiders)—C. punctorium*, *C. inclusum*, *C. mildei*, *C. diversum*, and *C. japonicum* are often implicated in envenomations. The most toxic venom fraction is said to be a protein of 60 kDa, and the venom is high in norepinephrine and serotonin. The patient usually describes the bite by *C. inclusum* as sharp and painful, and a reddened wheal

with a hyperemic border develops. Small petechiae may appear near the center of the wheal. Skin temperature over the lesion is often elevated, but body temperature is usually normal. Lymphadenitis and lymphadenopathy may develop. *C. japonicum* produces more severe manifestations, including severe local pain, nausea and vomiting, headache, chest discomfort, severe pruritus, and shock.

***Theraphosidae* Species (Tarantulas)—**True tarantulas are predators that feed on vertebrate and invertebrate prey that are captured after envenomation with venoms that act rapidly and irreversibly on the central and peripheral nervous systems. In humans, reported bites elicit mild to severe local pain, strong itching, and tenderness that may last for several hours. Edema, erythema, joint stiffness, swollen limbs, burning feelings, and cramps are common.

At least 33 peptide toxins from various tarantula venoms have a molecular weight of 3000 to 5700 Da, and targets include voltage-gated potassium, sodium, and calcium channels, tetrodotoxin-sensitive channels, and acid-sensing ion channels, which are sensitive to extracellular pH. Theraphosid spiders contain several toxins that are being evaluated for development as drugs. *Grammostola* mechanotoxin 4 from *G. spatulata* has considerable promise as an antiarrhythmic. Protoxin I and II from *Thrixopelma pruriens* have promise as analgesics because they inhibit the tetrodotoxin-resistant sodium channels.

Ticks

The saliva of certain ticks of the families Ixodidae, Argasidae, and Nuttalliellidae causes tick paralysis. Ticks are known to transmit the organisms causing Lyme disease, Rocky Mountain spotted fever, babesiosis, leptospirosis, Q fever, ehrlichiosis, typhus, tick-borne encephalitis, and others.

Saliva from *Ixodes scapularis* contains apyrase (ATP diphosphohydrolase), which hydrolyzes ADP that is released at the bite site, thereby inhibiting ADP-induced platelet aggregation; kininase (ACE-like protein or angiotensin-converting enzyme-like protein), which hydrolyzes circulating kinins and reduces the host inflammatory response; glutathione peroxidase; serine protease inhibitors, which inhibit coagulation enzymes; an anticomplement protein that inhibits an enzyme in the alternative pathway for complement; an amine-binding protein that binds serotonin, histamine, and other biogenic amines; and prostanoids (PGE_2 and $PGF_{2\alpha}$).

As tick bites are often not felt, the first evidence of envenomation may not appear until several days later, when small macules 3 to 4 mm in diameter develop that are surrounded by erythema and swelling. The patient often complains of difficulty with gait, followed by paresis and eventually locomotor paresis and paralysis. Problems in speech and respiration may ensue and lead to respiratory paralysis if the tick is not removed. The saliva of *Ixodes holocyclus* has yielded a peptide holocyclotoxin-1 that may cause paralysis. Symptoms resolve rapidly on removal of the tick.

CHILOPODA (CENTIPEDES)

A prevalent biting genus is a *Scolopendra* species. The venom is concentrated within the intracellular granules, discharged into vacuoles of the cytoplasm of the secretory cells, and

moved by exocytosis into the lumen of the gland; from thence ducts carry the venom to the jaws. Centipede venoms contain high-molecular-weight proteins, proteinases, esterases, 5-hydroxytryptamine, histamine, lipids, and polysaccharides. Such venom contains a heat-labile cardiotoxic protein of 60 kDa that produces, in humans, changes associated with acetylcholine release. The bite produces two tiny punctures, sharp pain, immediate bleeding, redness, and swelling often lasting for 24 hours. Localized tissue changes and necrosis have been reported, and severe envenomations may cause nausea, vomiting, changes in heart rate, vertigo, and headache.

DIPLOPODA (MILLIPEDES)

The repellent secretions expelled from the sides of their bodies contain a toxin of benzoquinone derivatives plus a variety of complex substances such as iodine and hydrocyanic acid, which the animal makes use of to produce hydrogen cyanide. Some species can spray these defensive secretions, and eye injuries are not uncommon. The lesions produced by millipedes consist of a burning or prickling sensation and development of a yellowish or brown-purple lesion; subsequently, a blister containing serosanguinous fluid forms, which may rupture. Eye contact can cause acute conjunctivitis, periorbital edema, keratosis, and much pain, such an injury must be treated immediately.

INSECTA

Heteroptera (True Bugs)

The clinically most important of the true bugs are the Reduviidae (the reduviids): the kissing bug, assassin bug, wheel bug, or cone-nose bug of the genus *Triatoma*. The venom of these bugs appears to have apyrase activity and it is fairly rich in protease properties. It inhibits collagen-induced platelet aggregation. Three peptides isolated from the saliva of predatory reduviids are 34 to 36 amino acid residues in size and are calcium channel blockers like ω-conotoxins. The bites of *Triatoma* species are painful and give rise to erythema, pruritus, increased temperature in the bitten part, localized swelling, and—in those allergic to the saliva—systemic reactions such as nausea, vomiting, and angioedema.

Hymenoptera (Ants, Bees, Wasps, and Hornets)

Formicidae (Ants)—Most ants have stings, but those that lack them can spray a defensive secretion from the tip of the gaster, which is often placed in the wound of the bite. Clinically important stinging ants are the harvesting ants (*Pogonomyrmex*), fire ants (*Solenopsis*), and little fire ants (*Ochetomyrmex*).

The venoms of the ants vary considerably. The venoms of the Ponerinae, Ecitoninae, and *Pseudomyrmex* are proteinaceous in character. The Myrmicinae venoms are a mixture of amines, enzymes, and proteinaceous materials, histamine, hyaluronidase, phospholipase A, and hemolysins. Formicidae ant venom contains about 60% formic acid. Fire ant venoms are rich in

alkaloids such as solenopsin. The sting of the fire ant gives rise to a painful burning sensation, after which a wheal and localized erythema develop, leading in a vesicle turns into a pustule. In multiple stings, there may be nausea, vomiting, vertigo, increased perspiration, respiratory difficulties, cyanosis, coma, and even death.

Apidae (Bees)—This family includes the bumble bees, honeybees, carpenter bees, and yellow jackets. The commonest stinging bees are *Apis mellifera* and the Africanized bee, *Apis mellifera adansonii*, and the incidence of Hymenoptera poisonings is increasing. The venom contains biologically active peptides, such as melittin, apamine, mast cell–degranulating peptide, and others, as well as phospholipases A_2 and B, hyaluronidase, histamine, dopamine, monosaccharides, and lipids. Melittin also forms tetramers with pores, thereby causing a breakdown of the resting potential and rapid depolarization of nociceptors, which induces pain. The compound apamine contains 18 amino acids cross-linked by two disulfide bridges. Apamine is a blocker of calcium-dependent potassium channels. In addition to apamine, mast cell–degranulating peptide stimulates the release of histamine, and specifically inhibits voltage-dependent potassium channels.

Bee stings typically produce immediate, sharp or burning pain, slight local erythema, and edema followed by itching. It is said that 50 stings can be serious and lead to respiratory dysfunction, intravascular hemolysis, hypertension, myocardial damage, hepatic changes, shock, and renal failure. With 100 or more stings, death can occur. Patients allergic to bee stings may have an immediate allergic reaction with the risk of anaphylactic shock.

Vespidae (Wasps)—This family includes wasps and hornets. These venoms contain a high content of peptides, which include mastoparan in wasps and hornets and crabolin in hornet venom. These peptides release histamine from mast cells. Wasp kinins cause immediate pain, vasodilation, and increased vascular permeability leading to edema. These venoms also contain phospholipases and hyaluronidases, which contribute to the breakdown of membranes and connective tissue to facilitate diffusion of the venom.

Lepidoptera (Caterpillars, Moths, and Butterflies)

The urticating hairs, or setae, of caterpillars are effective defensive weapons that protect some species from predators. The setae are attached to unicellular poison glands at the base of each hair. The toxic material found in the venom glands contains aristolochic acids, cardenolides, kallikrein, histamine, and a fibrinolytic peptide. The spicules of *Thaumetopoea pityocampa* contain thaumetopoein, which is a strong dermal irritant and highly allergenic peptide. The stings of several species of Lepidoptera give rise to a bleeding diathesis, often severe and sometimes fatal.

MOLLUSCA (CONE SNAILS)

The genus *Conus* is a group of approximately 500 species of carnivorous predators found in marine habitats that use venom for prey capture. Cone snails have a venom duct for synthesis and storage of venom and hollow harpoon-like teeth for injection of the venom. There are probably over 100 different venom components per species known as conotoxins. Molecular

targets include G protein–coupled receptors, neurotransmitter transporters, and ligand- or voltage-gated ion channels. Some components have enzymatic activity. Figure 26–10 provides an overview of peptidic *Conus* venom components, indicating gene superfamilies, disulfide bond characteristics, and general targets.

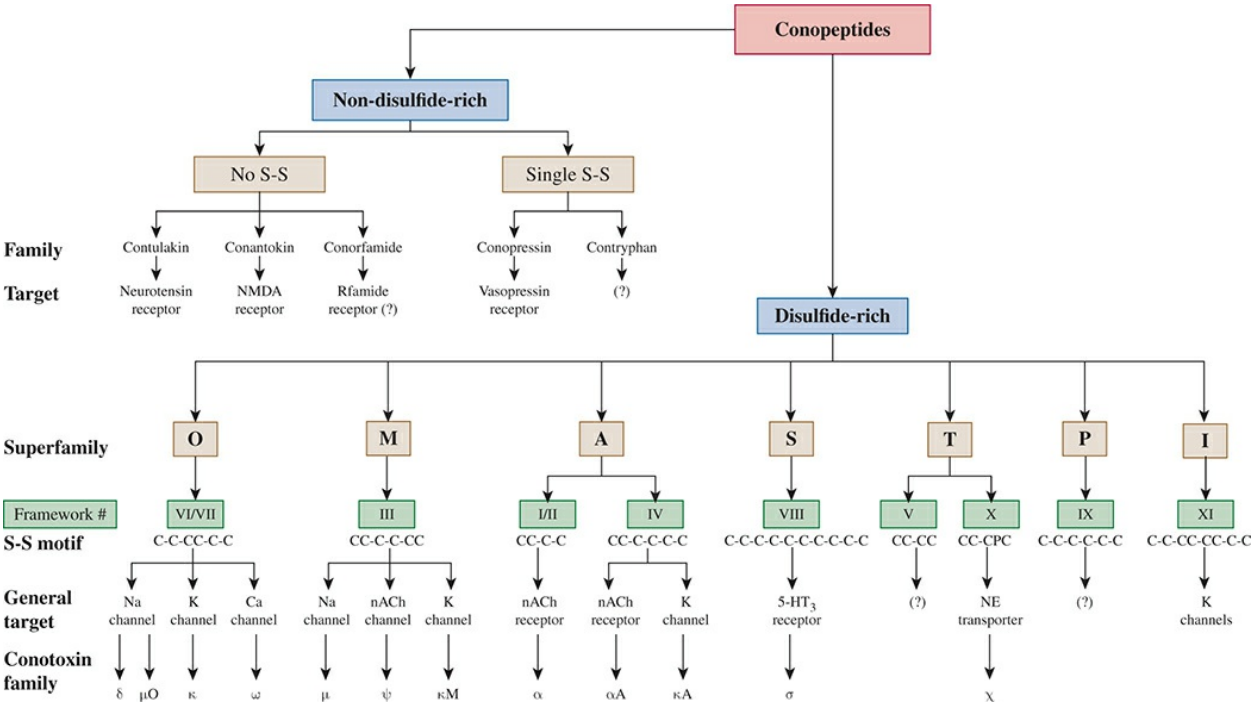

FIGURE 26–10 *Organizational diagram for Conus peptides, indicating gene superfamilies, disulfide patterns, and known pharmacologic targets.* Only the superfamilies of the disulfide-rich peptides are shown. (Reprinted with permission from Terlau H, Olivera BM. *Conus* venoms: a rich source of novel ion channel targeted peptides. *Physiol Rev*. 2004;84:41–68.)

Cone snails could be called sophisticated practitioners of combination drug therapy. After injection, multiple conopeptides act synergistically to affect the targeted prey. The term “toxin cabal” has been applied to this coordinated action of the conopeptide mixture. The fish-hunting species *Conus purpurascens* apparently has two distinct cabals whose effects differ in time and space. The “lightning-strike cabal” causes immediate immobilization of the injected prey because various venom components inhibit voltage-gated sodium channel inactivation and block potassium channels, resulting in massive depolarization of axons near the injection site and a tetanic state. The second physiologic cabal, the “motor cabal,” acts more slowly as conotoxins must distribute throughout the body of the prey producing total inhibition of neuromuscular transmission. Various conopeptides inhibit presynaptic calcium channels that control neurotransmitter release, the postsynaptic neuromuscular nicotinic receptors, and the sodium channels involved in the muscle action potential.

REPTILES

Lizards

The Gila monster (*Heloderma suspectum*) and the beaded lizards (*Heloderma horridum*) are far less dangerous than is generally believed. Their venom is transferred from venom glands in the lower jaw through ducts that discharge their contents near the base of the larger teeth. The venom is then drawn up along grooves in the teeth by capillary action. The venom of this lizard has serotonin, amine oxidase, phospholipase A, a bradykinin-releasing substance, helothermine, helodermin, helospectin I and II, gilatoxin, and low-proteolytic as well as high-hyaluronidase activities. The clinical presentation of a helodermatid bite can include algogenia, edema, hypotension, nausea, vomiting, weakness, and diaphoresis. No antivenin is commercially available. Treatment is supportive.

Snakes

General Information and Classification—Venomous snakes primarily belong to the following families: Viperidae (vipers), Elapidae, Atractaspididae, and Colubridae. The Colubridae are considered the largest venomous family and are composed of nearly 60% of all snakes. It is estimated that there are over 2.5 million snakebites annually, and that over 100,000 victims will die.

Snake Venoms—These venoms are complex mixtures: proteins and peptides, consisting of both enzymatic and nonenzymatic compounds. Snake venoms also contain inorganic cations such as sodium, calcium, potassium, magnesium, and small amounts of zinc, iron, cobalt, manganese, and nickel. The metals in snake venoms are likely catalysts for metal-based enzymatic reactions. For example, in some elapid venoms, zinc ions appear to be necessary for anticholinesterase activity, and calcium may play a role in the activation of phospholipase A and the direct lytic factor. Some proteases appear to be metalloproteins. Some snake venoms also contain carbohydrates (glycoproteins), lipids, and biogenic amines, such as histamine, serotonin, and neurotransmitters (catecholamines and acetylcholine) in addition to positively charged metal ions. The complexity of snake venom components is illustrated in Fig. 26–11.

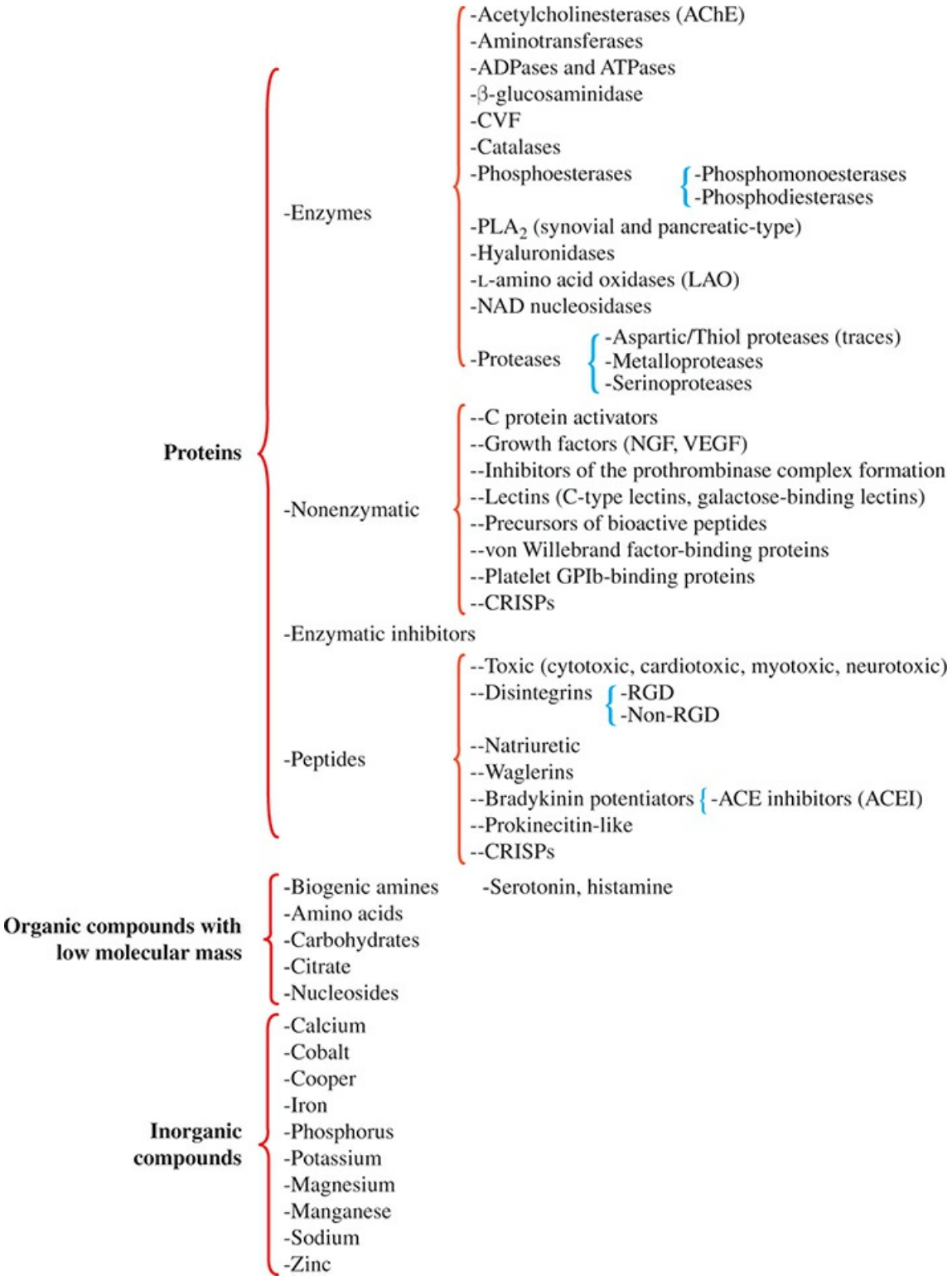

FIGURE 26–11 *Components of snake venoms.* ACE, angiotensin-converting enzyme; CRISP, cysteine-rich secretory protein; CVF, cobra venom factor–like proteins; LAO, L-amino acid oxidase; PLA_2, phospholipase A2; RGD, arginine–glycine–aspartate. (Reprinted from Ramos OHP, Selistre-de-Araujo HS. Snake venom metalloproteases—structure and function of

catalytic and disintegrin domains. *Comp Biochem Physiol C Toxicol Pharmacol*. 2006;142:328–346.)

Actions of snake venoms can be broad ranging in several areas. A simplistic approach would group toxin components as neurotoxins, coagulants, hemorrhagins, hemolytics, myotoxins, cytotoxins, and nephrotoxins. Neurotoxins produce neuromuscular paralysis ranging from dizziness to ptosis; to ophthalmoplegia, flaccid facial muscle paralysis, and inability to swallow; to paralysis of larger muscle groups; and finally, to paralysis of respiratory muscles and death by asphyxiation. Coagulants may have initial procoagulant action that uses up clotting factors leading to bleeding. Coagulants may directly inhibit normal clotting at several places in the clotting cascade or via inhibition of platelet aggregation. In addition, some venom components may damage the endothelial lining of blood vessels leading to hemorrhage. Bite victims may show bleeding from nose or gums, from the bite site, and in saliva, urine, and stools. Myotoxins can directly affect muscle contraction leading to paralysis or cause rhabdomyolysis or the breakdown of skeletal muscle. Myoglobinuria, or a dark brown urine, and hyperkalemia may be noted. Cytotoxic agents have proteolytic or necrotic properties leading to the breakdown of tissue. Typical signs include massive swelling, pain, discoloration, blistering, bruising, and wound weeping. Finally, nephrotoxins can cause direct damage to kidney structures leading to bleeding, damage to several parts of the nephron, tissue oxygen deprivation, and renal failure.

Enzymes—At least 26 different enzymes have been isolated from snake venoms. No single snake venom contains all 26 enzymes and some important snake venom enzymes are shown in Fig. 26–11. Proteolytic enzymes that catalyze the breakdown of tissue proteins and peptides include peptide hydrolases, proteases, endopeptidases, peptidases, and proteinases. Several proteolytic enzymes may be in a single venom. Collagenase is a specific proteinase that digests collagen. Hyaluronidase cleaves internal glycoside bonds in certain acid mucopolysaccharides resulting in a decrease in the viscosity of connective tissues. The breakdown in the hyaluronic barrier allows other fractions of venom to penetrate the tissues. Fibrin(ogen)olytic enzymes, the metalloproteinases and the serine proteinases, ultimately break down fibrin-rich clots and help to prevent further clot formation. Phosphodiesterase, found in the venoms of all families of poisonous snakes, acts as an exonucleotidase, attacking DNA and RNA. Phospholipase A_2 is widely distributed in snake venoms, and this enzyme family interacts with other venom components often resulting in synergistic reactions.

The snake venom metalloproteinases (SVMPs) are enzymes that disrupt the hemostatic system. The PII metalloproteases block the function of integrin receptors, a function that could alleviate a variety of pathological conditions such as inflammation, tumor angiogenesis and metastasis, and thrombosis. SVMPs degrade proteins such as laminin, fibronectin, type IV collagen, and proteoglycans from the endothelial basal membrane; degrade fibrinogen and von Willebrand factor enhancing the hemorrhagic action; and inhibit platelet aggregation and stimulate release of cytokines.

Polypeptides—Snake venom polypeptides are low-molecular-weight proteins that do not have enzymatic activity. More than 80 polypeptides with pharmacologic activity have been isolated from snake venoms. Most of the lethal activity of the poison of the sea snake *Laticauda semifasciata* involves erabutoxin-a and erabutoxin-b. Erabutoxin-a and α-cobratoxin are curamimetic at the neuromuscular junction. Disintegrins are a family of short cysteine-rich polypeptides that exhibit affinities for many ligand receptors. The myotoxin crotamine from

Crotalus durissus terrificus venom induces skeletal muscle spasms and paralysis by changing the inactivation process of sodium channels, leading to depolarization of the neuromuscular junction.

Toxicology—The venoms of rattlesnakes and other New World crotalids produce alterations in the resistances and often in the integrity of blood vessels, changes in blood cells and blood coagulation mechanisms, direct or indirect changes in cardiac and pulmonary dynamics, and—with crotalids such as *C. durissus terrificus* and *C. scutulatus*—serious alterations in the nervous system and changes in respiration. In humans, the course of the poisoning is determined by the kind and amount of venom injected; the site where it is deposited; the general health, size, and age of the patient; the kind of treatment; and those pharmacodynamic principles noted earlier in this chapter. Death in humans may occur within less than 1 hour or after several days, with most deaths occurring between 18 and 32 hours. Hypotension or shock is the major therapeutic problem in North American crotalid bites.

Snakebite Treatment—The treatment of bites by venomous snakes is now so highly specialized that almost every envenomation requires specific recommendations. However, three general principles for every bite should be kept in mind: (1) snake venom poisoning is a medical emergency requiring immediate attention and the exercise of considerable judgment; (2) the venom is a complex mixture of substances of which the proteins contribute the major deleterious properties, and the only adequate antidote is the use of specific or polyspecific antivenom; and (3) not every bite by a venomous snake ends in an envenomation. Venom may not be injected about a quarter of all bites. The incidence with the bites of cobras and perhaps other elapids is probably higher.

Snake Venom Evolution—Considerable efforts are being expended to examine the complex process by which snake venom components are thought to have changed over the years. In general, the toxins from ancestral proteins that were constructed of dense networks of cysteine cross-linkages are considered among the most diverse today in terms of toxicological insult.

ANTIVENOMS

Antivenoms have been produced against most medically important snake, spider, scorpion, and marine toxins. Antivenom consists of venom-specific antisera or antibodies concentrated from immune serum to the venom. Antisera contain neutralizing antibodies: one antigen (monospecific) or several antigens (polyspecific). Monovalent antivenoms have a high neutralization capacity, which is desirable against the venom of a specific animal. Polyvalent antisera are typically used to cover several venoms, such as snakes from a geographic region. Neutralization capacity of antivenom is highly variable as there are no enforced international standards. Antivenom may cross-react with venoms from distantly related species and may not react with venom from the intended species. Nevertheless, in general, the antibodies bind to the venom molecules, rendering them ineffective.

All antivenom products may produce hypersensitivity reactions. Once initiated, anaphylaxis may continue despite discontinuation of antivenom administration. The risks of anaphylaxis should always be considered when one is deciding whether to administer antivenom.

POTENTIAL CLINICAL APPLICATION OF VENOMS

Toxin specificities for receptors and channels that facilitate the interface and coordination of neuromuscular activity are utilized and manipulated to study, model, diagnose, and sometimes treat acute and degenerative conditions. On closer examination of α-bungarotoxin and candoxin nicotinic acetylcholine receptor specificity, plans are under way to utilize the reversible and irreversible receptor binding in muscular and neuronal tissues, respectively, in Alzheimer patients. In addition to treating neurological diseases, specific α-toxins (longer chained) are also studied for their antiangiogenic capabilities in treating malignant tumor growth in patients suffering from small cell lung carcinoma. In cases such as this, there is an inherent trade-off between promoting some degree of neurological deficit versus combating tumor growth. Toxins such as the snake venom thrombin–like enzymes are valuable tools in both research and therapeutic applications. Fibrin(ogen)olytic enzymes that break down fibrin-rich clots preventing further clot formation may be useful as controls in blood clotting research or to treat heart attacks and strokes.

Active research efforts have noted that animal venoms contain components that can reduce pain, can selectively kill specific cancers, may reduce the incidence of stroke via effects on blood coagulability, and function as antibiotics or in releaving pain. Another major area of investigation and success involves the venom components that act as enzyme inhibitors. In particular, venom peptides from *Bothrops jararaca* were initially called bradykinin-potentiating peptides and lowered blood pressure. Venom toxins can also be used as a component of the toxin–receptor–antibody complex for diagnosis of autoimmune disorders. Leeches, earthworms, helminths, snails, centipedes, spiders, and ticks all produce substances with potential clinical applications, such as osteoarthritis, deep vein thrombosis, antimicrobial action, inflammatory bowel disease, analgesia, and hyperlipidemia. Blood from the mongoose, hedgehog, and opossum contains proteins that inhibit hemorrhagins in snake venoms.

CONCLUSION

The myriad toxins produced by plants and animals range from the relative simplicity of small chemicals to the exceedingly complex proteinaceous toxins. The interplay between toxin and organism is often difficult to study due to difficulty involved in recreating the interaction in the laboratory. The interactions that arise between toxins and substances already present in the organism can worsen or reduce the poisonous outcome. As laboratory techniques become more sophisticated and new methods are developed, research concerning toxins and their effects will continue to grow.

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QUESTIONS

1. Which of the following statements regarding animal toxins is FALSE?
   - **a.** Animal venoms are strictly metabolized by the liver.
   - **b.** The kidneys are responsible for the excretion of metabolized venom.
   - **c.** Venoms can be absorbed by facilitated diffusion.
   - **d.** Most venom fractions distribute unequally throughout the body.
   - **e.** Venom receptor sites exhibit highly variable degrees of sensitivity.

2. Scorpion venoms do NOT:
   - **a.** affect potassium channels.
   - **b.** affect sodium channels.
   - **c.** affect chloride channels.
   - **d.** affect calcium channels.
   - **e.** affect initial depolarization of the action potential.

3. Which of the following statements regarding widow spiders is TRUE?
   - **a.** Widow spiders are exclusively found in tropical regions.
   - **b.** Both male and female widow spiders bite and envenomate humans.
   - **c.** The widow spider toxin decreases calcium concentration in the synaptic terminal.
   - **d.** Alpha-latrotoxin stimulates increased exocytosis from nerve terminals.
   - **e.** A severe alpha-latrotoxin envenomation can result in life-threatening hypotension.

4. Which of the following diseases is not commonly caused by tick envenomation?
   - **a.** Rocky Mountain spotted fever.
   - **b.** Lyme disease.
   - **c.** Q fever.
   - **d.** ehrlichiosis.
   - **e.** cat scratch fever.

5. Which of the following is NOT characteristic Lepidoptera envenomation?
   - **a.** increased prothrombin time.
   - **b.** decreased fibrinogen levels.
   - **c.** decreased partial thromboplastin time.

d. increased risk of hemorrhaging.
e. decreased plasminogen levels.

6. A species of which of the following animals produces a venom that contains 60% formic acid?
   a. snakes.
   b. lizards.
   c. ants.
   d. spiders.
   e. scorpions.

7. Which of the following animals has a venom containing histamine and mast cell-degranulating peptide that is known for causing hypersensitivity reactions?
   a. bees.
   b. ants.
   c. snakes.
   d. spiders.
   e. reduviids.

8. Which of the following enzymes is not typically found in snake venoms?
   a. hyaluronidase.
   b. lactate dehydrogenase.
   c. collagenase.
   d. phosphodiesterase.
   e. histaminase.

9. All of the following statements are true EXCEPT:
   a. genetic variability plays a role in the toxicity of a plant.
   b. plant toxins are most highly concentrated in the leaves.
   c. young plants may have a higher toxin concentration than older plants.
   d. the weather can influence the toxicity of plants.
   e. soil composition can alter a plant’s production of toxin.

10. Activation of a vanilloid receptor is characteristic of which of the following chemicals?
    a. acetylandromedol.
    b. capsaicin.
    c. colchine.
    d. ergotamine.
    e. linamarin.

CHAPTER 27

Food Toxicology: Fundamental and Regulatory Aspects[1]

Supratim Choudhuri

- Furocoumarins
- Gossypol
- Erucic Acid
- Lectins
- Phytic Acid (Phytate)
- Oxalic Acid
- Enzyme Inhibitors
- Phytoestrogens

Some Naturally Occurring Toxins in Food of Marine Origin

- Saxitoxin
- Okadaic Acid (OA), Dinophysistoxin (DTX), Pectenotoxin (PTX), Yessotoxins (YTX)
- Domoic Acid (DA)
- Brevetoxins
- Ciguatoxin
- Tetrodotoxin
- Scombroid Toxicity
- Palytoxin

Some Microbial Contaminants in Food

Some Foodborne Molds and Mycotoxins

- Aflatoxins
- Trichothecenes
- Fumonisins
- Ochratoxin A
- Ergot Alkaloids

Bovine Spongiform Encephalopathy

Some Toxicants Generated in Food During Cooking or Processing

- Polycyclic Aromatic Hydrocarbons
- Heterocyclic Amines
- *N*-Nitroso Compounds
- Acrylamide

KEY POINTS

- Food is an exceedingly complex mixture of nutrient and nonnutrient substances.
- A substance listed as Generally Recognized as Safe (GRAS) achieves this determination on the adequacy of safety, as shown through scientific procedures or through experience based on common use.
- An estimated daily intake (EDI) is based on two factors: the daily intake of the food in which the substance will be used and the concentration of the substance in that food.
- Food hypersensitivity (allergy) refers to a reaction involving an immune-mediated response, including cutaneous reactions, systemic effects, and even anaphylaxis.
- The vast majority of food-borne illnesses in developed countries are attributable to microbiologic contamination of food.

INTRODUCTION TO FOOD TOXICOLOGY

The practice of food toxicology involves detecting toxic substances in food, characterizing their properties, studying their fate in the body (absorption, distribution, metabolism, and excretion), and investigating their adverse health effects. Toxic substances can be naturally present in food, formed when the food is cooked, added directly to food, or they can find their way into food from the immediate environment, such as packaging. Typically, a food contains hundreds of

substances. Apart from the most obvious constituents, most substances in foods have not been fully characterized. The assumption that food is safe is based on the history of its consumption.

NATURE AND COMPLEXITIES OF FOOD

Food is a complex mixture of nutrient and non-nutrient substances, whether it is consumed uncooked or in a cooked and/or highly processed ready-to-eat form. Many non-nutrient substances are necessary for the growth and survival of the plant (from which they are derived). Some of these substances may act as antinutrients rather than frank toxins, such as trypsin and chymotrypsin inhibitors, phytates that bind minerals, anti-thiamines in fish and plants, etc. Nutrients in food are classified as calorigenic *macronutrients*, such as carbohydrates, proteins, and fats, and the non-calorigenic *micronutrients*, such as vitamins and minerals.

The Food and Nutrition Board of the Institute of Medicine (IOM) developed Dietary Reference Intakes (DRIs) that serve as a guide for good nutrition. The DRIs provide a set of four nutrient-based reference values: Estimated Average Requirement (EAR), Recommended Dietary Allowances (RDA), Adequate Intake (AI), and Tolerable Upper Intake Level (UL).

The EAR is the average daily nutrient intake level estimated to meet the requirements of 50% of healthy individuals in a group, and the RDA is the daily dietary intake level sufficient to meet the nutrient requirements of nearly all healthy individuals in a group. The RDA is the EAR plus two standard deviations (RDA = EAR + 2SD). If sufficient information is not available to establish an EAR and calculate the RDA, an AI level is developed; AI represents the approximate estimates of nutrient intake by healthy people. The UL is the highest level of daily nutrient intake that is not likely to pose any risk of adverse health effects. Therefore, nutrient intake levels beyond the UL represent a transition from nutrition to potential toxicity in an exposure-dependent manner.

IMPORTANCE OF THE GASTROINTESTINAL TRACT

The gastrointestinal (GI) tract is a large, complex, and dynamic organ with a vast absorptive surface. The digestive system consists of the GI tract, and the salivary glands, liver, pancreas, gallbladder, and the gut microbiome, which in adult humans is roughly 40 trillion organisms.

The enterocytes of the intestinal epithelium are capable of extensive metabolism of xenobiotics that gain entry through the GI tract, as it possesses a full complement of phase I and phase II xenobiotic metabolizing enzymes and transporters. The GI tract is also an immunologic organ and is constantly exposed to antigens in food. The immune system of the GI tract is referred to as gut-associated lymphoid tissue (GALT). It is heavily populated by lymphocytes, macrophages, and other cells that participate in immune responses. One cell layer away from these antigens is the lamina propria of the GI tract, which contains the mucosal-associated lymphoid tissue (MALT), comprising lymphocytes and antigen-presenting cells, as well as unique dendritic cells, which interact with dietary antigens and ultimately determine whether an antigen is tolerated or an immune response is initiated.

Transporters and Absorption

The constituents of food and other ingesta (e.g., drugs, contaminants, inhaled pollutants dissolved in saliva and swallowed) are physicochemically heterogeneous and, various membrane transport proteins are involved in regulating the absorption, distribution, and excretion of food, drugs, and other substances. The role of some transporters in the absorption of various macro- and microingredients in food is shown in Table 27–1. The strategic localization of transporters on the apical versus the basolateral side of the cells is crucial for proper directional transport. In *primary active transport* (1° active), ATP directly provides the energy needed to move a molecule against its concentration gradient. In *secondary active transport* (2° active), the electrochemical gradient of an ion (e.g., Na^+ or H^+), already established through primary active transport, is utilized to move a molecule across the membrane. In *facilitative (facilitated) or passive transport*, transporters passively move substrates down the concentration gradient from high to low concentration.

TABLE 27–1 An Overview of Some Nutritionally Relevant Transporters

Transporter	*Slc* (Gene) Family Designation	Known Substrates
Sugar Transporters		
SGLT (2° Active)	*SLC5A*	Glucose, galactose
SGLT1—high-affinity but low-capacity glucose transporter that also transports galactose		
SGLT2—low-affinity but high-capacity glucose transporter that does not transport galactose		
GLUT (Facilitative)	*SLC2A*	Glucose, fructose, myo-inositol, glucosamine, dehydroascorbate
The major glucose transporter across the basolateral membrane		
Amino Acid Transporters		
AA TRANSPORTERS	*SLC 1A, 6A, 7A, 16A, 36A, 38A, 43A*	Amino acids, monocarboxylates (e.g., lactate, pyruvate)
(2° Active or Facilitative depending on the transporter)		
Peptide Transporters		
PEPT (2° Active)	*SLC15A*	Dipeptides, tripeptides
Fatty Acid Transporters		
MCT (2° Active)	*SLC16A*	Short-chain fatty acids (monocarboxylates, e.g., acetate, lactate, pyruvate, butyrate, propionate, succinate)
FATP	*SLC27A*	Long-chain fatty acids
Nucleoside Transporters		
CNT (2° Active)	*SLC28A*	Nucleosides
ENT (Facilitative)	*SLC29A*	Nucleosides
Water-Soluble Vitamin Transporters		
SVCT (2° Active)	*SLC23A*	Vitamin C (L-ascorbate)
SMVT (2° Active)	*SLC5A6*	Biotin, pantothenate, α-lipoate
PCFT (2° Active)	*SLC46A1*	Folate, antifolate (e.g., methotrexate)
RFC1 (2° Active)	*SLC19A1*	Folate, antifolate (e.g., methotrexate)
THTR (Facilitative)	*SLC19A2 & SLC19A3*	Thiamine
Fat-Soluble Vitamin Transporters		
SR-B1		Vitamin D, vitamin E, lutein, lycopene, cholesterol, cholesteryl esters
NPC1L1		Vitamin D, vitamin E, cholesterol
Other Transporters		
OCTN, CT2 (2° Active or Facilitative depending on the transporter)	*SLC22A*	L-Carnitine
TAUT	*SLC6A6*	Taurine
ZIP	*SLC39*	Metals (e.g., Zn)
ZnT	*SLC30*	Metals (e.g., Zn)
Nramp (Nramp1; Nramp2 also known as DCT1/DMT1)	*SLC11A*	Divalent metals and iron

Adapated from Choudhuri S, Chanderbhan RF. The biology of nutrients: genetic and molecular principles. In: Gupta RC, ed. *Nutraceuticals: Efficacy, Safety and Toxicity*. London: Elsevier; 2016:209–225.

Like food, water is absorbed by special means in the GI tract. Fluid transport is a major function of the GI tract, with more than 9 L of fluid being absorbed or secreted across epithelia in human salivary gland, stomach, the hepatobiliary tract, pancreas, small intestine, and colon.

The movement of water across the cell membrane can occur by several mechanisms. The membrane phospholipid bilayer is basically permeable to small polar molecules like water. Large volumes of water are cotransported along with ions and solutes. Water is also transported by water channels (aquaporins) in response to osmotic gradients created by active solute transport. Aquaporins also serve in the permeation of small molecules such as glycerol and urea.

FOOD CONSTITUENTS AND GENOTYPIC VARIATIONS: NUTRITIONAL IMPLICATIONS OF INTER-INDIVIDUAL GENETIC DIFFERENCES

There are inter-individual genetic differences in taste preference, food tolerance, metabolism, absorption of food constituents, and other nutrition-related characteristics in human population. Many genotypic variations that conferred selective advantage in an environment were fixed in a population. Just as a genotypic variation could determine the ability to utilize food constituents, food habits could also drive nutritional adaptation.

Many genotypic variations are allelic variants created through single nucleotide polymorphisms (SNPs) and copy number variations (CNVs). The inter-individual differences in the ability to produce functional enzymes, transporters, and other proteins that aid in the digestion, transport, and assimilation of nutrients are important determinants of personalized nutrition.

Genotypic Variations and the Ability to Utilize Food Constituents

The lack of ability to utilize nutrients often results in mild-to-severe pathological conditions. For example, two polymorphisms in the methylenetetrahydrofolate reductase (*MTHFR*) gene [C677T → Ala222Val (A222V) and A1298C → Glu429Ala (E429A)] are associated with higher homocysteine levels in the blood. Mutations downregulating glutathione *S*-transferase M1 (GSTM1) expression are associated with more rapid excretion of sulforaphane, an isothiocyanate with anti-tumor effects obtained from cruciferous vegetables, such as broccoli, brussel sprout, and cabbage. Likewise, polymorphisms in the genes encoding UDP-glucuronosyltransferase (*UGT*) and sulfotransferase (*SULT*) may contribute to variability in phytochemical (e.g., flavonoid) clearance and efficacy. Lactose malabsorption is due to the cessation of expression of the enzyme lactase with age in certain populations, with other populations maintaining normal lactase expression throughout life.

Mutations in Zn transporters may limit the uptake of zinc (Zn). Mutations in ZIP4, an uptake transporter involved in intestinal zinc uptake, can cause acrodermatitis enteropathica (AE). In contrast, mutations in ZnT2 cause transient neonatal zinc deficiency (TNZD) with symptoms similar to AE. ZnT2 is responsible for supplying human milk with zinc. AE is fatal if zinc is not supplied to the infant after weaning, whereas TNZD is a genetic defect of the mother limiting the

supply of zinc in the milk, and the infant usually will obtain enough zinc once weaned.

Food Habits Driving Nutritional Adaptation During Evolution

The genes coding for amylase (*AMY1*), lactase (*LCT*), and alcohol dehydrogenase (*ADH*) provide three of the most interpretable examples of genetic adaptations to dietary specializations in human metabolism.

Starch Digestion—The alpha-amylase (*AMY1*) gene encodes salivary alpha-amylase that helps digest starch. Salivary amylase can persist in the stomach and intestine after swallowing, thereby augmenting the enzymatic activity of pancreatic amylase.

Lactose Digestion—The lactase gene (*LCT*) in the intestine produces the enzyme lactase that digests the lactose in milk. Most mammals, including humans, lose the ability to digest lactose with age due to reduced lactase expression. The persistence of the lactase expression phenotype (LP), and the continuing ability to digest milk lactose, is inherited as a dominant genetic trait.

Alcohol Metabolism—Ethanol is oxidized to acetaldehyde by alcohol dehydrogenase (ADH), and acetaldehyde is subsequently oxidized to acetic acid by aldehyde dehydrogenase (ALDH). One of the best-studied polymorphisms influencing ethanol metabolism is *ADH1B* Arg47His. The *ADH1B*47His* allele (previously called *ADH2*2*) causes enhanced catalytic activity, resulting in an increased metabolism of ethanol, and increased blood levels of acetaldehyde. If acetaldehyde is not metabolized rapidly by ALDH, chronic tissue exposure to acetaldehyde could result in significant adverse consequences including cancer. Therefore, ALDH deficiency, which is seen among East Asians, together with an increased catalytic activity of ADH results in an increased blood level of acetaldehyde. This causes flushing, intolerance, and discomfort associated with alcohol drinking. Molecular dating suggests that the emergence of this allele coincides with the origin of rice domestication in East Asia. It has been proposed that the rise of *ADH1B*47His* allele frequency was an adaptation to rice domestication and the subsequent production and consumption of fermented food and/or beverages.

FOOD CONSTITUENTS AND REGULATION OF GENE EXPRESSION

Growing *Escherichia coli* in lactose instead of glucose led to the de novo transcription and expression of three enzymes: β-galactosidase, *trans*-acetylase, and permease that are all involved in the uptake and utilization of lactose as the carbon source. Likewise, nutrients such as glucose, amino acids, fatty acids, as well as vitamins and minerals are known to impact gene expression.

Carbohydrates and Gene Expression

Red blood cells and the brain primarily rely on a direct supply of glucose as their fuel source. Therefore, for most genes regulated by a rich carbohydrate diet, the induction or inhibition of their mRNAs is substantial, relatively rapid, and reversible once the carbohydrate availability

decreases. Both insulin and glucose contribute to the coordinated regulation of carbohydrate and lipid metabolism in the liver.

Glucose-6-phosphate (G6P) is a likely signaling molecule regulating the glucose-induced transcription of the target genes. Two transcription factors—the sterol regulatory element (SRE)-binding protein 1c (SREBP-1c) and the carbohydrate response element (ChRE)-binding protein (ChREBP)—play crucial roles in insulin- and glucose-mediated transcriptional regulation of genes involved in carbohydrate metabolism.

SREBP-1c is a major mediator of insulin action on hepatic glucokinase and lipogenic gene expression. It is rapidly and robustly induced by a carbohydrate-rich diet (resulting in a high-insulin level) and downregulated by fasting (resulting in a low-insulin level). The induced SREBP-1c may preferentially regulate genes involved in de novo lipogenesis, whereas SREBP-2 may regulate genes in cholesterol synthesis and uptake.

ChREBP is predominantly expressed in the liver, kidney, and white and brown adipose tissue. ChREBP forms a heterodimer with Max-like protein X (Mlx) that binds to the carbohydrate response element (ChRE) located in the promoters of glucose target genes, such as the *L-PK*, *FAS*, and *ACC*. The ChREBP–Mlx complex is able to discriminate between the E-box sites that are glucose-responsive and those that are not. Figure 27–1 shows possible signaling pathways for transcriptional regulation of target genes in the liver mediated by glucose and insulin.

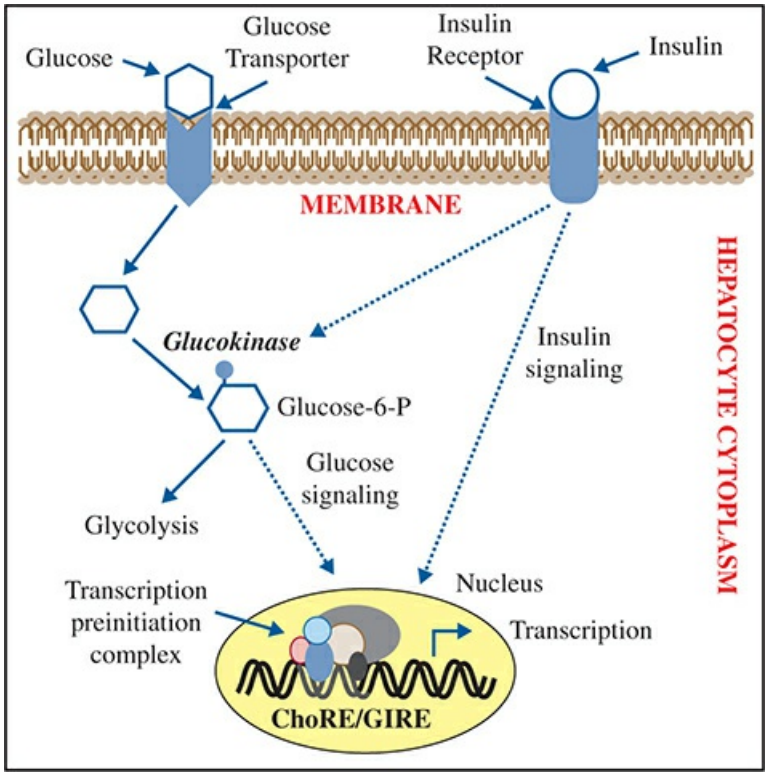

FIGURE 27–1 *Transcriptional regulation by glucose and insulin signaling in the liver.* Glucose-6-phosphate is the most likely signaling molecule regulating glucose-induced transcription, and its formation is an important step in glucose-signaling pathway. Insulin-signaling pathway may involve insulin-responsive transcription factor SREBP-1c.

Amino Acids and Gene Expression

Amino acid availability regulates the expression of many genes both in vivo and in vitro. Some examples of genes upregulated by amino acid starvation include the enzymes of amino acid synthesis pathway (e.g., asparagine synthetase, glutamine synthetase), enzymes of the urea cycle (e.g., argininosuccinate synthetase), the rate-controlling enzyme in polyamine biosynthesis pathway (e.g., ornithine decarboxylase), transporters (e.g., sodium-coupled neutral amino acid transporter SNAT2, cationic amino acid transporter CAT1, amino acid transporter ATA2), ribosomal proteins (e.g., L17, L35, S13, and S25), transcriptional activator proteins, such as CCAAT/enhancer-binding protein (C/EBP) and C/EBP homologous protein (CHOP), insulin-like growth factor-binding protein-1 (IGFBP-1), and proto-oncogenes (jun and myc).

Fatty Acids and Gene Expression

Fatty acids and their acyl-CoA derivatives serve as metabolic energy sources, substrates for membranes, signaling molecules for metabolic regulation, and regulators of gene expression. The uptake of fatty acid across the plasma membrane is mediated by distinct transport proteins (Table 27–1). Once within the cell, most fatty acids are non-covalently bound to fatty acid-binding proteins (FABPs), which are involved in reversibly binding intracellular hydrophobic ligands, and trafficking them throughout cellular compartments, including the peroxisomes, mitochondria, endoplasmic reticulum, and nucleus.

The regulation of gene expression by fatty acids is dictated by the type, quantity, and the duration of exposure to dietary fats. Among short-chain fatty acids (SCFAs), such as acetate, propionate, and butyrate, butyrate halts DNA synthesis, arrests cell proliferation, alters cell morphology, and increases or decreases gene expression. Butyrates were found to increase gene expression by inhibiting histone deacetylase activity, thereby increasing histone acetylation, which promotes transcriptional activation. The long-chain fatty acids (LCFAs) potentially regulate gene expression by functioning with peroxisome proliferator-activated receptor (PPAR) and prostanoid-regulated processes. Three different PPAR subtypes—PPARα, PPARβ (or δ), and PPARγ—bind polyunsaturated fatty acids (PUFAs), such as arachidonic acid (ARA), docosahexaenoic acid (DHA), and others.

Surprisingly, circulating free fatty acids (FFAs) that primarily originate from adipose tissue lipolysis (so-called *old fat*) cannot activate PPARα in the liver. In contrast, dietary and endogenously synthesized fatty acids (so-called *new fat*) can. This difference may be related to the existence of (as yet unknown) differential signaling properties between the two forms of FFAs. However, the plasma FFAs (the *old fat*) can activate hepatic PPARγ.

Vitamins and Gene Expression

Like the macronutrients (protein, carbohydrate, and fat), micronutrients (vitamins and minerals) can also modulate gene expression. Retinoic acid, a bioactive form of vitamin A, is a ligand for the nuclear retinoic acid receptor (RAR). Ligand-activated RAR heterodimerizes with RXR; the RAR–RXR complex binds to the cognate retinoic acid response element (RARE) in the regulatory region of the target genes and regulates gene expression. The expression of *PEPCK* gene is a target of retinoic acid. Although high levels of retinoic acid exposure can be teratogenic to the developing embryo, retinoic acid targets many genes including the developmentally

important homeobox (*Hox*) genes, such as *Hoxa1*, *Hoxb1*, *Hoxb4*, and *Hoxd4*. All the target genes harbor RARE. Dietary vitamin A levels can also modulate the expression of genes encoding insulin-like growth factor I (IGF-I), IGF-I receptor (IGF-IR), and insulin receptor (IR) in many different tissues. Serum IGF-I concentrations and tissue *IGF-I* mRNA are downregulated by vitamin A deficiency.

The model of vitamin D (1,25-dihydroxyvitamin D_3)-mediated gene expression involves similar steps of events as for vitamin A, that is, vitamin D binding–mediated activation of vitamin D receptor (VDR), formation of VDR–RXR complex, its binding to VDRE, and modification of transcriptional output. Genome-wide mapping of vitamin D receptor binding revealed that vitamin D could influence over 200 genes. Vitamin D plays an important role in regulating genes involved in bone remodeling and the maintenance of bone health, such as *VDR*, *TNFSF11*, *LRP5*, *CBS*, and *CYP24a1*.

Minerals and Gene Expression

Dietary non-heme ferric iron Fe(III) is first reduced by enterocyte membrane-bound ferrireductases into the ferrous Fe(II) form. The apical transporter divalent metal transporter 1 (DMT1) transports Fe(II) from the intestine into the enterocytes. The Fe(II) is translocated through the enterocyte and is exported into the circulation by the basolateral exporter ferroportin. During export, Fe(II) is oxidized by hephaestin, which is a membrane-bound ferrooxidase. Thus, hephaestin is necessary for effective iron transport from enterocytes into the circulation. In the circulation, Fe(III) binds to plasma transferrin (Tf) to be distributed throughout the body, and taken up primarily by the liver. In the liver, the Fe(III)–Tf complex binds to the membrane transferrin receptor (TfR). The Fe(III)–Tf–TfR complex is endocytosed inside the cell. The acidic pH of the endosome releases the Fe(III), which is again reduced to Fe(II) by the intracellular ferrireductase STEAP3 (Fig. 27–2).

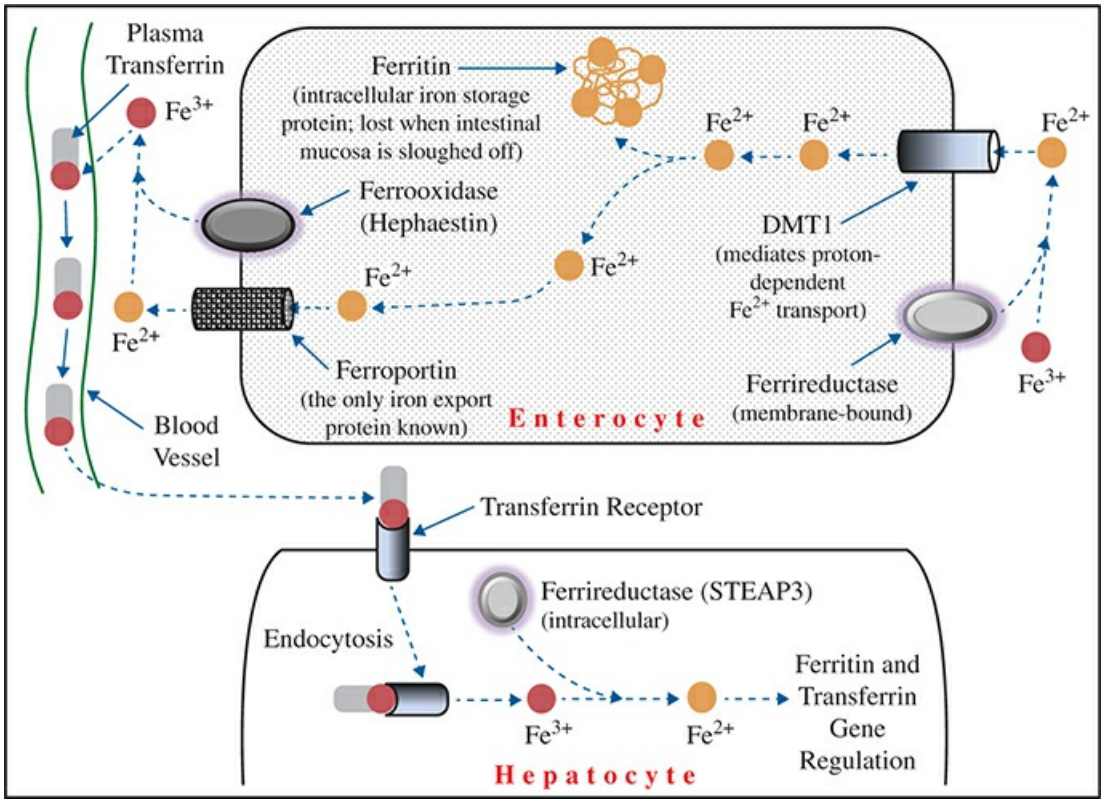

FIGURE 27–2 *The transport of dietary iron from the intestine to the liver.* Non-heme ferric iron Fe(III) is first reduced by enterocyte membrane–bound ferrireductases into ferrous Fe(II) form. The apical transporter divalent metal transporter 1 (DMT1) transports Fe(II) into the enterocytes. The Fe(II) is translocated through the enterocyte and is exported into the circulation by the basolateral exporter ferroportin. During export, Fe(II) is oxidized by hephaestin, which is a membrane-bound ferrooxidase. In the circulation, Fe(III) binds to plasma transferrin to be distributed throughout the body, and taken up primarily by the liver. In the liver, the Fe(III)–Tf complex binds to the membrane transferrin receptor (TfR). The Fe(III)–Tf–TfR complex is endocytosed inside the cell. The acidic pH of the endosome releases the Fe(III), which is again reduced to Fe(II) by the intracellular ferrireductase STEAP3.

The intracellular iron storage is regulated at multiple levels. When body iron stores and erythropoiesis are adequate, iron export from enterocytes into the circulation is reduced by hepcidin-mediated internalization and degradation of Fe–ferroportin complex. The iron released from ferroportin is stored in ferritin. Under such conditions, the hepatic iron uptake is also reduced by downregulating the expression of the transferrin receptor through degradation of the transferrin receptor mRNA. In the enterocytes, the stored iron in ferritin is lost after 3 days by desquamation of intestinal cells. Iron regulation of gene expression requires the presence of a 30-nucleotide long iron response element (IRE) in the 5′-untranslated or 3′-untranslated region of the target mRNAs. The IRE binds the iron regulatory protein (IRP), also called IRE-binding protein (IRE-BP). The IRPs are cellular iron sensors. The mRNAs associated with iron transport, distribution, and storage all contain IREs.

Dietary Polyphenols, Indoles, Carotenoids, and Gene Expression

Dietary polyphenolic constituents present in fruits, vegetables, and spices can mediate nutrient–gene interactions by activating or inhibiting specific transcription factor pathways, including both flavonoid polyphenols (e.g., quercetin, kaempferol, apigenin, tangeretin), and non-flavonoid polyphenols (e.g., resveratrol, curcumin). Indole-3-carbinol (I3C), present in cruciferous vegetables, and dietary carotenoids (e.g., canthaxanthin, astaxanthin) also mediate nutrient–gene interactions. I3C is derived from glucobrassicin (glucosinolate). These dietary constituents regulate gene expression through modulation of the aryl hydrocarbon receptor (AhR) pathway.

FOOD–DRUG INTERACTIONS

Food–drug interactions can alter pharmacokinetic and pharmacodynamic parameters of the drug. For drugs with a narrow therapeutic index and the need for dose titration, even small changes in the dose–response effects can have great consequences. Some food–drug interactions, such as the effects of dietary constituents on the induction or inhibition of CYP3A4, may be potentially important because CYP3A4 metabolizes more than 50% of all drugs consumed by humans. Thus, induction of CYP3A4 might enhance the clearance and reduce the efficacy of a drug. In contrast, inhibition of CYP3A4 might prevent the drug's metabolism, resulting in a gradual build-up of its serum concentration and causing toxicity. Examples of foods affecting some phase I and phase II enzymes, and transporters are provided in Table 27–2.

TABLE 27–2 Food–Drug Interactions (Activity May be Enhanced or Inhibited)

Enzyme Or Transporter	Food	Drug
CYP1A2	Caffeine, theophylline, grapefruit juice (naringenin and furanocoumarins bergamottin and dihydroxybergamottin), grape juice, cruciferous vegetables, apiaceous vegetables, cooked meat	Clozapine, fluvoxamine, imipramine
CYP2E1	Watercress and possibly other isothiocyanate-containing cruciferous vegetables; polyunsaturated fatty acids (corn oil, menhaden oil)	Ethanol, halothane, enflurane
CYP3A4	Grapefruit juice, orange juice, red wine, possibly other polyphenol-containing substances, St Johns wort, garlic	Ketoconazole, cyclosporin, erythromycin, protease inhibitors, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors
UGT and GST	Brussel sprouts, cabbage, watercress, broccoli	Acetaminophen, oxazepam, morphine, ibuprofen
P-glycoprotein and OATP	Vegetables, fruit juice, St Johns wort	Digoxin, cyclosporine, pravastatin

Abbreviations: GST, glutathione-S-transferases; OATP, organic anion–transporting polypeptides; UGT, uridine diphosphate glucuronosyltransferases.

FOOD SAFETY REGULATIONS IN THE UNITED STATES

The U.S. Food and Drug Administration

The FDA has a wide responsibility for protecting public health that includes ensuring the safety of the nation's food supply. Within FDA, the Center for Food Safety and Applied Nutrition (CFSAN) is responsible for:

- The safety of substances added to food, e.g., food additives (including sources of radiation) and color additives
- The safety of foods and ingredients developed through biotechnology
- Seafood and juice Hazard Analysis and Critical Control Point (HACCP) regulations
- Regulatory and research programs to address health risks associated with foodborne, chemical, and biological contaminants
- Regulations and activities dealing with the proper labeling of foods (e.g., ingredients, nutrition health claims)
- Regulations and policy governing the safety of dietary supplements, infant formulas, and medical foods
- Food industry postmarket surveillance and compliance
- Industry outreach and consumer education
- Cooperative programs with state, local, and tribal governments
- International food standard and safety harmonization efforts

Chemical risk assessment is a critical aspect of food toxicology; the chemicals of interest are natural toxicants found in food, substances intentionally added to food, substances that unintentionally become part of food, and contaminants.

Historical Perspective on the Science of Food Toxicology

The history of food toxicology as it relates to substances intentionally added to food or becoming part of food via food processing or contamination began in the last decades of the 19th century.

History of Food Law

The current federal law governing food safety is the result of incremental legislative actions over a period spanning more than 100 years. It began with the passage of the Pure Food and Drug Act of 1906, which declared a food to be adulterated if it contained “any added poisonous or other added deleterious ingredient which may render such article injurious to health.” Two concepts, adulterated and misbranded, still provide the legal basis for food safety regulations.

The Food, Drug, and Cosmetic Act (FD&C Act) of 1938 clarified the regulation that foods under this statute are considered adulterated if they contain added poisonous or deleterious substances that “may render the food injurious to health.” The FD&C Act of 1938 introduced the concept of premarket approval, which was required only for drugs, not food ingredients.

Current U.S. Food Law to Ensure Safety of Foods, Food Ingredients, and Food Contaminants

Recent amendments to the FD&C Act include the Food Additives Amendment of 1958, the Color Additive Amendments of 1960, and FDA Modernization Act of 1997.

FD&C Act, FDA Regulations, and FDA Guidance—The FD&C Act is a federal law enacted by Congress and contained in Title 21 of the United States Code. The Act along with all its

provisions can be accessed at the United States Code website (http://uscode.house.gov/browse/&edition=prelim).

FDA follows the procedures required by the Administrative Procedure Act (5 U.S.C.) to issue FDA regulations. This typically involves a process known as “notice and comment rulemaking” that allows for public input on a proposed regulation before FDA issues a final rule. The rules are first published in the *Federal Register*. Rules are then codified into regulations (laws) in the code of federal regulations (CFR). Therefore, FDA regulations are also federal laws, but they are not part of the FD&C Act. FDA regulations can be found in Title 21 of the Code of Federal Regulations (21 CFR). In contrast to FDA regulations that are federal laws, FDA guidance describes the agency’s current thinking on a regulatory issue, and its guidance is not legally binding on the public or FDA.

Food Additives

The 1958 Food Additives Amendment covers both direct and indirect additives. Direct additives are substances added deliberately to food for a specific technical purpose; such substances usually remain in food (Table 27–3). The technical effects could be to maintain or improve safety and freshness; improve or maintain nutritional value; or, improve taste, texture, and appearance. Secondary direct additives are added to food or food components during their processing, but which are usually removed from the food before the product is consumed. Indirect additives that may become components of food, include materials that may migrate from food contact surfaces during production, manufacturing, packing, processing, preparing, treating, packaging, transporting, or holding food. Both indirect and some secondary direct additives are now referred to as food contact substances (FCSs).

TABLE 27–3 Some Direct Food Additives by Functionality

Number	Designation	Description	Examples
170.3(o)(1)	Anticaking agents and free-flow agents	Substances added to finely powdered or crystalline food products to prevent caking, lumping, or agglomeration	Glucitol, sodium ferrocyanide, silicon dioxide
(3)	Antioxidants	Substances used to preserve food by retarding deterioration, rancidity, or discoloration due to oxidation	Butylated hydroxyanisole, butylated hydroxytoluene, propyl gallate
(9)	Enzymes	Enzymes used to improve food processing and the quality of the finished food	Papain, rennet, pepsin
(12)	Flavor agents and adjuvants	Substances added to impart or help impart a taste or aroma in food	Cinnamon, citral, *p*-cresol, thymol, Zingerone
(19)	Non-nutritive sweeteners	Substances having less than 2% of the caloric value of sucrose per equivalent unit of sweetening capacity	Aspartame, neotame, sucralose, saccharin

Substances Generally Recognized as Safe (GRAS)—Many foods and food ingredients have a long history of safe use, such as butter, egg, salt, milk, vinegar, sugar, and others, which are generally recognized as safe (GRAS). The GRAS status of an ingredient can be based on either a safe history of "common use in food prior to 1958" or "scientific procedures" that demonstrate safety.

Color Additives

Color additives are defined in 21 CFR, Section §70.3 and general restrictions on their use are

listed in Section §70.5. The use of an unlisted color additive, improper use of a listed color additive, or use of a color additive that does not conform to the purity and identity specifications of the listing regulation may cause a product to be adulterated and subject to enforcement action by FDA.

The Delaney Clause and Its Applications—The Delaney clause states that no cancer-causing substance, as demonstrated in humans or animals, may be added to food. The 1960 Color Additive Amendments also contained the Delaney clause that prohibited the use of a color additive shown to be a carcinogen in animal feed or human food.

The Food Quality Protection Act (FQPA) of 1996 amended the zero-risk standard of the Delaney clause as it applies to pesticide residues in food. The new standard was based on quantitative risk assessment and determination of the lifetime risk of developing cancer from exposure to pesticide residues in food. In practice, EPA targets a risk level of less than one in one million people (10^{-6}) over the course of a typical human life span. Except for the situation of pesticide residues in food, the Delaney clause still applies to food safety in general.

A number of substances (e.g., butylated hydroxyanisole [BHA], xylitol, methylene chloride, sorbitol, trichloroethylene, nitrilotriacetic acid [NTA], diethylhexyl phthalate, melamine, formaldehyde, bentonite) are listed in the CFR as food additives, but they are also listed as carcinogens by various agencies. The reasoning applied by FDA in almost every case is based on secondary carcinogenesis. To restrict the applicability of the Delaney clause, FDA narrowed the definition of food additive to exempt constituents and contaminants that pose a de minimis risk. According to FDA's constituents' policy, food additive refers to the added substance as a whole and not to each of its individual constituents. For example, the constituents' policy was employed to approve the use of FD&C Green No. 5 and Green No. 6, both of which contain a nonfunctional contaminant *p*-toluidine that by itself causes cancer.

For new animal drugs, safety assessment is concerned primarily with residues that occur in animal food products (milk, cheese, etc.) and edible tissues (muscle, liver, etc.). Toxicity studies require data on the pharmacokinetics, metabolism, and the nature of any/all metabolites. The parent drug and its metabolites are evaluated both qualitatively and quantitatively in the animal products of concern (eggs, milk, meat, etc.).

To address the issue of residues of metabolites of animal drugs known to induce cancer in humans or animals, the FDA considered threshold assessment, which combines information on the structure and in vitro biological activity of a metabolite for the purpose of determining whether carcinogenicity testing is necessary. If testing is necessary and if the substance is found to induce cancer, the FDA's definition states that a lifetime risk of one in a million is equivalent to the meaning of "no residue" as intended by Congress.

The Safety Standard and Toxicological Testing Recommendations for Food Ingredients —The safety standard for food and color additives and other food ingredients is reasonable certainty of no harm. In 1982 the FDA published a document titled *Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food*. Popularly known as "the Redbook," FDA provided its updated recommendations for the minimum set of toxicological data normally needed for evaluating the safety for the use of direct food additives. The Redbook is available on the world-wide-web.

The Redbook utilizes the concept of structure–activity relationships of chemicals to assign substances added to food to categories. Additives with functional groups with a high order of

toxicity are assigned to category C, those with low potential for toxicity are assigned to category A, while those of unknown or intermediate toxicity are assigned to category B. Further, assigned concern levels are relative measures of the degree to which the use of an additive may present a health hazard. Concern level III represents the highest probable risk to human health, Concern level I represents the lowest probable risk, and Concern level II is intermediate between high and low risk. Listed in Table 27–4 are the recommended initial, minimum testing batteries for substances assigned to Concern levels I, II, and III. More extensive testing may be needed to resolve scientific questions that may arise during research and development.

TABLE 27–4 Recommended Toxicological Testing Based on Concern Level Assignment

Toxicity Studies	Concern Levels		
	I	II	III
Genetic toxicity tests	X	X	X
Short-term toxicity tests in rodents	X	X	X
Subchronic toxicity studies in rodents		X	X
Subchronic toxicity studies in nonrodents		X	X
One-year toxicity studies in nonrodents			X
Chronic toxicity or combined chronic toxicity/ carcinogenicity tests in rodents			X
Carcinogenicity study in rodents			X
Reproduction studies with teratology phase		X	X
Developmental toxicity studies		X	X
Metabolism and pharmacokinetic studies		X	X
Human studies			X

Data from The Redbook. Available at: http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/IngredientsAdditivesGRASPackaging/ucm054658.htm.

ADI, EDI, and Reasonable Certainty of No Harm—The goal of the toxicological testing of a food additive is to determine its acceptable daily intake (ADI). The ADI is compared to the estimated daily intake (EDI) of the additive to determine whether the proposed use of the additive falls within the bounds of "reasonable certainty of no harm." In order to calculate the ADI, FDA uses the longest duration study in the most appropriate animal species to establish the no-observed-adverse-effect-level (NOAEL). The NOAEL is the highest dose that does not cause any observed adverse effects. By applying an appropriate safety factor to this NOAEL, the ADI is obtained. The standard safety factor is usually 100, which is derived by multiplying a factor of 10 to account for interspecies variation with an additional factor of 10 to account for intraspecies

variation in the sensitivity of response to a chemical. Hence, ADI = NOAEL /100.

For micronutrients and macroingredients, it may not be possible to apply a 100-fold safety factor to a NOAEL and have an adequate dietary intake to allow them to fulfill their physiological roles or their technological purpose, respectively. In such instances, 10-fold or lower safety factors are not uncommon. Typically, if the EDI is less than the ADI (in the same units, such as mg/kg body weight/day), use of the additive or a GRAS ingredient is considered to lie within the bounds of "reasonable certainty of no harm," for its intended use in food.

Dietary Intake Estimate—Dietary exposure is an important contributor to the development of the concern level. Estimates of intake of an ingredient or chemical constituent of food require two key pieces of information: (1) the concentration of the substance in a food, and (2) consumer intake of foods that will or might contain the substance. Sources that provide intake estimates of substances in the diet include (1) food consumption surveys; (2) food/ingredient disappearance data; (3) total diet study; and (4) body burden/excretion measurements, or "biomarkers." Food consumption data may be collected at the national, household, or individual level.

The relationship of substance concentration data, portion size, and food consumption frequency to the estimated daily intake (EDI) of a substance X for a single individual is captured in the following equation:

$$\mathrm{EDI}_x = \sum_{f=1}^{F} \frac{\mathrm{Freq}_f \times \mathrm{Port}_f \times \mathrm{Conc}_{xf}}{N}$$

where

F = Total number of foods in which substance "x" can be found
Freq$f$ = Number of eating occasions of food "f" over "N" survey days
Port$f$ = Average portion size for food "f"
Concxf = Concentration of the substance "x" in food "f"
N = Number of survey days

Threshold of Regulation (TOR)—TOR policy established procedures for exempting indirect food additives (now called FCSs) from premarket evaluation if the likely dietary exposure to humans from the migrating substances is below 0.5 ppb. The TOR policy is based upon the notion that below certain dietary exposure levels, additives migrating into food will present only negligible risk.

Food from New Plant Varieties—FDA regulates the safety of food for humans and animals produced from new plant varieties, including genetically engineered (GE) plants. Foods from GE plants must meet the same safety requirements as foods derived from traditionally bred plants.

In the United States, new plant varieties are regulated not only by FDA, but also by the EPA and USDA. FDA is responsible for the safety and labeling of foods and feeds derived from crops, irrespective of the method used to produce the new plant variety. The EPA is responsible for assuring the safety of pesticides; thus, if a pesticidal protein is produced in a new plant variety, this product would also fall under EPA's jurisdiction. The USDA's Animal and Plant Health Inspection Service (APHIS) has responsibility for the environmental safety of field testing and commercial planting of new plant varieties. The developer of a biotechnology-derived crop variety must obtain registration from not only the country of origin but importing

countries as well.

The Food Allergen Labeling and Consumer Protection Act (FALCPA)—FALCPA requires manufacturers to identify the presence of ingredients that contain proteins derived from milk, eggs, fish, crustacean shellfish, tree nuts, peanuts, wheat, or soybeans, and also specify the source when possible. Because the source of ingredients such as lecithin, flour, or whey may not be obvious to the consumer, the source must follow the name of the ingredient. For example, lecithin, flour, and whey must be given on the labels as follows: lecithin (soy), flour (wheat), and whey (milk); alternatively, a "contains" statement "Contains wheat, milk, and soy" may be used.

Food Safety Through the Hazard Analysis and Critical Control Point (HACCP) System —The HACCP is a management system in which food safety is addressed through the analysis and control of biological, chemical, and physical hazards from raw material production, procurement, and handling, to manufacturing, distribution, and consumption of the finished product. The seven principles of HACCP are (1) hazard analysis, (2) identification of CCPs, (3) establishing the critical limits, (4) monitoring control of the CCPs, (5) establishing corrective actions, (6) establishing verification procedures, and (7) establishing documentation and record-keeping systems.

The Food Safety and Modernization Act (FSMA)—FSMA aims to ensure the safety of the U.S. food supply by shifting the focus from responding to contamination to preventing it. The major elements of FSMA include (1) preventive controls, (2) inspection and compliance, (3) imported food safety, (4) response, and (5) enhanced partnerships. The new food safety plan requires the identification and prevention of all reasonably foreseeable food safety hazards that could be natural or unintentionally introduced into the food facility. The Hazard Analysis and Risk-Based Preventive Controls (HARPC) plan is the process that a food company uses in each of its facilities to implement its food safety plan.

Regulation of Dietary Supplements—A dietary supplement is a product intended for ingestion that contains a dietary ingredient intended to add further nutritional value to (supplement) the diet. A dietary ingredient may be one, or any combination, of the following substances: a vitamin, a mineral, an herb or other botanical, an amino acid, a dietary substance for use by people to supplement the diet by increasing the total dietary intake, a concentrate, metabolite, constituent, or extract. The Dietary Supplement Health and Education Act of 1994 (DSHEA) makes manufacturers and distributors responsible for evaluating the safety and labeling of their products before marketing to ensure that they meet all the requirements of DSHEA and FDA regulations.

Global Food Safety Authoritative Bodies

In parallel with U.S. FDA's effort to assure the safety of food additives, similar efforts internationally include the Food Standards Australia and New Zealand (FSANZ), European Food Safety Authority (EFSA), and others.

TOXIC SUBSTANCES IN FOOD

The requirement for food varies with age and activity level. The average daily consumption of food is about 2 kg/day. Obviously, human beings consume a lot of materials as food daily. Thus, any hazard present in foods, such as toxic substances, antinutrients, or contaminants may have adverse health consequences.

Some Naturally Occurring Toxic Substances in Food

Plants produce a stunning array of chemicals to protect themselves from invading pathogens, insects, and herbivorous animals. Many of these chemicals are secondary metabolites that are not essential for growth and reproduction. Some of these secondary metabolites may have beneficial health effects on humans, usually at a low or moderate level of consumption. However, many others are toxic to animals and humans, particularly when consumed at high levels. The secondary metabolites usually belong to one of the three major chemical classes: *terpenoids* (contain isoprene units), *phenolics* (contain phenol group), and *alkaloids* (contain nitrogen). There are also nitrogen-containing non-alkaloids. Some of these toxic substances are fatty acids or proteins.

Cycasin—Cycasin is an azoxyglycoside found in cycads (e.g. *Cycas circinalis*) that produces a complex neurodegenerative disease known as amyotrophic lateral sclerosis-parkinsonism-dementia (ALS-PDC). The β-d-glucosidase of intestinal bacteria hydrolyzes cycasin into its toxic aglycone metabolite methylazoxymethanol.

Pyrrolizidine Alkaloids—Pyrrolizidine alkaloids (PAs) are found in many species of flowering plants. Some examples include *heliotrine*, *lycopsamine*, *lasciocarpine*, *retrorsine*, and *senecionine*. PAs are metabolized in the liver to electrophilic pyrrole derivatives, which cause hepatotoxicity in animals and veno-occlusive disease in humans. Many PAs also have genotoxic and carcinogenic properties.

Ptaquiloside—Ptaquiloside is a norsesquiterpene glucoside that is the major carcinogen in bracken fern (*Pteridium aquilinum*). It causes bladder and intestinal cancer in livestock, mammary and intestinal tumors in rats, as well as neurotoxicity in mice and sheep. In addition to the ptaquiloside (PTA), bracken fern also contains prunasin (a cyanogen glycoside) and thiaminase. The thiaminase splits thiamine (vitamin B1) into its two inactive components, pyrimidine and thiazole, causing thiamine deficiency.

Safrole—Safrole is typically extracted from the root-bark or the fruit of sassafras plants in the form of sassafras oil. The metabolic intermediates of safrole directly react with DNA forming adducts. Safrole is mutagenic and also genotoxic in various in vitro mammalian cell systems. Safrole is a precursor in the synthesis of the illegal recreational drug methylenedioxymethamphetamine (MDMA) or “Ecstasy.”

Cyanogenic Glycosides—Cyanogenic glycosides produce hydrogen cyanide (HCN; also known as prussic acid), which inhibits cellular respiration and causes cytotoxic hypoxia. Many common food plants contain cyanogenic glycosides, such as *linamarin* from cassava and *amygdalin* from cherry, apple, and peach pits.

Glucosinolates—Glucosinolates (nitrogen-containing non-alkaloid) are sulfur-containing

goitrogenic glycosides found in cruciferous vegetables. Enzymatic hydrolysis of glucosinolates produces several different products, such as isothiocyanates, thiocyanates, oxazolidinethiones, and nitriles that are thought to be responsible for the goitrogenic or antithyroid activity. Thiocyanate and oxazolidinethione anions compete with iodine by inhibiting its uptake by the sodium iodide symporter and its binding to tyrosine residues of thyroglobulin at high concentrations. Some examples of glucosinolates are *sinigrin*, *dehydroerucin*, and *glucobrassicin*. Symptoms of poisoning include depressed growth, goiters, enlarged livers, perosis (leg deformity in birds), and poor egg production in chicken.

Glycoalkaloids (α-Solanine and α-Chaconine)—Glycoalkaloids in potato are closely associated with greening of the peel due to exposure to light, mechanical damage, and improper storage conditions. The content of α-solanine and α-chaconine is not affected much by cooking but peeling significantly reduces these glycoalkaloids. α-Chaconine and α-solanine act as inhibitors of acetylcholinesterase and disruptors of cell membranes. Effects on the nervous system include increased heart, pulse, and respiratory rates, sedation, and coma. Effects resulting from cell membrane disruption included internal hemorrhaging, edema, diarrhea, constriction of the abdominal muscles, and lesions of the stomach and duodenum.

Tannins—Tannins are a group of plant phenolics. Tannins are of two types: condensed tannins and hydrolyzable tannins (HT). HTs are polymers of gallic or ellagic acid that are potentially toxic to ruminants. Pyrogallol is a product of HT degradation by ruminal microbes that has hepatotoxic and nephrotoxic effects. Epigallocatechin-3-gallate (EGCG) and epicatechin gallate (ECG) are the main tannins in green tea. Hepatotoxicity in humans has been associated with the consumption of high doses of green tea–containing dietary supplements (10 to 29 mg/kg bw/d). All cases resolved following cessation of supplement consumption.

Furocoumarins—Furocoumarins are another group of plant phenolics found mainly in plants belonging to the Rutaceae (e.g., citrus fruits) and Umbelliferae (e.g., parsnip, parsley, celery, carrots) families. Furocoumarins, such as *psoralen*, 5-*methoxypsoralen* (5-MOP, bergapten), and *8-methoxypsoralen* (8-MOP, xanthotoxin or methoxsalen), can form DNA adducts and DNA crosslinks in the presence of UV light (UVA, 320 to 380 nm).

Gossypol—Gossypol (polyphenolic aldehyde) is found in *Gossypium* sp. and is concentrated in the cottonseed, hulls, leaves, and stems. The free form of gossypol is toxic, whereas the protein-bound form is relatively nontoxic. The concentrations of free gossypol contained in feedstuffs such as whole cottonseed and cottonseed meals vary considerably, but gossypol toxicity limits cottonseed use in animal feed. Gossypol poisoning is usually chronic and cumulative. The signs of gossypol poisoning include labored breathing, decreased growth rate, and anorexia. Postmortem findings include generalized edema and congestion of lungs and liver, fluid-filled thoracic and peritoneal cavities, and degeneration of heart fibers. Gossypol also has antifertility effects in many non-ruminant species.

Erucic Acid—Erucic acid is a 22-carbon monounsaturated fatty acid with a single double bond at the omega 9 position (C22:1, ω-9) found in the oil extracted from rape (*Brassica napus* or *B. campestris*) and mustard (*Brassica nigra*) seeds. Through selective breeding, *B. napus* and *B. campestris* varieties with low erucic acid content are popularly known as canola (less than 2% of the total fatty acids as erucic acid). Erucic acid is digested, absorbed, and metabolized like other

fatty acids, but is poorly oxidized and causes myocardial lipidosis.

Lectins—Lectins are glycoproteins that are also known as agglutinins (agglutinates cells) and hemagglutinins (agglutinates erythrocytes). Lectins are present in high levels in legumes (e.g., black beans, soybeans, lima beans, kidney beans, and lentils) and grain products. Lectins selectively bind the carbohydrate moieties of cell-surface glycoproteins. Ricin from castor beans is one of the most toxic substances known. The seeds of precatory bean *Abrus precatorius* contain the highly toxic lectin *abrin*. The combination of soaking and cooking is the best way to reduce the lectin content and its activity in all bean varieties.

Phytic Acid (Phytate)—Phytic acid is inositol hexaphosphate and is found in edible seeds, nuts, beans/legumes, and grains (mostly in the bran). It serves as the main storage form of phosphorus. When seeds sprout, phytic acid provides the phosphorus needed by the young plant. Phytic acid strongly binds minerals (calcium, magnesium, iron, copper, and zinc) making them unavailable for intestinal absorption. The phytic acid content in plants is highly variable. Phytate has also been shown to inhibit digestive enzymes such as pepsin, trypsin, α-amylase, and α-glucosidase. Phytate-rich foods are digested at a slower rate. Phytic acid content in food can be reduced by fermentation, soaking, germination, and enzymatic treatment of grains with phytase.

Oxalic Acid—Oxalic acid is one of the antinutrients found in the leaves, stems, and underground bulbotubers of many plants, including rhubarb, spinach, beet greens, purslane, taro, and others. Ingestion of foods containing high concentrations of oxalic acid results in the formation of insoluble calcium oxalate and oxalates of other minerals in the systemic circulation. Calcium oxalate precipitates in the kidneys, resulting in renal failure. About 65% of kidney stones consist of calcium oxalate.

Enzyme Inhibitors—Enzyme inhibitors present in many plants (e.g., beans, peas, cereal grains, alfalfa, sunflower, potatoes) and animal foods can inhibit the action of proteases, amylases, and lipases.

Phytoestrogens—Phytoestrogens include lignans, flavonoids (subgroups isoflavones, coumestans, and prenylflavonoids), and stilbenes. Lignans are found in many cereals, fruits, and vegetables. Most phytoestrogens bind both estrogen receptors, ERα and ERβ, and activate ER-dependent gene transcription. Isoflavones act like selective estrogen receptor modulators (SERMs). However, the data in humans are not conclusive.

Some Naturally Occurring Toxins in Food of Marine Origin

Toxicity from seafood is due to algal or bacterial toxins that accumulate in shellfish and fish or from the production of histamine by spoilage bacteria in fish. Many of these toxins act on sodium channels—some toxins block the channels inducing muscle paralysis; others open the channels, prolong depolarization of the membrane and spontaneous firing causing symptoms like involuntary muscle spasms, tingling paresthesia, etc.

Saxitoxin—Saxitoxin and related toxins are tetrahydropurines mainly produced by the marine dinoflagellate genus *Alexandrium* spp., but also from *Gymnodinium catenatum* and *Pyrodinium bahamense*. Saxitoxin causes *paralytic shellfish poisoning* (PSP), which is a severe, rapid onset,

life-threatening neurotoxicity caused by the consumption of seafood contaminated with saxitoxin or closely related marine toxins. Saxitoxin inhibits nerve impulse conduction by blocking sodium channels. Symptoms of PSP, such as headache, nausea, vomiting, and diarrhea, appear within 30 minutes. In fatal cases respiratory arrest occurs 2 to 12 hours following consumption of the contaminated shellfish.

Okadaic Acid (OA), Dinophysistoxin (DTX), Pectenotoxin (PTX), Yessotoxins (YTX)—OA, DTX, PTX, and YTX are lipophilic polyether compounds that all cause *diarrheic shellfish poisoning* (DSP), which is a rapid-onset but self-resolving illness involving the GI system. The toxins are produced by marine dinoflagellates of the genus *Dinophysis* spp. OA strongly inhibits the eukaryotic serine/threonine phosphatases 1 and 2A, resulting in hyperphosphorylation of many cellular proteins including those that control sodium secretion by intestinal cells, thereby causing diarrhea. Symptoms of DSP, such as diarrhea and abdominal cramps, usually appear within 30 minutes after exposure but typically resolve within 3 to 4 days.

Domoic Acid (DA)—DA is a water-soluble, heat-stable amino acid that causes *amnesic shellfish poisoning* (ASP). DA is produced by *Pseudonitzschia* spp. and *Chondria* spp., particularly when there are severe algal blooms. Because DA toxicity does not always involve amnesia, it is also known as *domoic acid poisoning*. Symptoms of ASP included vomiting, nausea, diarrhea, and abdominal cramps within 24 hours of ingestion; neurological symptoms developed within 48 hours. Both DA and kainic acid (KA) are excitatory amino acid analogues of glutamic acid that act through the ionotropic glutamate receptors (GluR). Hyperexcitation of the receptors leads to excessive stimulation of the neurons, which ultimately "burn out" and die off. The damage of hippocampal neurons results in permanent loss of short-term memory in susceptible individuals (hence the name amnesic shellfish poisoning).

Brevetoxins—These lipophilic cyclic polyether toxins that cause *neurotoxic shellfish poisoning* (NSP) are produced by the marine dinoflagellate *Karenia brevis* (formerly known as *Gymnodinium breve*). Brevetoxins open voltage-gated sodium channels causing uncontrolled Na^+ influx into the cell, prolonged depolarization of neuronal and muscle cell membranes, and spontaneous firing. The GI symptoms of NSP include vomiting, diarrhea, and pain in the abdomen. Neurological symptoms include paresthesia, reversal of hot and cold perception, vertigo, and ataxia. Symptoms typically occur within 30 minutes to a couple of hours after consuming contaminated shellfish and resolve within 2 days.

Ciguatoxin—Ciguatera toxin, gambiertoxin, and maitotoxin are mostly lipophilic, heat-stable polyethers. Produced by the benthic dinoflagellate *Gambierdiscus toxicus*, gambiertoxin is biotransformed by the fish to produce ciguatera toxin. Ciguatoxins cause *ciguatera fish poisoning* (CFP), which is primarily associated with the consumption of large predatory fishes that have accumulated ciguatoxins by feeding on smaller contaminated fishes. Ciguatoxins open voltage-gated sodium channels at the neuromuscular junction, resulting in membrane hyperexcitability, spontaneous repetitive neurotransmitter release, blockage of synaptic transmission, and depletion of synaptic vesicles. Maitotoxin is water-soluble and it opens calcium channels of the cell plasma membrane. Symptoms of CFP include GI (e.g., vomiting, diarrhea, nausea), neurological (e.g., tingling, itching), and cardiovascular (e.g., hypotension, bradycardia) effects. In severe cases the symptoms may begin as soon as 30 minutes after ingestion of contaminated fish, while in milder cases they may be delayed for 24 to 48 hours.

Symptoms may last even for months.

Tetrodotoxin—Tetrodotoxin is a heat-stable, water-soluble molecule produced by symbiotic bacteria concentrated in the liver and gonad of puffer fish (Fugu), and in many other marine organisms. Tetrodotoxin is a potent sodium channel blocker; it reduces membrane excitability and blocks conduction of nerve impulses, thereby causing muscle paralysis. Symptoms of toxicity include perioral paresthesia (burning or prickling sensation) that may spread to the entire body, that could be followed by vomiting, lightheadedness, and dizziness. In non-fatal poisoning, symptoms usually begin within 30 minutes after exposure and completely resolve within 24 hours after onset. In fatal cases, progression is precipitous, with death due to respiratory muscle paralysis occurring within 6 hours after onset of symptoms.

Scombroid Toxicity—Scombroid fish poisoning is mostly caused by the consumption of fish (Scombroidae family) with high histamine levels, such as tuna, mackerel, blue fish, sardines, anchovies, *and* mahi-mahi. Symptoms are essentially the same as those associated with histamine toxicity. If the fish with high levels of tissue histidine are not refrigerated properly, the bacteria proliferate and convert the tissue histidine into histamine, which is heat-stable. Symptoms of toxicity include tingling and burning sensations around the mouth, headache, facial flushing, palpitations, profuse sweating, truncal rash and pruritis, abdominal cramps, nausea, and diarrhea. In most people, these symptoms are self-limiting, but circulatory collapse, shock, and acute pulmonary edema have been described in severe cases. Scombroid poisoning is the most common cause of ichythyotoxicosis worldwide.

Palytoxin—Produced by the zoantharians of the genus *Palythoa* (cnidarians found in coral reefs), palytoxin has been reported in mackerel, parrotfish, and several species of crabs. It binds to the Na^+/K^+-ATPase and turns it into a nonspecific ion channel resulting in K^+ efflux, Na^+ influx, and prolonged membrane depolarization. Victims exposed to palytoxin report nausea, vomiting, and diarrhea. Within several hours, symptoms include myoglobinuria, a burning sensation around the mouth and extremities, muscle spasms, paresthesia, bradycardia, dyspnea, and dysphonia (difficulty in speaking). Cause of death may be the result of myocardial injury. Palytoxin is known in vitro to be a powerful hemolysin.

Some Microbial Contaminants in Food

Food can serve as the vector for the transfer of microbial toxins or live pathogenic microbes into the human GI tract. Bacterial toxins are of two types: exotoxin and endotoxin. *Exotoxins* are soluble, usually heat-labile proteins found in the cytoplasm that are easily secreted into the surrounding medium, usually by gram-positive bacteria. Exotoxins enhance bacterial virulence and are further subdivided according to their mode of action, such as emetic toxins that trigger severe vomiting, enterotoxins that cause diarrhea, cytotoxins that kill host cells, neurotoxins that interfere with normal transmission of nerve impulse. Table 27–5 lists some important foodborne bacterial exotoxins and their mode of action in causing toxicity. In contrast, *endotoxins* are lipopolysaccharides and membrane constituents. Endotoxins are not secreted but released from dead or dying gram-negative bacteria. Humans have background exposure to endotoxins by the oral and inhalation route, which may vary significantly depending on the living and working conditions. Endotoxins are pyrogenic and are not easily destroyed. Endotoxins also stimulate

inflammatory responses. Some bacteria, such as *Shigella* spp., *Staphylococcus aureus*, or *Escherichia coli*, can elaborate both endotoxin and exotoxin.

TABLE 27–5 Some Foodborne Bacterial Exotoxins and Their Mode of Action

Bacterial Exotoxin	Producing Organism	Toxicity to the Host	Mode of Action
Cereulide (emetic toxin)	*Bacillus cereus*	Vomiting (emesis)	Toxin binds to the 5-HT3 receptors in the stomach/small intestine and stimulates the vagus nerve. Stimulation of the afferent vagal neurocircuit triggers the vomiting center in the hindbrain
Cholera toxin (enterotoxin)	*Vibrio cholerae*	Diarrhea	Stimulates the efflux of ions and water from intestinal epithelial cells. The mechanism involves overstimulation of G proteins, which results in persistent activation of adenylate cyclase to produce cAMP. Very high levels of intracellular cAMP prolong the opening of membrane channels that cause the efflux of Cl^- and other ions and water from the infected cells
Staphylococcal enterotoxin (superantigen)	*Staphylococcus aureus*	Vomiting and diarrhea	Stimulates the vomiting center in the brain. Penetrates the gut lining and also activates immune response in the gut. The inflammatory response and the released inflammatory mediators are associated with inflammatory injury to the GI tract, and electrolyte dysregulation
Listeriolysin O (enterotoxin)	*Listeria monocytogenes*	Diarrhea	Pore-forming cytolysin that directly binds to the membranes in a cholesterol-dependent manner, which is followed by oligomerization of monomers, and formation of pores of 20- to 30-nm diameter that facilitate the efflux of ions and water
CPE (enterotoxin)	*Clostridium perfringens*	Diarrhea	Pore-forming cytolysin. Binds to the claudin receptors (tight junction membrane proteins) on enterocytes, forms large complex that forms pores in the membrane, causes a strong influx of calcium, and apoptotic cell death. Enterocyte death disrupts villus integrity, and causes intestinal damage and electrolyte dysregulation
Diphtheria toxin (cytotoxin)	*Corynebacterium diphtheriae*	Cytotoxicity (cell death)	Inhibits protein synthesis by inhibiting the eukaryotic elongation factor 2 (eEF2)
Shiga toxin (cytotoxin)	*Shigella dysenteriae*; shiga toxin–producing *Escherichia coli* (STEC)	Cytotoxicity (cell death)	Inhibits protein synthesis by inhibiting 28S ribosomal RNA (28S rRNA). The same mechanism is also utilized by ricin in castor beans
Botulinum toxin (neurotoxin)	*Clostridium botulinum*	Neurotoxicity	Interferes with normal transmission of nerve impulse by blocking neurotransmitter (e.g., acetylcholine) release at the nerve terminals

Data from Schmitt CK, Meysick KC, O'Brien AD. Bacterial toxins: friends or foes? *Emerg Infect Dis*. 1999;5:224–234; Dramsi S, Cossart P. Listeriolysin O: a genuine cytolysin optimized for an intracellular parasite. *J Cell Biol*. 2002;156:943–946; Pinchuk IV, Beswick EJ, Reyes VE. Staphylococcal enterotoxins. *Toxins (Basel)*. 2010;2:2177–2197; Kotsonis FN, Burdoch GA. Food toxicology. In: Klaassen CD, ed. *Casarett and Doull's Toxicology: The Basic Science of Poison*. 8th ed. New York: McGraw Hill; 2013;1305–1356.

***Clostridium botulinum, C. butyricum, and C. baratti*—**Food botulism is caused by eating foods contaminated with botulinum toxins. All Clostridia are gram-positive, spore-forming anaerobes, and they produce a total of seven serotypically and antigenically distinct botulinum neurotoxins (serotypes A–G). Human botulism is caused mainly by types A, B, E, and (rarely) F. Types C, D, and E cause toxicity in other mammals, birds, and fish. Type G has not caused any human illness. Botulinum toxin interferes with neural transmission by blocking the release of acetylcholine at peripheral nerve endings and causing muscle paralysis. Clinical illness is characterized by cranial nerve palsies, followed by descending flaccid muscle paralysis, which can involve the muscles of respiration. Recovery often takes weeks to months. The toxins are heat-labile (may be rendered harmless at 80°C to 100°C for 5 to 10 minutes) but the spores are among the most heat-resistant.

***Escherichia coli*—**Enterohemorrhagic *E. coli* (EHEC) serotype O157:H7 is a human pathogen responsible for outbreaks of bloody diarrhea and hemolytic uremic syndrome worldwide. Four main categories of *E. coli* are associated with diarrheal disease: enteropathogenic, enterotoxigenic, enteroinvasive, and verocytotoxin-producing *E. coli* (VTEC). VTEC includes "shiga toxin"–producing *E. coli* (STEC) and "shiga-like toxin"–producing *E. coli*. EHEC is a serotype of VTEC. The reference to shiga toxin results from the clinical similarity of the bloody diarrhea caused by EHEC to that caused by *Shigella*. The symptoms of *E. coli* STEC infections commonly include severe stomach cramps, diarrhea (often bloody), and vomiting. About 5% to 10% of those diagnosed with STEC infections develop hemolytic uremic syndrome, a potentially life-threatening complication.

Like the diarrhea-causing *E. coli*, *Shigella* spp. (*S. dysenteriae, S. flexneri, S. boydii*, and *S. sonnei*) cause an estimated 165 million cases of *Shigella* diarrhea annually, with over 90% occurring in developing countries resulting in over a million deaths annually. Contamination of food by unhygienic food handlers and consumption of raw vegetables raised in contaminated soils are two main contributors to the incidence of *Shigella* diarrhea.

Salmonella sp.*—Salmonella enterica* serotype Enteritidis can cause salmonellosis. Feces of infected humans, animals, and birds containing *Salmonella* can contaminate raw fruits and vegetables as well as meat and milk products. Consuming contaminated food causes nausea, vomiting, abdominal cramps, diarrhea, fever, and headache. The interval between exposure and the onset of the symptoms may be as short as a few hours or it can take a couple of days. The illness is generally self-limiting and resolves within a week.

Cronobacter sakazakii (Enterobacter sakazakii)*—C. sakazakii*, previously known as *E. sakazakii*, is found in milk powder, rice, vegetables, cheese, sausage meat, teas, and various spices. Most of the attention to *C. sakazakii*–related contamination has focused on powdered infant formula. This emerging opportunistic bacterium is associated with rare but life-threatening cases of meningitis, necrotizing enterocolitis, and sepsis in premature and full-term infants.

Some Foodborne Molds and Mycotoxins

Molds have served humans for centuries in the production of foods (e.g., ripening cheese) and have provided various fungal metabolites with important medicinal uses. Frank growth of fungi on animal hosts produces *mycoses*, whereas dietary, respiratory, dermal, and other exposures to toxic fungal metabolites produce *mycotoxicoses*. Mycotoxins are secondary fungal metabolites (i.e., not essential for survival of the mold) secreted into the microenvironment around the mold.

Prolonged exposure to small quantities of mycotoxin may lead to deleterious effects including growth retardation, birth defects, impaired immunity, decreased disease resistance, and tumor formation in humans and decreased production in farm animals. Importantly, the presence of a toxigenic mold does not guarantee the presence of a mycotoxin, which is elaborated only under certain conditions (Table 27–6).

TABLE 27–6 Selected Mycotoxins Produced by Various Molds and Some of Their Effects and Commodities Potentially Contaminated

Mycotoxin	Source	Effect	Commodities Contaminated
Aflatoxins B1, B2, G1, G2	*Aspergillus flavus*, *A. parasiticus*	Acute aflatoxicosis, carcinogenesis	Corn, peanuts, and others
Aflatoxin M1	Metabolite of AFB1	Hepatotoxicity	Milk
Fumonisins B1, B2, B3, B4, A1, A2	*Fusarium verticillioides*	Renal and liver carcinogenesis	Corn
Trichothecenes (e.g., T-2, deoxynivalenol, diacetoxyscirpenol)	*Fusarium*, *Myrothecium*	Hematopoietic toxicity, meningeal hemorrhage of brain, "nervous" disorder, necrosis of skin, hemorrhage in mucosal epithelia of stomach and intestine, emesis, feed refusal, immune suppression	Cereal grains, corn
Zearalenones	*Fusarium*	Estrogenic effect	Corn, grain
Cyclopiazonic acid	*Aspergillus*, *Penicillium*	Muscle, liver, and splenic toxicity	Cheese, grains, peanuts
Kojic acid	*Aspergillus*	May be hepatotoxic?	Grain, animal feed
3-Nitropropionic acid	*Arthrinium sacchari*, *A. saccharicola*, *A. phaeospermum*	Central nervous system impairment	Sugarcane
Citreoviridin	*Penicillium citreoviride*, *P. toxicarium*	Cardiac beriberi	Rice
Cytochalasins E, B, F, H	*Aspergillus*, *Penicillium*	Cytotoxicity	Corn, cereal grain
Sterigmatocystin	*Aspergillus versiolar*	Carcinogenesis	Corn
Penicillinic acid	*Penicillium cyclopium*	Nephrotoxicity, abortifacient	Corn, dried beans, grains
Rubratoxins A, B	*Penicillium rubrum*	Hepatotoxicity, teratogenic	Corn
Patulin	*Penicillium patulum*	Carcinogenesis, liver damage	Apple and apple products
Ochratoxin	*Aspergillus ochraceus*, *A. carbonarius*, *Penicillium verrucosum*	Endemic nephropathy, carcinogenesis	Grains, peanuts, grapes, green coffee
Citrinin	*Aspergillus*,*Penicillium*	Nephrotoxicity	Cereal grains
Penitrem(s)		Tremors, incoordination, bloody diarrhea, death	Moldy cream cheese, English walnuts, hamburger bun, beer
Ergot alkaloids	*Claviceps purpurea*	Ergotism	Grains

Aflatoxins—Generally, aflatoxins occur in susceptible crops as mixtures of aflatoxins B1, B2, G1, and G2, with only aflatoxins B1 and G1 demonstrating carcinogenicity. A carcinogenic hydroxylated metabolite of aflatoxin B1 (termed aflatoxin M1) can occur in the milk from dairy cows that consume contaminated feed. The two major sources of aflatoxin contamination of commodities are field contamination, especially during times of drought and other stresses, which allow insect damage that opens the plant to mold attack, and inadequate storage conditions. Aflatoxin B1 is acutely toxic and death typically results from hepatotoxicity. Aflatoxin B1 is also highly mutagenic, hepatocarcinogenic, and possibly teratogenic. The hepatocarcinogenicity of aflatoxin B_1 is associated with the formation of covalent adducts with DNA, RNA, and protein.

Trichothecenes—The trichothecenes constitute more than 60 sesquiterpenoid metabolites produced by *Fusarium*, *Myrothecium*, *Phomopsis*, *Stachybotrys*, *Trichoderma*, *Trichothecium*, and others. Commonly found as food and feed contaminants, consumption of these mycotoxins can result in alimentary hemorrhage and vomiting, direct contact causes dermatitis. Alimentary toxic aleukia (ATA) is a frequently fatal mycotoxicosis, caused by the ingestion of contaminated

overwintered grain or grain by-products. ATA can be caused by trichothecene toxins (e.g., T-2 toxin) in humans and is characterized by leukopenia, agranulocytosis, necrotic angina, a hemorrhagic rash, sepsis, exhaustion of the bone marrow, bleeding from the nose, throat, and gums, and fever. Trichothecenes bind to ribosomes and inhibit protein synthesis.

Fumonisins—Fumonisins are produced by several *Fusarium* species, notably *Fusarium verticillioides* (formerly *F. moniliforme*), *F. proliferatum*, and *F. nygamai. F. verticillioides* is present in virtually all corn samples. Most strains do not produce the toxin, so the presence of the fungus does not necessarily mean that fumonisin is also present. The most abundantly produced member of the family is fumonisin B1. Fumonisins interfer with sphingolipid metabolism. They cause leukoencephalomalacia (hole in the head syndrome) in equines and rabbits; pulmonary edema and hydrothorax in swine; and hepatotoxic and hepatocarcinogenic effects, and apoptosis in the liver of rats. In humans, there is a probable link with esophageal cancer. The underlying mechanism is a disruption of lipid metabolism by inhibition of ceramide synthetase, an enzyme integral to the formation of complex lipids for use in membranes.

Ochratoxin A—Ochratoxin A is primarily produced by *Aspergillus ochraceus*, *A. carbonarius*, and *Penicillium verrucosum*. It binds tightly to albumin in the blood and has a long half-life. Ochratoxin A is nephrotoxic, hepatotoxic, immunotoxic, teratogenic, and carcinogenic. It inhibits the enzymes involved in phenylalanine metabolism including phenylalanine-tRNA synthetase and mitochondrial ATP production, but stimulates lipid peroxidation.

Ergot Alkaloids—Ergot poisoning (ergotism) in humans and domestic animals is caused by ergot alkaloids that are produced by *Claviceps purpurea* and other genera that infect rye and cereals. Ergot alkaloids cause vasoconstriction and reduced blood flow by direct action on the smooth muscles of the arterioles and uterus; the most affected regions being the lower extremities. The disruption of blood flow causes gangrene, loss of hands and feet, as well as miscarriage of fetuses. Ergot alkaloids also stimulate the central nervous system, resulting in hallucinations, and itchy and burning skin.

Bovine Spongiform Encephalopathy

BSE is a form of neurodegenerative transmissible spongiform encephalopathies (TSEs) (in humans, the diseases kuru and Creutzfeldt–Jakob disease) caused by infectious pathogenic forms of prions. The infective agent can be transferred using preparations of neural tissue from infected animals across species barriers. Prions can be transferred to humans in meat that is improperly handled. BSE presents as neurological deterioration and wasting, leading to death. Characteristic histological lesions in the CNS cord are vacuolation and “spongiform” changes.

Some Toxicants Generated in Food During Cooking or Processing

Polycyclic Aromatic Hydrocarbons—PAHs are largely formed when food is cooked using high-temperature methods, such as frying, roasting, barbecuing, grilling, and smoking directly over an open flame. Fat and juices from meat being grilled directly over an open fire drip onto the fire, causing flames, which contain PAHs that then adhere to the surface of the meat. One

widely studied food-associated PAHs is benzo[*a*]pyrene, which is classified as a human carcinogen.

Heterocyclic Amines—HCAs are generated during cooking meat and fish by pan frying or grilling. The more than 20 HCAs are formed when amino acids, sugars, and creatine react at high temperatures. HCAs are metabolized to bioactivated genotoxic carcinogens that form DNA adducts. HCAs are rapidly absorbed by the GI tract, distributed to all organs, and decline to undetectable levels within 72 hours. Unreacted HCAs are subject to phase II detoxication reactions and are excreted via the urine and feces.

N*-Nitroso Compounds—N*-nitroso compounds (NOCs) contain an *N*-nitroso group and consist of nitrosamines and nitrosamides. Nitrosamines are metabolically activated by hydroxylation and additional steps to ultimately form a carbonium ion that alkylates DNA. Nitrosamides spontaneously decompose to a carbonium ion at physiological pH by a similar mechanism. The NOCs in food could be volatile or non-volatile. Volatile NOCs, such as *N*-nitrosodimethylamine (NDMA), *N*-nitrosodiethylamine (NDEA), *N*-nitrosopyrrolidine (NPYR), and *N*-nitrosopiperidine (NPIP) are known to be carcinogenic, whereas the non-volatile NOCs, such as *N*-nitrosoproline (NPRO) are not mutagenic or carcinogenic.

Acrylamide—Cooking starchy foods (e.g., potato) at high temperatures (120°C or higher) causes a chemical reaction between certain sugars and the amino acid asparagine present in the food to form acrylamide. Most acrylamide is formed in the final stages of baking, grilling, or frying as the moisture content of the food falls and the surface temperature rises. Acrylamide (the monomer) is a known neurotoxin and probable human carcinogen. Interestingly, the polymer of acrylamide, polyacrylamide, does not have significant toxicity.

Furan—Furan occurs in many foods that undergo heat treatment and is possibly carcinogenic to humans. Furan is hepatotoxic and shows clear evidence of carcinogenicity in both sexes in mice and rats. Furan is produced in a variety of experimental systems, including heating of sugars (e.g., glucose, lactose, fructose, xylose, rhamnose), heating sugars in the presence of amino acids or protein (e.g., alanine, cysteine, casein), and thermal degradation of vitamins (ascorbic acid, dehydroascorbic acid, thiamine).

Some Heavy Metal Contaminants in Food

Lead—Lead is a toxic metal present in our environment in small amounts. Everyone is exposed to lead from daily actions such as inhaling dust, eating food, or drinking water. Lead poisoning in children is a potential cause for concern.

Cadmium—Cadmium moves easily through soil layers and is taken up into the food chain by uptake into plants such as leafy vegetables, root crops, cereals, and grains (e.g., rice and wheat). Cadmium is primarily toxic to the proximal tubular cells of the kidney and tends to accumulate in the cortex of the kidney, eventually leading to renal failure.

Mercury—Methylmercury is formed in aquatic environments by bacterial action. Methylmercury becomes concentrated in fish including bonito (*Sarda* spp.), halibut (*Hippoglossus* spp.), mackerel (*Scomberomorus* spp.), marlin (*Makaira* spp.), shark (all species),

swordfish (*Xiphias gladius*), and bluefin tuna (*Thunnus* spp.). Human exposure to mercury can occur through the consumption of these fish species.

Arsenic—Food is the largest source of arsenic exposure, although much of this is likely to be in the organic form, which is less toxic. The highest levels of total arsenic (inorganic plus organic) in foods can be found in seafood, rice, rice cereal and other rice products, mushrooms, poultry, and other foods. Rice is particularly concerning because it is a major part of the diet in many parts of the world, and a major component of cereals consumed by infants and young children.

FOOD ALLERGY

Definition

The World Health Organization (WHO) proposed that adverse nontoxic reactions to food in general should be termed *food hypersensitivity*. If there is no role of the immune system, it should be called *non-allergic food hypersensitivity* (e.g., lactose intolerance, hypersensitivity toward sulfites). If an immunologic mechanism has been demonstrated, the appropriate term is *food allergy*, which could be specified as *IgE-mediated food allergy* (e.g., allergy caused by milk, egg, peanut, etc.) or *non-IgE-mediated food allergy* (e.g., celiac disease). The IgE-mediated food allergy involves the humoral immune response (antibody-mediated), whereas the non-IgE-mediated food allergy involves the cell-mediated immune response.

Occurrence and Symptoms

Food allergies are abnormal responses of the immune system to certain food components (allergens) in some individuals. The allergens in foods are typically naturally occurring proteins. Genetic risk factors include a family history of atopic disorders. Environmental factors include the extent and duration of exposure to the allergen from the environment, route of exposure, prior exposure to similar antigens (determining cross-reactivity), and others. The evidence of environmental factors modulating the expression of food allergy is provided by increasing peanut allergy in children. Early exposure through the GI tract may be tolerogenic, because clinical findings suggest that early consumption of food, such as peanuts, fish, or wheat is associated with a lower incidence of food allergy. Exposure to an increased diversity of foods in early life is inversely associated with allergic diseases including food allergy.

In young children the most common food allergies are those to cow's milk (2.5%), egg (1.3%), peanut (0.8%), wheat (about 0.4%), soy (about 0.4%), tree nuts (0.2%), fish (0.1%), and shellfish (0.1%). Early childhood allergies to milk, egg, soy, and wheat usually resolve by school age (about 80%). Although peanut, tree nut, and seafood allergies are generally considered permanent, about 20% of young children with peanut allergy experience resolution by 5 years of age. Adults are more likely to have allergies to shellfish (2%), peanut (0.6%), tree nuts (0.5%), and fish (0.4%). Reactions to fruits and vegetables are common (approximately 5%) but usually not severe. Allergy to seeds (e.g., sesame) is being increasingly reported.

The symptoms of food allergies may range from mild discomfort to severe, life-threatening reactions that require immediate medical attention. Symptoms may involve itching, redness, swelling in the skin; itching and swelling of the eyes; swelling of oral cavity; pain in the GI tract;

nausea, vomiting, diarrhea; itching and swelling of the nose and throat; asthma; chest pain, abnormal heart rhythm, and very low blood pressure causing fainting. Allergic reactions to foods generally occur within a few minutes to one hour (immediate hypersensitivity) after eating the offending food. Symptoms can last for days or even weeks. The specific symptoms and severity of an allergic reaction are affected by the amount of the allergen consumed and by the sensitivity of the allergic person.

Eosinophilic esophagitis (EoE) is a newly recognized chronic disease that can be associated with food allergies. It is characterized by inflammation and accumulation of eosinophils in the esophagus. Symptoms include nausea, vomiting, and abdominal pain after eating and can resemble acid reflux. Calpain 14 is an IL-13-induced protease that mediates esophageal epithelial barrier impairment. EoE involves T-helper type 2 (Th2) response in esophageal mucosal epithelium.

Foods That Elicit Allergic Reactions

Although more than 170 foods cause food allergies, the eight most common foods are eggs, milk, fish, crustacean shellfish (e.g., crab, lobster, and shrimp), tree nuts (e.g., almonds, walnuts, and pecans), peanuts, wheat, and soybeans. Food-induced anaphylactic reactions are possible, but the more prevalent adverse immunological reactions include eczema, urticaria, angioedema, nausea, vomiting, diarrhea, rhinoconjunctivitis, and asthma.

Mechanism of IgE and Non-IgE-Mediated Food Allergic Reactions

IgE-mediated reactions are type I hypersensitivity responses that typically affect the cutaneous, respiratory, GI and/or cardiovascular systems. IgEs are produced by B cells, and the response usually occurs within an hour of exposure and can result in anaphylaxis. Most allergens, including food allergens, are proteins. More details of the immune response are in Chapter 12.

Non-IgE-mediated food allergy is less frequent, and it occurs in the absence of detectable food-specific IgE antibodies in the serum or skin. The mechanisms of non-IgE-mediated food allergy are not well understood, but are thought to be cell-mediated (see Chapter 12). They occur at least 2 hours after exposure (or later) and mostly involve acute or chronic inflammation in the GI tract. The delayed onset of symptoms makes the clinical association between offending food and the clinical symptoms difficult.

Diagnosis and Treatment of Food Allergy

The first step in diagnosing a food allergy is to obtain/develop a detailed medical history. The next step is to conduct certain tests in order to identify food allergy. The tests may include (1) skin prick test, (2) blood test, (3) oral food challenge, and (4) elimination diet.

The skin prick test (SPT) is conducted by placing a drop of solution (or mashed fresh material, such as fruits or vegetables) containing the food allergen on the forearm or back of the person. Then the area of the skin where the material has been applied is gently pricked to let the applied material enter just below the surface. A positive reaction is indicated by a wheal (a raised bump surrounded by a small circle of itchy red skin) within 30 minutes or so. A positive SPT may

prompt further tests by the physician. *The blood test* measures the presence of IgE antibodies to a specific food. If the SPT and blood test are not sufficient for the physician to arrive at a conclusion, an *oral food challenge (OFC)* may be required; OFC is a highly accurate diagnostic test for food allergy. Because OFC has the potential to cause a serious reaction, it should be performed under the advice and supervision of a qualified allergy specialist doctor. In the *elimination diet* trial, the person is advised to avoid the suspected offending food(s) for about a month.

The best remedy for food allergies is to avoid the foods that cause the allergy. In general, antihistamines should work for mild reactions and epinephrine for serious reactions. However, a person who is suffering from confirmed or suspected food allergy should always consult the doctor about the necessary steps he/she should take and follow the doctor's advice.

CONCLUSION

Many foods that have been consumed for hundreds of years by humans may naturally contain antinutrients/toxicants that, if consumed in large amounts, could cause health concerns. Microbial food contamination is another problem. There is also the issue of food allergy among a seemingly increasing fraction of the population. The FD&C Act provides some thoughtful and pragmatic solutions through the creative use of specifications, process and manufacturing controls, action levels, tolerances, warning labels, advisories, and outright prohibitions.

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QUESTIONS

1. Which of the following statements regarding food complexity is FALSE?
   - **a.** Many flavor additives are nonnutrient substances.
   - **b.** Foods are subjected to environmental forces that alter their chemical composition.
   - **c.** There are more nonnutrient chemicals in food than nutrient chemicals.
   - **d.** A majority of nonnutrient chemicals are added to food by humans.
   - **e.** Food is more variable and complex than most other substances to which humans are exposed.

2. Which of the following foods contains the most nonnutrient chemicals?
   - **a.** beef.

b. banana.
c. tomato.
d. orange juice.
e. Cheddar cheese.

3. Which of the following is considered an indirect food additive?
a. nitrites.
b. plastic.
c. food coloring.
d. EDTA.
e. citric acid.

4. Estimated daily intake (EDI) is based on which of the following?
a. metabolic rate.
b. daily intake.
c. substance concentration in a food item.
d. body mass index.
e. concentration of substance in a food item and daily intake.

5. Which of the following is NOT characteristic of IgE-mediated food allergies?
a. urticaria.
b. wheezing.
c. hypertension.
d. nausea.
e. shock.

6. Which of the following wheat proteins is famous for being allergenic?
a. casein.
b. ovalbumin.
c. livetin.
d. gluten.
e. glycinin.

7. Which of the following foods contains a chemical that causes hypertension by acting as a noradrenergic stimulant?
a. cheese.
b. peanuts.
c. shrimp.
d. chocolate.
e. beets.

8. What is the mechanism of saxitoxin, found in shellfish?
a. interference with ion channels.
b. direct neurotoxicity.

c. interference with DNA replication.
d. binding to hemoglobin.
e. interference with a stimulatory G protein.

9. Which of the following foods can cause a reaction that mimics iodine deficiency?
a. chocolate.
b. shellfish.
c. peanuts.
d. fava beans.
e. cabbage.

10. Improperly canned foods can be contaminated with which of the following bacteria, causing respiratory paralysis?
a. *C. perfringens.*
b. *R. ricketsii.*
c. *S. aureus.*
d. *C. botulinum.*
e. *E. coli.*

[1] The opinions expressed in this chapter are the author's personal opinions and do not necessarily reflect those of FDA, DHHS, or the federal government.

CHAPTER 28

Toxic Effects of Calories

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KEY POINTS

- Nutrients can broadly be defined as chemical substances found in food that are necessary for proper growth, development, reproduction, and repair.
- Energy in the body is derived from three main nutrient classes: carbohydrates, protein, and fat, which in turn are made up of sugars, amino acids, and free fatty acids, respectively.
- Hormonal messages generated by the pancreas, adipose tissue, and GI tract orchestrate multiple responses associated with caloric intake and utilization.
- The “set-point” hypothesis proposes that food intake and energy expenditure are

coordinately regulated in the central nervous system to maintain a relatively constant level of energy reserve and body weight.

- Dieting is defined as the use of a healthy, balanced diet that meets the daily nutritional needs of the body and that reduces caloric intake with increased moderate exercise.

BIOLOGY OF EATING AND DIGESTION

Biotic organisms derive energy from food to sustain life. This energy "drives" cellular functions, including digestion, metabolism, pumping blood, and muscle contractions. Nutrients are chemical substances (typically found in foods or supplements) that are necessary for proper growth and development, reproduction, cellular function and maintenance, and repair following injury. Because most bacteria and higher organisms cannot carry out photosynthesis, they derive their energy by metabolism of preformed organic molecules, such as carbohydrates.

Digestion of Foods

Digestion is a remarkable orchestration of many complex biochemical and physiological events that occurs throughout the gastrointestinal (GI) tract, involving mechanical and chemical breakdown of food into simpler nutrients that are absorbable. Breakdown of food begins in the mouth via the actions of enzymes in saliva. In the stomach, food is acted upon by gastric juices, which contain high amounts of hydrochloric acid. This along with enzymes such as pepsin and α-amylase act upon proteins and carbohydrates, respectively, to generate polypeptides and simpler sugars (such as dextrins from starch). Digestion in the small intestine is aided by enzymes supplied by the pancreas to the duodenum. Bile made in the liver and stored in the gall bladder until secreted aids in the absorption of dietary fats. Pancreatic juice contains digestive enzymes including carboxypeptidases, lipases, amylases, and nucleases, plus bicarbonate and divalent cations (mostly sodium and potassium) that help neutralize the gastric acid. The small intestine secretes many enzymes including aminopeptidases, lipases, and disaccharidases.

The jejunum and ileum are primary sites of nutrient absorption. The surface area of the intestinal mucosa available for absorption is greatly increased due to a combination of folds called valvulae conniventes (folds of Kerckring) and finger-like projections (villi) that are lined with enterocytes. The luminal surface of the enterocytes is further lined by microvilli. The luminal and basolateral surfaces of the enterocytes are rich in transporters that mediate nutrient uptake into enterocytes. Commensal gut microbiota also play important roles in breakdown of complex carbohydrates (such as dietary fiber) into substrates that can be utilized by the host. Monosaccharides are transported into the enterocytes by active transport (glucose, galactose) or via facilitated diffusion (fructose). Digestion of proteins begins in the stomach and continues in the lumen of the small intestine. The jejunum is the site of absorption of amino acids and dipeptides and tripeptides by specific carriers in the enterocyte brush border. Dietary lipids are hydrolyzed by pancreatic and intestinal lipases to free fatty acids or monoacylglycerol. Bile salts along with phospholipids facilitate the absorption of lipids. Macronutrient molecules (amino acids, sugars, and fatty acids) that enter the circulation undergo metabolism in various tissues to be either oxidized to extract energy or stored for future utilization.

Integrated Fuel Metabolism

Energy in the body is derived from three main nutrient classes, carbohydrates, protein, and fat, which in turn are made up of sugars, amino acids, and free fatty acids, respectively. The principal circulating fuels in the body, glucose and free fatty acids, are stored as glycogen and triglycerides, respectively. Triglycerides are stored in adipose depots in specialized cells (adipocytes) within large lipid droplets. Proteins are critical in maintaining structure and function and are catabolized for energy only under extreme conditions.

Maintaining a stable supply of substrate for utilization by the brain is required because the brain has little to no stored energy in the form of glycogen or triglycerides. Unlike the brain, the heart and to some degree the liver and skeletal muscles derive most of their energy needs through the oxidation of fatty acids. Maintenance of protein reserves and replenishment of proteins (enzymes, cytoskeletal proteins, contractile proteins) following feeding are important functions of fuel metabolism.

Hormonal messages generated by the endocrine cells of the pancreas, adipose tissue (adipokines), and GI tract (gut neuropeptides) are critical to orchestrating the multiple processes associated with fuel flux and metabolism. Insulin is the principal hormone required to manage nutrient fuels in both fed and fasted states. Through its actions on various signaling pathways, insulin promotes glucose uptake in peripheral tissues, glycogen synthesis in the liver and muscle, lipid synthesis in adipocytes, and amino acid and protein synthesis in most cells. Insulin also acts to restrain catabolic processes such as lipolysis, gluconeogenesis, and protein degradation, which classically occur under states of low insulin (such as fasting). A rise in glucagon and glucocorticoids (such as cortisol) promote lipolysis and breakdown of glycogen.

Set-Point Theory and Neural Control of Energy Balance

Several redundant feedback mechanisms that maintain the homeostasis of energy in living systems regulate the balance between food intake and energy expenditure to maintain fuel reserves at pre-established levels. Energy is normally utilized to maintain basic metabolic rate, thermogenesis, and to carry out cellular processes, organ-specific functions, and muscle contractions. Excess fuels are converted to triglycerides and stored in adipose tissues. Because adipose tissue is the major depot for preserving energy, signals derived from the periphery signal regions in the brain that coordinate energy balance. When total energy consumed equals the total energy required to meet basal metabolic needs, growth, thermogenesis, and physical activity, the individual is in energy balance, and maintaining this balance will result in relatively stable weight and healthy body composition.

The “set-point” theory proposes that food intake and energy expenditure are coordinately regulated by defined regions in the central nervous system that signal to maintain a relatively constant level of energy reserve and body weight. The model requires the existence of four major components of an energy homeostasis system: afferent signals relaying the levels of energy stores, efferent processes regulating energy storage and expenditure, efferent mechanisms controlling ingestion, and integrative centers in the brain to coordinate these processes. The hypothalamus plays a central role in the control of energy balance, especially food intake. The hormone leptin, secreted in proportion to body fat stores from the adipose tissue, is a homeostatic regulator of energy balance. Leptin acts on metabolic-sensing neurons in the brain with receptor-mediated actions regulating signaling pathways that in effect decrease food intake.

Two populations of neurons involved in appetite control in the brain are sensitive to leptin's actions (among other neuropeptides), one expressing orexigenic peptides neuropeptide-Y and agouti-related peptide and the other expressing anorexigenic peptides proopiomelanocortin and cocaine- and amphetamine-regulated transcript. In addition to the hypothalamic control of appetite per se, reward and hedonic processes of "liking" and "wanting" food occur in the ventral striatum of the midbrain in conjunction with the mesolimbic dopamine system. The corticolimbic system of reward is controlled by areas in the prefrontal cortex, which integrates sensory, emotional, and cognitive information to coordinate behavioral responses. Hence, the homeostatic control of energy balance fits into the larger decision scheme of choice behavior via a complex neural system.

METHODS TO ASSESS ENERGY BALANCE

Assessing Caloric Intake

In animal studies, caloric intake can be quantitatively monitored by measuring the amount of food consumed by animals in metabolic cages. Caloric intake can be derived by multiplying the quantity (grams per day) of diets consumed with the caloric density of the diet. A prospective method to collect information about current intake is maintenance of *food records* for a specific duration of time (3 to 7 days, generally including both week and weekend days). Details may include portion sizes, cooking methods, and patterns of eating.

Assessing Caloric Content of Foods

Accurate assessment of the caloric value of foods is essential for effective nutritional management in clinical and public policy arenas. The general calorie factors of 4, 9, and 4 for the major sources of energy—carbohydrate, fat, and protein—have been widely used. The heat released by combustion of a food in a bomb calorimeter is a measure of its gross energy. The truly metabolizable energy can be derived by accounting for lost energy in urine (mainly from nitrogen) and on the body surface. Protein content is mainly determined via estimating nitrogen content. Fat content can be assessed by measuring the sum of methanol–chloroform extractable total fatty acids that can be expressed as triglyceride equivalents. Carbohydrate content is generally measured by difference as the remaining energy after accounting for protein, fat, alcohol, and ash.

Assessing Energy Expenditure

The process of oxidative phosphorylation couples the oxidation of nutrients with the synthesis of high-energy molecules (viz. adenosine triphosphate [ATP]). The total energy expenditure or metabolic cost for an average adult is primarily composed of three components: (1) basal energy expenditure, (2) thermic effect of food (TEF), and (3) energy expenditure associated with physical activity (EEPA). Basal energy expenditure, also called resting energy expenditure, is the energy expended when the individual is lying down and at complete rest, generally after sleep in the postabsorptive state. The energy expenditure from physical activity consists of (1)

expenditure related to planned exercise and (2) nonexercise activity thermogenesis.

Components of energy expenditure can be measured using either direct or indirect calorimetry. The basic principle in direct calorimetry is to measure the actual heat produced by the organism in a highly controlled environment as an estimate of energy expenditure. Most commonly used methods estimate energy expenditure by indirect calorimetry, such as measurement of oxygen consumption as a reliable surrogate for heat production. The ratio of carbon dioxide expired and oxygen consumed is called the respiratory quotient, and estimates can be useful in understanding both energy expenditure and overall substrate oxidation.

Assessing Body Composition

Body composition assessments describe the overall mass of an individual organism in terms of its molecular or nutrient components: water, fat mass, lean mass, protein, and minerals. In a simple two-compartment model of body composition assessment, total body mass is divided into fat mass (essential and nonessential fat) and fat-free mass (including lean mass and water). Lean mass in this scenario includes protein, carbohydrate, and minerals.

Anthropometric Analysis—While individuals with greater body weight (mass) per height tend to have greater fat mass, total body weight may also be determined by increased muscle mass. The simplest indirect measure of body fatness is the relative proportion of weight to height, more commonly referred to as body mass index (BMI). BMI is derived by ratio of mass (in kilograms) and square of height (in meters). BMI, however, is only an estimate and because BMI does not always reflect fat mass. Other anthropometric estimates of body composition include measurement of skin folds and circumference at various sites (abdomen, waist, hip). The general assumption is that total body fat is proportional to the fat deposited beneath the skin.

Hydrodensitometry—Using the density of the whole body and correcting for residual air in the lungs and GI tract, the relative body fat can be estimated using derived equations. This procedure is also known as underwater weighing.

Air Displacement Plethysmography—This procedure employs the same principles as underwater weighing, except rather than the body displaces air. This allows the body volume to be calculated and then body fat can be calculated. This is probably the most accurate, precise, and cost-effective measure of total body fat.

Absorptiometry—Imaging is performed throughout the entire body by a photon beam: a dual-photon source (gadolinium) or x-ray at two different energy levels (dual-energy x-ray absorptiometry) allows imaging of both soft tissues and bone. Attenuation of the signal received on the detector is proportional to tissue density. Percentage of body fat, lean tissue, and bone mineral density can be computed for the whole body or specific sites based on the analysis of images.

Computerized Tomography—The ability to generate three-dimensional “slices” or cross-sectional images allows regional localization of adipose tissues, muscles, and organs (liver). Using the image data, percent body fat and lean mass can be calculated.

Nuclear Magnetic Resonance—NMR works by interpreting radio-frequency signals of excited

nuclei in an external magnetic field. The physical characteristics of the hydrogen atom differ when the hydrogen is located on protein, fat, or water and this can be detected and quantitated to determine body composition. Images acquired are three-dimensional allowing detailed analysis of body composition and regional fat and lean mass distribution with depots and ectopic deposition within tissues.

Electrical Impedance—Bioelectrical impedance analysis (BIA) and total body electrical conductivity (TOBEC) measure total body composition based on measuring electrical impedance (the inverse of conductance) of an electric current passed through the body. The conductance of a weak painless current through the body is utilized. Lean mass has more water and greater conductivity than fat mass. Predictive equations are employed to derive fat and lean body mass.

Total Body Water—Body fat and lean mass can be calculated by estimating total body water using stable isotopes (either deuterium or ^{18}O). Because body water occupies 73.2% of lean mass, fat-free mass can be computed based on total body water, which is significantly influenced by hydration status and water present in fat tissue.

Assessing Physical Activity

Devices such as accelerometers and pedometers can be utilized to empirically estimate activity. An important challenge in utilizing accelerometers is to convert the count data into energy expenditure, which is done using different regression models.

BIOLOGY OF OBESITY

Obesity Risk: Genes, Epigenetics, and Fetal Environment

Access to plentiful food has been unpredictable throughout most of human (and animal) history. Fitness and survival of an individual were likely to be closely related to the ability to maximally seek, acquire, consume, and store energy (as fat) when food was available, and to select for mechanisms that reduce energy expenditure during times when food was scarce. Thus, so-called thrifty genes orchestrate anabolic processes over energy-consuming ones and provide selective advantage to those who possessed them during periods of food deprivation. The advent of agrarian lifestyle and recent industrialization has meant that much of the developed and emerging world now has a drastically altered environment. Food is generally available for most people and our lifestyles require less physical activity and exertion. Hence, the once beneficial thrifty genes placed in an environment of caloric abundance now produce weight gain, obesity, and its associated metabolic dysfunction.

Recently, natural variation and random mutation (genetic drift) in genes controlling hypothalamic energy balance set-points occurred as human beings developed fire and social behaviors and were released from risk of predation. This “drifty gene” hypothesis better explains why even in societies where obesity is high not everyone becomes obese. Obesity is a highly heritable trait and studies comparing monozygotic with dizygotic twins indicate that 40% to 75% of the interindividual difference in trait is accounted for by genetic variability. Several genes whose disruption causes severe monogenic forms of familial obesity have been described.

Remarkably, most of these genes impair central control of food intake.

As the incidence of obesity continues to rise, even among infants, it is widely accepted that increased susceptibility to obesity can be programmed in utero and early postnatal life. Epidemiological studies have extended the initial associations between birth weight and later cardiovascular disease to include associations between early growth patterns and risk for hypertension, insulin resistance, type 2 diabetes, and obesity in later life. Another important influence on risk of obesity in later life is maternal body composition (fat mass) at conception and gestational weight gain. Maternal diet and body composition during pregnancy influence aspects of metabolism and appetite regulation in the offspring.

TOXICITY RELATED TO EXCESS CALORIC INTAKE/OBESITY

Many adaptive, physiological responses to a positive energy balance produced as a result of overeating and inadequate physical activity result in toxicity over the long term. Short-term coordinated changes in metabolic pathways in white adipose tissue in response to overfeeding result in excess energy storage in the form of triglycerides and hypertrophy of preexisting adipocytes and formation of new adipocytes (hyperplasia). Under such conditions, the efficiency of energy storage in adipose tissue is decreased and the body stores energy in additional ectopic sites. Triglycerides begin to accumulate in nonadipose tissues, such as the liver, skeletal muscle, and pancreas as lipid droplets, resulting in inflammation, and tissue damage.

In addition, adipose tissue from obese individuals releases chemokines and cytokines, the so-called adipokines, which contribute to a state of “metabolic inflammation.” Nonesterified fatty acids and other factors released from adipose tissue contribute to the development of the metabolic syndrome in some overweight and obese individuals, which leads to insulin resistance, disruptions in lipid homeostasis (dyslipidemia), and elevated blood pressure that substantially increase the risk for development of cardiovascular disease and type 2 diabetes.

Adaptation of Liver and Adipose Tissue to Excess Calories

Triglycerides and glycogen are used by the body to store excess caloric energy. Free fatty acids that are toxic are immediately conjugated with acetyl CoA and are bound to intracellular fatty acid–binding proteins before reesterification with glycerol to form triglyceride lipid droplets in the cytosol. Such droplets are highly dynamic and are coated with PAT proteins (named after *p*erilipin, *a*dipophilin, and the *t*ail-interacting protein). Adipophilin and perilipin have important roles in droplet stabilization and regulation of triglyceride turnover. Small hepatic lipid droplets function as a temporary energy storage site, whereas in adipocytes the small lipid droplets fuse to form a single large storage droplet and can serve as a longer-term storage site. In the hours after a meal, triglycerides from the hepatic droplets are incorporated into the lipoprotein VLDL, which is secreted into the plasma and transports hepatic triglycerides to the adipose tissue for storage.

Obesogenesis can also develop from excessive caloric intake of any food energy source, including carbohydrates and proteins. Simple carbohydrates are converted to monosaccharides, mainly glucose and fructose, which are further metabolized to glycogen and de novo fatty acid

and triglyceride synthesis.

Recent DNA microarray analysis of gene expression in human adipose tissue biopsies suggests that coordinated upregulation of lipogenesis rapidly occurs in fat as a result of increased caloric intake. The increased triglyceride synthesis after consumption of excess calories involves activation of two important diet-sensitive transcription factors, sterol regulatory element–binding protein 1c (SREBP-1c) and carbohydrate response element–binding protein (ChREBP), which ultimately drives adipose tissue hypertrophy. In addition to hypertrophy, excess calories also trigger proliferation and differentiation of preadipocytes into new adipocytes.

In some individuals, there is a limit to which adipocytes can expand safely and when this limit is reached, toxicity results. This limit appears to depend on which fat depot the adipocytes are in. Subcutaneous adipocytes can attain a greater size than adipocytes in visceral fat and this may explain why metabolic dysfunction and diseases such as type 2 diabetes are linked to visceral adiposity. Hypertrophic adipocytes develop ultrastructural abnormalities including calcium accumulation and cholesterol crystals and show evidence of oxidative and endoplasmic reticulum (ER) stress ultimately leading to cell death and release of intracellular components that can serve as metabolic damage–associated molecular patterns (DAMPs).

When fat mass increases excessively, adipose tissue also undergoes extensive structural remodeling. An extracellular matrix (ECM) with high concentrations of collagen fibrils and fibronectin appears to be essential for maintenance of the structural integrity of adipocytes and for preadipocyte differentiation. Once adipocytes reach a certain size limit within a particular fat pad, hypoxia triggers expression of the transcription factor hypoxia-inducible factor 1α (HIF-1α), which regulates inappropriate ECM remodeling and development of fibrosis in adipose tissue in response to hypoxia and obesity. Fibrosis can be measured by staining collagen fibrils and by analysis of col6a3 gene expression. Secreted protein acidic and rich in cysteine (SPARC) is required for appropriate collagen synthesis during ECM remodeling. Both $SPARC^{-/-}$ mice and obesity-prone ob/ob mice where the collagen VI gene has been deleted display increased adipocyte and fat pad size, loose ECM structure, and reduced inflammation and metabolic disturbances after high-fat feeding. Complex interactions between enlarging adipocytes and a fibrotic extracellular matrix trigger activation of MAP kinase pathways such as c-Jun N-terminal kinase (JNK), resulting in development of adipocyte insulin resistance (see Fig. 28–1).

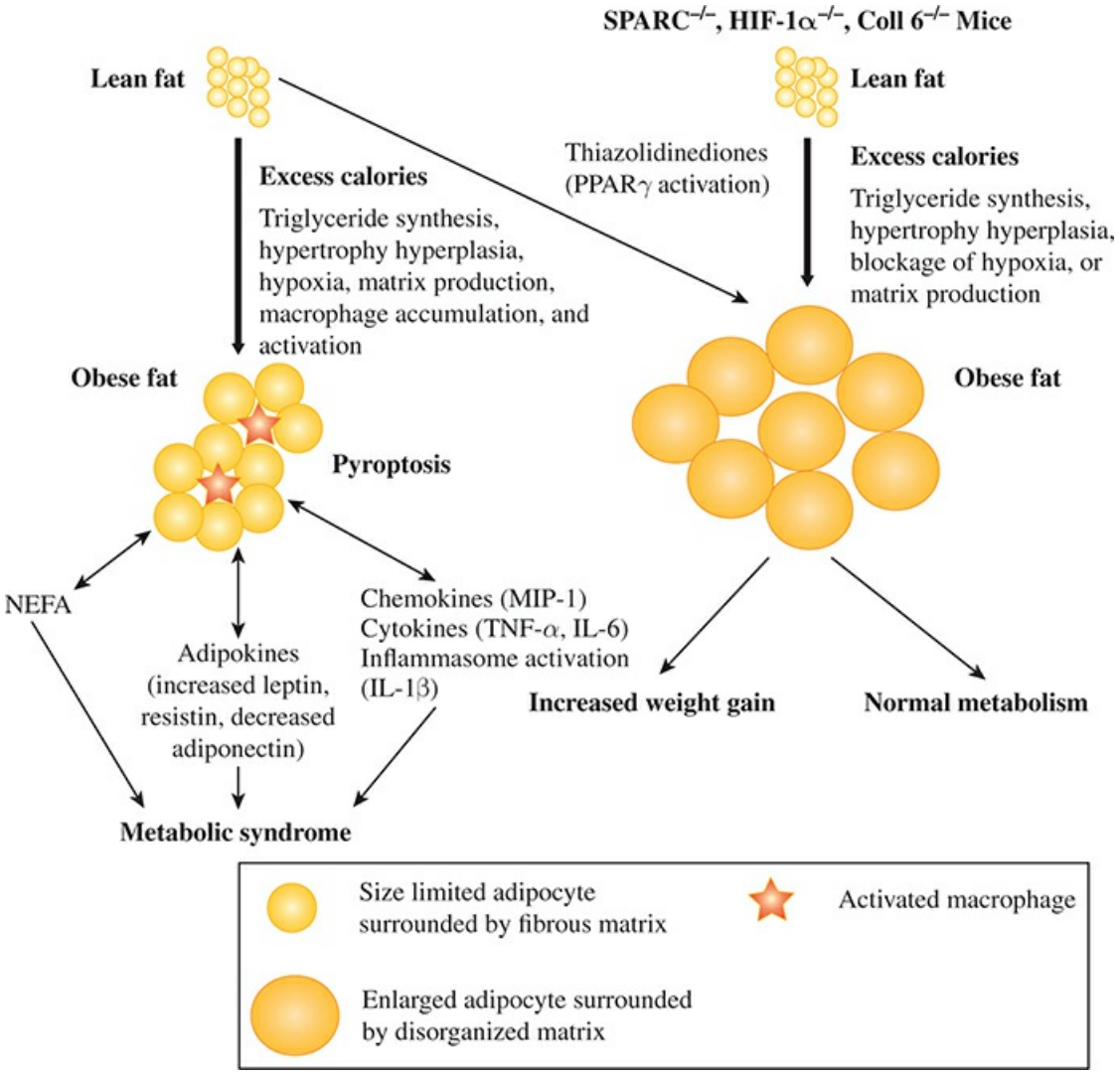

FIGURE 28–1 *Effects of excess calories (energy) on fat morphology under conditions leading to metabolic syndrome (left) or following stimulation of adipocyte differentiation and hyperplasia by thiazolidinedione treatment in knockout mice incapable of normal responses to hypoxia (HIF-1α–/–) and in knockout mice incapable of normal extracellular matrix production (SPARC–/–, Coll 6–/–) (right).*

Ectopic Fat Deposition

The major sites for ectopic fat deposition are the liver and skeletal muscle. Correlation between central (visceral) adiposity, waist circumference, and ectopic fat deposition is better than BMI and is also highly correlated with progressive insulin resistance. Intrahepatocellular lipid accumulation, also known as fatty liver, or steatosis, is defined as an increase in hepatic lipid content above 5% by weight. To confirm that steatosis is actually present, additional staining of frozen sections for triglycerides using stains such as Oil Red O is required. Abnormal lipid accumulation in the liver in the absence of heavy alcohol usage is referred to as nonalcoholic fatty liver disease (NAFLD) and is associated with a wide spectrum of hepatic dysfunction.

Simple steatosis is generally reversible with weight loss and/or lifestyle modification (diet and exercise). Hepatic lipid accumulation can occur as a result of one or more of the following: (1) increased fatty acid supply to the liver and increased fatty acid transporter expression, (2) increased de novo fatty acid and triglyceride synthesis, (3) decreased fatty acid oxidation, and (4) decreased synthesis and/or secretion of VLDL. Which of these processes predominates depends on the degree of obesity, total caloric intake, and diet composition. Reduced serum concentrations of adipokine adiponectin that accompany development of obesity will result in increased hepatic fatty acid synthesis and reduced fatty acid degradation and thus contribute to development of steatosis.

The other major site of ectopic fat deposition in obesity is skeletal muscle in the form of intramyocellular lipid (IMCL). Skeletal muscle contains an intracellular pool of stored triglyceride that exchanges with circulating free fatty acids. IMCL has been shown to positively correlate with visceral adiposity.

Metabolic Syndrome

The development of central obesity as a result of overnutrition and a sedentary lifestyle leads to a clustering of metabolic and physiological components in some individuals that is associated with a doubling of cardiovascular disease risk and a fivefold increase in incidence of type 2 diabetes. Metabolic syndrome characteristics include central obesity (waist circumference), insulin resistance (increased fasting glucose above 100 mg/dL and increased fasting insulin), dyslipidemia (decreased serum HDL below 40 mg/dL in men and 50 mg/dL in women, and increased serum triglycerides above 150 mg/dL), and hypertension (blood pressure higher than 130/85).

The relationship between obesity and whole-body insulin resistance appears to be mediated through increased circulating fatty acids and ectopic fat deposition particularly in intramyocellular lipid (IMCL) in skeletal muscle (Fig. 28–2). Consumption of a very low-calorie diet in obese subjects for as little as 5 days can produce marked decreases in IMCL and enhanced insulin sensitivity without significant changes in body fat mass. Reductions in glucose import into skeletal muscle with IMCL result from inhibition of translocation of the glucose transporter GLUT-4 from cytosolic- to membrane-associated compartments through the action of IMCL metabolites such as diacylglycerol, long-chain fatty acid CoAs, ceramides, and oxidized lipids. Reduced glucose transport also occurs in insulin-resistant adipose tissue itself, and the negative effects of obesity are exacerbated because reduced insulin signaling in adipocytes also enhances expression of hormone-sensitive and adipose triglyceride lipases to further increase the release of nonesterified free fatty acids (NEFA).

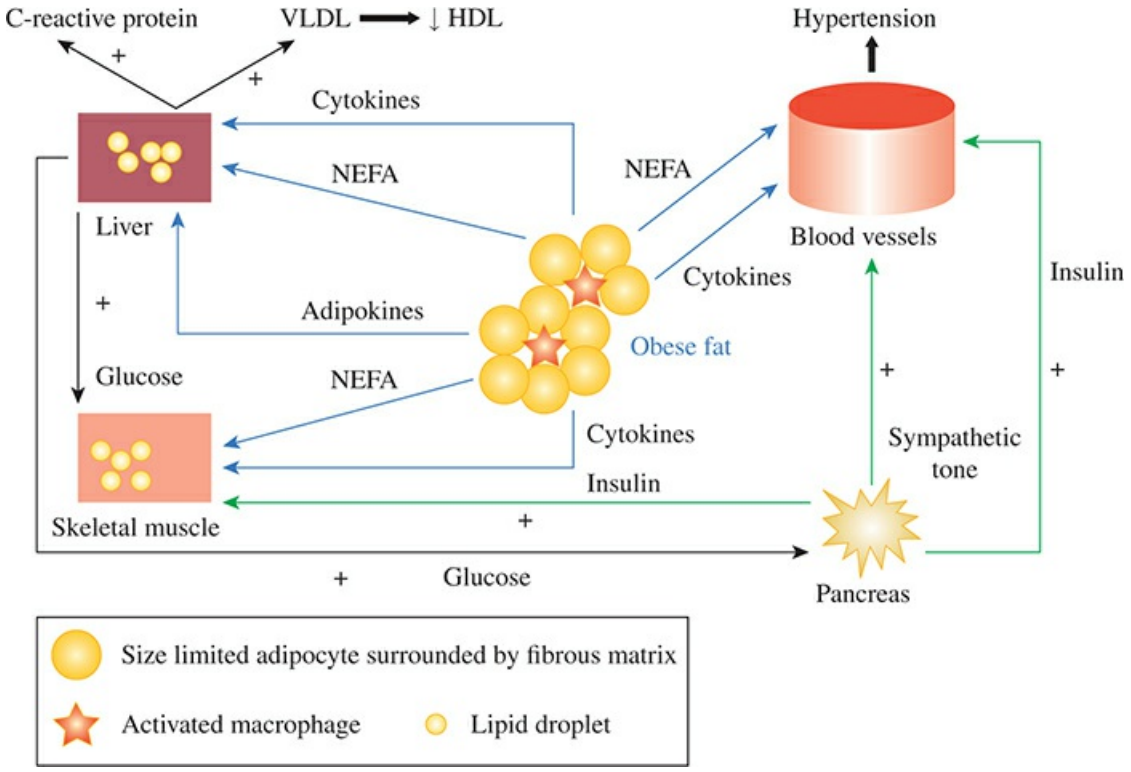

FIGURE 28–2 *Pathogenesis of metabolic syndrome.* NEFA – nonesterified free fatty acids.

Insulin resistance in the liver leads to excess glucose production as a result of a reduced ability of insulin to suppress the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK). This contributes to systemic hyperglycemia and increased pancreatic insulin production. Insulin resistance and steatosis are strongly correlated, and interventions that lead to lower plasma insulin levels also decrease liver triglyceride content.

Therapeutic Options for Managing Metabolic Syndrome

Lifestyle modifications including diets producing stable weight loss and long-term increased physical activity or bariatric surgery are of benefit in treating all the components of metabolic syndrome, but suffer from limited compliance and significant risk of complications in the case of surgery. Therefore, routine clinical management has focused on pharmaceutical therapies for insulin resistance/hyperglycemia, dyslipidemia, and hypertension to reduce the risks of cardiovascular disease and type 2 diabetes.

Metformin acts on the liver to reduce hepatic glucose production as a result of activation of the AMP kinase pathway. Thiazolidinediones such as pioglitazone also improve insulin sensitivity and slow progression of diabetes in about 50% of patients as a result of activation of the transcription factor PPARγ in extrahepatic tissues and increasing circulating levels of adiponectin. Increased triglycerides and low HDL levels are often treated with statins, inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA), the rate-limiting step in cholesterol biosynthesis. Statins are often used in combination with ezetimibe or niacin to further reduce LDL and triglycerides and raise HDL levels. High doses of 2 to 4 g of fish oil omega-3 fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) may reduce serum

triglycerides by 20% to 40%. Control of hypertension with various classes of drugs is also key to prevention of cardiovascular events. Nevertheless, each of these treatments has additional actions and adverse effects that might limit use.

Nonalcoholic Steatohepatitis

Ectopic fat deposition in the liver is strongly correlated with obesity and insulin resistance. The disease progression of NAFLD begins with simple steatosis, which progresses to nonalcoholic steatohepatitis (NASH), which is characterized by cell death, inflammation, and fibrosis, then to cirrhosis in which liver function is significantly impaired, and ultimately to hepatocellular carcinoma (Fig. 28–3).

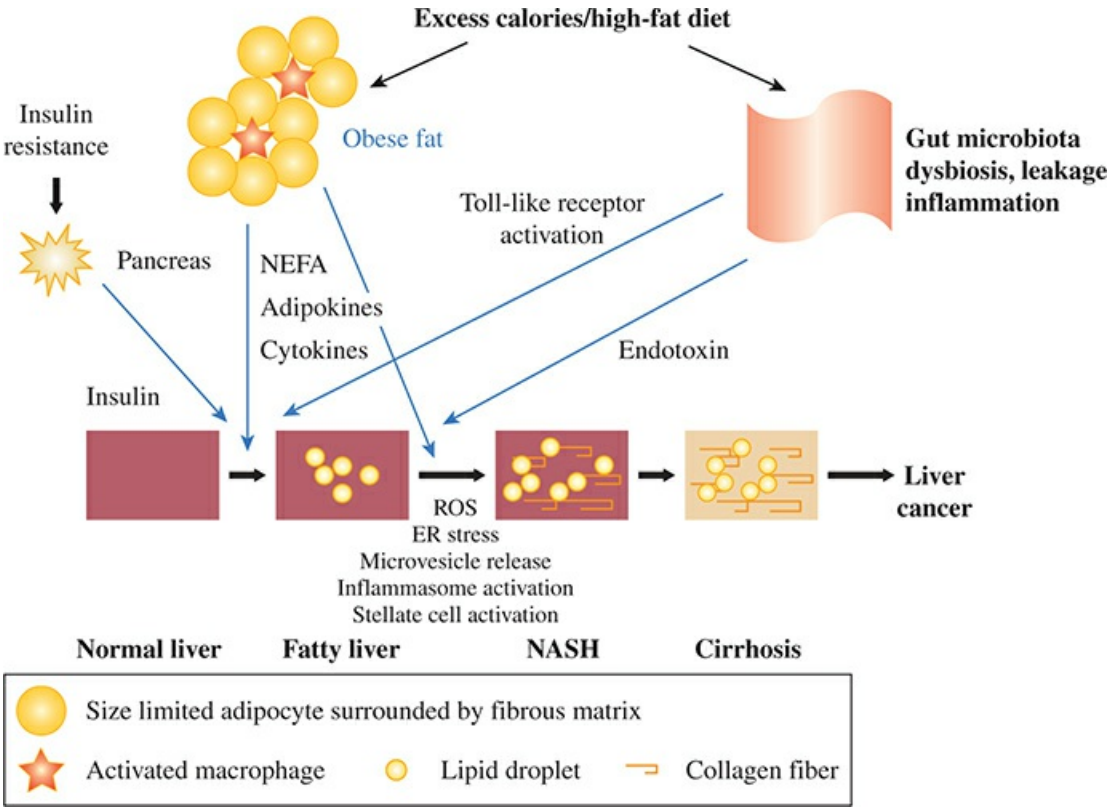

FIGURE 28–3 *Progression of nonalcoholic fatty liver disease (NAFLD).* ER stress, endoplasmic reticulum stress; ROS, reactive oxygen species.

Factors such as adipokines and the proinflammatory cytokine tumor necrosis factor (TNF-α), activation of the endogenous immune system through Toll-like receptors, ER stress, and signals from the gut microbiota can all increase hepatic fat accumulation. The progression of steatosis to necroinflammatory injury and fibrosis in NASH livers appears to depend on oxidative stress mediated by reactive oxygen species generated by cytochrome P450 CYP2E1 and NADPH-oxidase enzymes. Damage to hepatocytes in steatotic livers may also be linked to release of extracellular microvesicles/exosomes containing micro RNAs, and to release of damage-associated molecular patterns (DAMPs), which appear to play a role in development of inflammation including inflammasome activation in other parenchymal cells and resident

macrophages. Development of fibrosis after NASH appears to involve activation of hepatic stellate cells leading to inappropriate secretion of matrix components, particularly collagen. Proliferative regenerative responses also ultimately promote development of hepatocellular carcinomas in NASH patients.

Alteration in Drug Pharmacokinetics and Metabolism in Obesity and NAFLD

Obesity and fatty liver disease can affect drug pharmacokinetics, metabolism, and therapeutic efficacy with the potential to result in adverse drug reactions. The most significant change associated with obesity is likely to be in volume of distribution (V_d). The V_d of lipid-soluble drugs that partition readily into adipose tissue is likely to be increased in obesity because obese individuals have a larger fat compartment into which the drug can distribute. In contrast, V_d for hydrophilic drugs that do not readily partition into fat may be relatively unaffected.

Obesity and NAFLD appear to have variable effects on hepatic phase I and II drug metabolism and on transporters. They increase expression of the cytochrome P450 enzyme CYP2E1 in both animal models and human clinical studies. In contrast, hepatic expression of CYP1A2 appears to be consistently suppressed. Sulfotransferase expression is reduced in NASH patients, but effects on drug glucuronidation and glutathione conjugation are inconsistent. Recent studies in experimental rat models of NAFLD suggest decreased expression of hepatic uptake transporters such as the sodium/taurocholate co-transporting polypeptide (NTCP) and the organic anion transporting polypeptides (OATPs) and increased expression of efflux transporters, such as the multidrug resistance–associated proteins Mrp2, Mrp3, and Mrp4.

Endocrine Dysfunction in Obesity, Metabolic Syndrome, and NAFLD

Obesity in pregnancy can result in complications of gestational diabetes, pregnancy-associated hypertension, preeclampsia, and fetal abnormalities including neural tube defects, spina bifida, heart defects, and cleft palate. Delays in milk production and decreased duration of breast-feeding have been associated with obesity in women.

Growth hormone (GH) secretion is dramatically suppressed by obesity in both adults and children. Mechanisms appear to involve direct feedback effects of insulin on the pituitary and a reduction in secretion of the endogenous GH-releasing peptide ghrelin that is produced by the stomach and hypothalamic centers. Hyperinsulinemia appears to increase adrenal androgen production and reduce plasma concentrations of sex hormone–binding globulin (SHBG) in obese prepubertal girls, resulting with early pubertal development. Hyperandrogenization also may explain the increased incidence of polycystic ovary syndrome (PCOS) in obese adolescent girls, anovulatory cycles, and subfertility in obese women of childbearing age. In obese boys, puberty may be delayed. This may also be related to increased aromatization of androgens in adipose tissue because negative feedback of estrogens at the level of the hypothalamic–pituitary axis may result in reduced luteinizing hormone secretion and reduced testosterone production (hypogonadotropic hypogonadism). In addition to suppressed GH and gonadotropin secretion, hypothyroidism is common in individuals with metabolic syndrome. Reduced thyroid hormone

concentrations may exacerbate NASH progression in the liver by increasing triglyceride synthesis, reducing fatty acid oxidation, and increasing hepatic cholesterol concentrations by reducing conversion to bile acids.

Obesity and Cancer Risk

Increased BMI is associated with significantly increased risk of several cancers. These include sex-steroid-dependent endometrial, breast, and prostate cancer, GI tract cancers such as esophageal adenocarcinoma, and colon cancer and renal cancer (Table 28–1). Increased sex steroid concentrations in obesity promote growth of tumors in the mammary gland, endometrium, and prostate. Leptin increases as fat mass increases, and is proproliferative, antiapoptotic, proinflammatory, and angiogenic. Additional potential associations between obesity and cancer may involve depletion of cellular antioxidant systems as a result of the low-grade chronic systemic inflammation that accompanies morbid obesity and the possibility that mesenchymal stromal cells arising from expanding white adipose tissue may be recruited to tumors to promote angiogenesis and drive tumor progression.

TABLE 28–1 Estimated Risk Ratios* for Cancer in Relation to BMI

Cancer Type	Men	Women
Colon cancer	1.24	1.09
Gallbladder cancer	—	1.59
Leukemia	1.08	1.17
Malignant melanoma	1.17	—
Multiple myeloma	1.11	1.11
Esophageal adenocarcinoma	1.52	1.51
Renal cancer	1.24	1.34
Thyroid cancer	1.33	1.14
Prostate cancer	1.03	—
Postmenopausal breast cancer	—	1.12
Endometrial cancer†	—	1.73

*Per increase in BMI by 5 kg/m^2 (Data from Roberts DL, Dive C, Renehan AG. Biological mechanisms linking obesity and cancer risk: new perspectives. *Annu Rev Med.* 2010;61:301–316).
†For BMIs above 27 kg/m^2.

HEALTH BENEFITS AND LIFE EXTENSION ASSOCIATED WITH CALORIC RESTRICTION

The opposite of overfeeding is caloric restriction (CR). Experimental CR has been repeatedly shown to increase life span and reduce age-related disease in comparison with ad libitum feeding in organisms including yeast, nematodes, fruit flies, fish, many rodent species, dogs, and rhesus monkeys. Intensive studies are underway to identify CR mimetics that might have the same benefits as CR on long-term health and longevity without the necessity for imposed food restriction. The grape polyphenol resveratrol has been shown to activate Sir2 and to mimic CR effects to increase life span in yeast, *C. elegans*, *Drosophila*, and fish in the absence of nutrient alteration. A second CR mimetic rapamycin is an inhibitor of the nutrient signaling mediator target of rapamycin (TOR).

TREATMENT OF OBESITY

Lifestyle Modification: Dieting and Exercise

Attaining a healthy body composition usually requires trimming excess body fat by reductions in total caloric intake, modifications in diet composition, and increases in total energy expenditure (TEE) via increased physical activity. This combined strategy is intended to develop a metabolic state in which energy expenditure exceeds energy intake, and which requires total body energy requirements to come from utilization of body fat stores.

Adhering to almost any available diet plan can achieve body weight loss and reduction of total fat mass. The major problem is sustaining a healthy body composition. Most dieters return to their predieting weight and body composition (or worse) because they never achieve a true lifestyle change that meets their expectations. Comprehensive lifestyle changes that promote sustainability include two major and closely linked components: (1) learning to consume only the number of calories from high-quality foods necessary to support basal body energy requirements plus the energy to maintain physical activity and (2) selecting a reasonable physical activity plan that fits into the dieter's lifestyle. Thus, losing weight and getting a healthy body composition comes down to energy balance, that is, consuming only enough energy to maintain a given body composition and assuring that energy intake equals energy expenditure.

The energy in food eaten can be "cost accounted" roughly as follows: (1) energy required to digest and absorb food; (2) energy utilized to support basal functions such as pumping blood and breathing; (3) energy for body functions other than basal metabolism, such as walking and playing golf; and (4) nonutilized food calories, such as food components not fully digested or absorbed. The energy expenditure of a body at rest is termed the basal metabolic rate (BMR) and energy expenditures differ by age, gender, and body size (height and weight). The way to reduce body fat (adipose tissue) is to consume less calories than expended and the rate of fat loss is directly related to the level of the energy deficit (i.e., caloric intake vs. caloric expenditure).

Insulin has a major influence on carbohydrate, fat, and protein metabolism. Under conditions

of adequate carbohydrate intake, insulin causes excess sugar not utilized as fuel to be stored as fat and prevents utilization of fat as an energy source.

Although obesity can be reversed by the combination of consuming a healthy diet and reducing caloric intake to below calories expended, *maintaining a healthy body composition* with a caloric intake equal to energy expenditure often is as complicated as the factors leading to obesity in the first place. Adequate physical activity is essential to maintaining good physical health.

Toxic Effects of Dieting

If the diet does not include all the required nutrients (an imbalanced diet), metabolism will suffer and with time result in health problems. This is true with nutrient deficiency disorders such as anemia or osteoporosis or toxicity caused by nutrient excess such as thyroid impairment, vitamin deficiencies, and mental confusion. Popular diet plans that call for excess intake of a particular food can not only alter metabolism but also interfere with medications.

Drug Therapy for Weight Loss

Appetite suppressants attempt to lessen the psychological motivation for food, usually by acting on central nervous system appetite control centers, such as those in the hypothalamus. The appetite-reducing effects of sympathomimetics tend to decrease after a few weeks in many people. Often used in the early stages of a weight loss program, the weight loss is generally temporary without modifications in diet composition, eating behavior, and physical activity. Short-term use is usually accompanied by minor side effects such as thirst, irritability, constipation, stomach pain, dizziness, dryness of mouth, heightened sense of well-being, headache, irritability, nausea, nervousness or restlessness, trembling or shaking, and trouble sleeping. Adverse effects of long-term use include intracerebral hemorrhage, acute dystonia, myocardial injury, psychosis, cerebral arteritis, cardiac arrhythmias, heart valve damage, and even fatal pulmonary hypertension.

Surgical Interventions

Bariatric surgery may limit food intake by reducing the capacity of the stomach, and also having patients feel satiated. Weight loss is achieved by resecting and linking the small intestines to a small stomach pouch (gastric bypass surgery), removal of a portion of the stomach (sleeve gastrectomy), or reducing the size of the stomach with an implanted medical device (gastric banding) or sutures (gastric plication). These procedures are generally credited with significant long-term loss of body fat and body weight. Malabsorption and nutritional deficiency can occur because of reduced absorptive area. This can reduce calcium and vitamin absorption. Other deficiencies including iron and micronutrients such as vitamin B_{12}, fat-soluble vitamins, thiamine, and folate are common. Because patients cannot eat a large quantity of food, physicians typically recommend a diet relatively high in protein and low in fats and alcohol.

Liposuction is another surgical procedure widely used to improve body composition by physically and surgically removing body fat. The significant risks with this procedure are mainly associated with (1) the aggressiveness with which the procedure is performed, especially the

amount of tissue sucked from the body; (2) the venues in which the procedures are performed; and (3) the amount of anesthesia used to sedate patients during increasingly lengthy procedures.

ECONOMIC, SOCIOLOGICAL, AND LEGAL ASPECTS OF THE OBESITY EPIDEMIC

Health Insurance and Obesity

Obesity is not considered an illness for most insurance purposes. However, obesity can affect the cost of health insurance because as a group, obese people have a significantly greater risk of cardiovascular disease, hypertension, type 2 diabetes, and other health issues than lean people. Thus, insurance companies factor obesity into the cost of first-time insurance purchasers. Obesity can result in high premiums and in the case of morbidly obese individuals, insurers may decline their application. Obesity is also regarded by insurance companies as a substantial risk for both life and disability policies.

Changing the Environment: Family and Community Approaches to Healthy Eating and Physical Activity

The development of obesity within an individual, a community, or a country is complex and the central cause(s) or reason(s) underlying the rapid rise in obesity is not well understood. The end results in terms of body composition and secondary medical problems associated with obesity are better understood than the root causes of the obesity epidemic.

Prior to 1970, the average BMI was 25.1 for men and 24.9 for women in the United States. Physical education (PE) classes were a regular feature of school curriculums; most meals were prepared at home using fresh produce, meats, and dairy products. School lunches were prepared at schools. It was common for children to walk or ride bicycles to school and to participate in games requiring physical activity during school recess and after school and on weekends. By 2002, average BMI for U.S. men had risen to 27.8 and average BMI for U.S. women to 28.1.

To fight the obesity issue, there are initiatives to establish community gardens, build community walking and riding trails, and teach people cooking and shopping skills that lead to healthier meal preparation. School systems are starting to return to PE classes on a regular basis and remove high-density foods and drinks from vending machines. Providing access to higher-quality fresh food instead of highly refined food will improve nutrition. Education on food selection and preparation and linking to some form of physical activity may improve body composition and health.

Food Labels

The Food and Drug Administration (FDA) is responsible for assuring that foods sold in the United States are properly labeled. This applies to foods produced domestically and foods from foreign countries. The FDA requires food labels that bear nutrient content claims and certain health messages to comply with specific requirements. Food labeling is required for most

prepared foods, such as breads, cereals, canned and frozen foods, snacks, desserts, and drinks. Nutrition labeling for raw produce (fruits and vegetables) and fish is voluntary.

Federal law requires that ingredients and nutrition data be listed on food packaging. The FDA monitors labeling language for such issues as fat, protein, carbohydrate, and other nutrient contents and the purported ability of a particular food to prevent medical problems. In fact, there are requirements for nutrition labeling of standard menu items for chain restaurants, similar retail food establishments, and chain vending machine operators. The most important information that must be provided for standard menu items is the number of calories in each menu item and a statement on the menu that puts the calorie information in the context of a recommended total daily caloric intake.

Governmental and Corporate Issues

Local, state, and federal governments regulate various aspects of food production and marketing as a means of promoting health and reducing obesity and the secondary consequences of obesity. Food labeling is just one example of government intervention, whereby food processors and restaurants must provide a measure of nutrient and/or caloric content.

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QUESTIONS

1. Humans consume food to provide energy needed to
   - **a.** drive cellular functions including digestion, metabolism, pumping blood, nerve activity, and muscle contractions.
   - **b.** promote photosynthesis.
   - **c.** synthesize oxygen in the lungs.
   - **d.** prepare minerals for use in the body.
   - **e.** produce carbon dioxide to fuel body functions.

2. Neural control of energy balance
   - **a.** may be defined as the action of leptin on CNS function.
   - **b.** may be defined as the action of hypothalamic cholinergic control of appetite and hedonic control.
   - **c.** may involve a balance between food intake and energy expenditure.
   - **d.** may involve a balance between leptin’s action on orexigenic versus anorexigenic peptide expression.
   - **e.** may involve adrenocortical control of hepatic function.

3. Body composition may be assessed by
   a. electrical impedance because lean mass has more water and greater conductivity than fat mass.
   b. anthropometric analysis of the body mass index.
   c. hydrodensitometry, which uses the density of the whole body and corrects for residual air in the lungs and GI tract to determine relative body fat.
   d. nuclear magnetic resonance.
   e. all of the above.

4. Ectopic fat deposition includes
   a. adipose tissue.
   b. skeletal muscle.
   c. Lungs.
   d. Heart.
   e. GI tract.

5. Excess calories may be
   a. stored as glucose in adipose tissue.
   b. stored as triglycerides in CNS tissue.
   c. stored as glycogen in CNS tissue.
   d. stored as glycogen in the liver.
   e. stored as triglycerides in the GI tract.

6. Metabolic syndrome is a constellation of actions including
   a. typically results from elevated fasting glucose, increased HDL, and hypertension.
   b. typically results from elevated fasting glucose, increased LDL, and hypertension.
   c. typically results from elevated fasting glucose, hypertriglyceridemia, and hypotension.
   d. typically results from elevated fasting glucose, hypotriglyceridemia, and truncal obesity.
   e. typically results from elevated fasting glucose, hypertriglyceridemia, and truncal obesity.

7. Excess caloric intake
   a. may lead to nonalcoholic steatohepatitis.
   b. is always correlated with obesity and insulin resistance.
   c. is characterized by elevations of serum ALT concentrations in all cases.
   d. leads to hepatic cirrhosis and liver cancer in almost all cases.
   e. is readily reversible by dieting.

8. Although dieting may effectively reduce body weight,
   a. toxicity may result from stimulation of adipokine release.
   b. toxicity may result from inhibition of drug metabolizing enzymes.
   c. toxicity may result from a loss of required nutrients.
   d. toxicity may result from extreme mental illness.

e. toxicity may result from weight cycling.

9. Body mass index
   a. may be used as an indicator of sufficient caloric and essential nutrient intake.
   b. may be defined as body height divided by body weight squared.
   c. has risen insignificantly over the past thirty years in the United States.
   d. may not be used in the estimation of cancer risk in humans.
   e. may be defined as body weight divided by height squared.

10. Which of the following definitions is false?
   a. The set-point hypothesis proposes that food intake and energy expenditure are coordinately regulated by defined regions in the brain that signal to maintain a relatively constant level of energy reserve and body weight.
   b. Hormonal messages generated by the endocrine cells of the pancreas, adipose tissue, and GI tract are involved in orchestrating multiple responses associated with caloric intake and caloric utilization.
   c. Caloric content of foods generally assumes factors of 4, 9, and 4 for carbohydrate, fat, and protein.
   d. The body mass index (BMI) is an accurate method for assessing body composition.
   e. Liver, adipose, muscle, and other tissues adapt to excess caloric loads.

CHAPTER 29

Nanoparticle Toxicology

David B. Warheit, Günter Oberdörster, Agnes B. Kane, Scott C. Brown, Rebecca D. Klaper, and Robert H. Hurt

KEY POINTS

- Nanotechnology is the understanding and control of matter at nanoscale dimensions between approximately 1 and 100 nm, where unique phenomena enable novel applications.
- Nanotoxicology can be defined as the study of adverse effects of nanomaterials on living organisms and the environment.
- Surface properties are major determinants of biological reactivity due to high surface area, surface charge, dissolution and release of metal ions, and redox activity leading to generation of reactive oxygen species (ROS).
- The respiratory tract is the major route for humans to exposure of nanomaterials.
- Surface properties are major determinants of biological reactivity due to high surface area, surface charge, hydrophobicity and partitioning into lipid membranes, dissolution and release of metal ions, and redox activity.
- Dosemetric defines a dose in terms of an inherent property (physical, chemical, reactivity, etc.)

INTRODUCTION

Engineered nanomaterials (ENMs) such as nanosilver, fullerenes, quantum dots, carbon nanotubes (CNTs), graphene-based materials, and metal oxide nanoparticles (NPs) are continually emerging with potential for significant commercial applications in energy generation, environmental sensing and remediation, aerospace and defense, and medical diagnosis and therapy. Examples of nanoscale materials are depicted in Fig. 29–1.

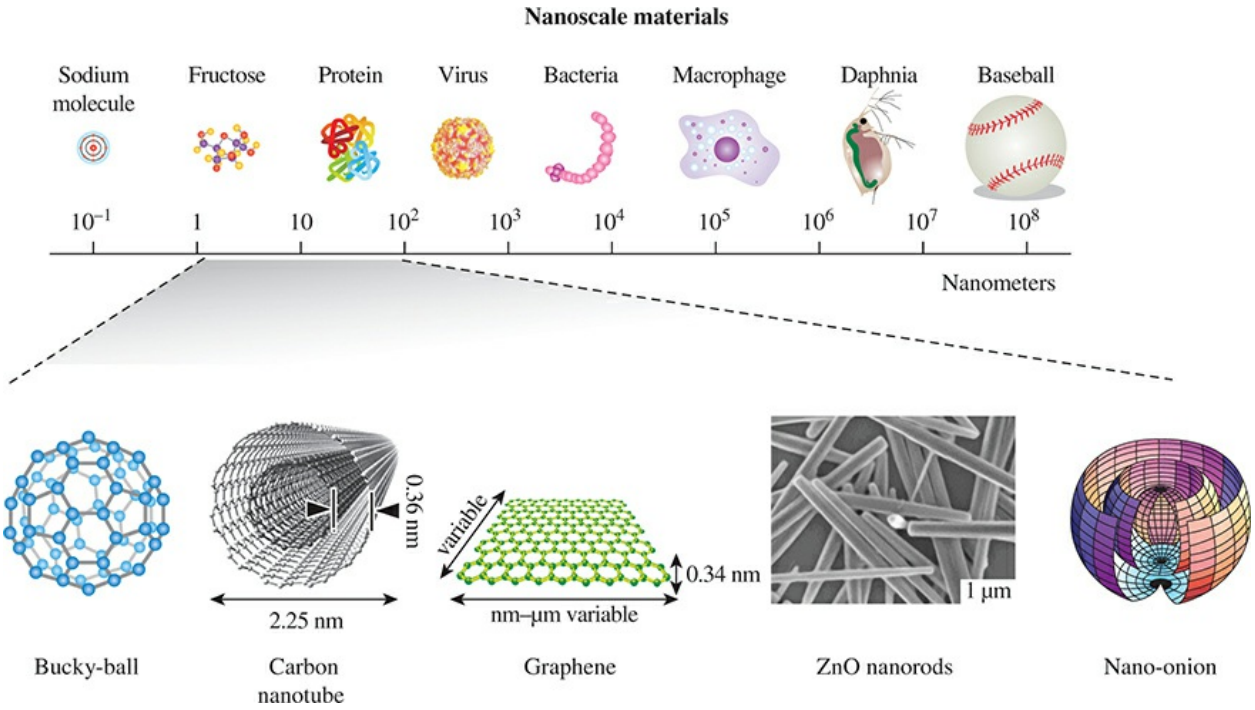

FIGURE 29–1 *Length scales for natural and synthetic structures (above) and some examples of engineered nanomaterials of varying size and shape (below).*

Nanotechnology may be defined as the understanding and control of matter at the nanoscale at dimensions between approximately 1 and 100 nm, where unique phenomena enable novel applications. The continuing introduction of nanoscale materials into consumer products, such as TiO_2 in sunscreen creams, antibacterial Ag in textiles, quantum dots in televisions, multiwalled carbon nanotubes (MWCNTs) in sports equipment, together with increasing numbers of publications reporting toxic responses mostly observed in in vitro studies, has led to increasing concerns and more public awareness about potential adverse health effects.

Nanotoxicology studies the adverse effects of nanomaterials on living organisms and the environment. Nanotoxicology considers the impact of dose, dose rate (influenced by dosing method in vitro and in vivo), and dosimetry when assessing nanomaterial toxicity. The goals of nanotoxicology are to identify and characterize a hazard of ENMs for purposes of risk assessment for humans and the environment.

REGULATORY OVERSIGHT

The Working Party of Manufactured Nanomaterials (WPMN) established by the Organization for Economic Co-Operation and Development (OECD) remains the leading international forum on regulatory policy and oversight for nanomaterials and addresses issues related to the risk assessment and risk management of nanomaterials regarding human and environmental health and safety. The WPMN comprises international governmental policymakers and subject matter experts including observers from environmental NGOs, unions, UN groups, the International Organization Standardization (ISO), and industry. Member countries have integrated

nanomaterials into their chemical regulatory programs. Several jurisdictions have implemented information collections rules or registries for nanomaterials to acquire information on their prevalence and uses in commerce. The regulatory actions that have taken place to date are largely monitoring activities, in part due to marked uncertainty in the sciences as well as the uses, quantities, and diversity of nanomaterials in commerce.

NANOMATERIAL BASICS

Perspectives: Engineered Nanoparticles versus Ambient Particulate Matter

Airborne ambient particulate matter (PM) can elicit adverse effects not only in the respiratory tract but also in secondary organs and systemically. Exposure to the smallest fraction of PM, referred to as UFPs or $PM_{0.1}$, particles smaller than 100 nm, has been associated with effects in the cardiovascular and central nervous system. Epidemiological studies have demonstrated that a major factor for inducing adverse effects from ambient particulate air pollution—including UFP—is susceptibility such as pre-existing disease (asthma, cardiovascular disease, diabetes), age (very young, elderly), or genetic background (polymorphism).

Properties and Behaviors of ENPs versus Larger Particles

Table 29–1 contrasts differences between NPs (<100 nm) and larger particles (>500 nm) in terms of some general characteristics, translocation propensity, interactions with cells, and effects, assuming inhalation exposure and the respiratory tract as the portal of entry. Biological systems do not perceive a precise boundary at the 100 nm size threshold, but rather a gradual transition between NPs and larger-sized particles.

TABLE 29–1 What Is Different: Nanoparticles versus Larger Particles (respiratory tract as portal-of-entry)

	Nanoparticles (<100 nm)	Larger Particles (>500 nm)
General characteristics		
Ratio: number or surface area/volume or mass	High	Low
Agglomeration in air, liquids	Likely (dependent on medium and surface)	Less likely
Deposition mechanism in respiratory tract	Diffusion; throughout resp. tract	Sedimentation, impaction, interception; throughout resp. tract
Protein/lipid adsorption in vitro	Very effective and important	Less effective
Protein/lipid adsorption in vivo	Yes	Some
Translocation to secondary target organs	Yes	Generally not (to liver under "overload")
Clearance		
• Mucociliary	Probably yes	Efficient
• By alveolar macrophages	Poor	Efficient
• Into or across lung epithelium	Yes	Mainly under overload
• Lymphatic	Yes	Under overload
• Blood circulation	Yes	Under overload
• Sensory neurons (uptake + transport)	Yes	Not likely
Cell entry/uptake	Yes (caveolae; clathrin; lipid rafts; diffusion)	Yes (primarily phagocytic cells)
• Mitochondria	Yes	No
• Nucleus	Yes (<40 nm)	No
Effects (*caveat: dose!*)		
At secondary target organs	Yes	(No)
At portal of entry (resp. tract)	Yes	Yes
• Inflammation	Yes	Yes
• Oxidative stress	Yes	Yes
• Activation of signaling pathways	Yes	Yes
• Genotoxicity, carcinogenicity	Probably yes	Some

Data from Oberdörster G, Elder A, Rinderknecht A. Nanoparticles and the brain: cause for concern? *J Nanosci Nanotechnol.* 2009;9:4996–5007.

Mucociliary and alveolar macrophage-mediated elimination following deposition in the respiratory tract is efficient for both NPs and larger particles once they are internalized by macrophages. NPs inhaled and deposited as singlets are too small to be efficiently recognized and phagocytized by alveolar macrophages unless they are aggregated or agglomerated to form larger particles. Overall alveolar macrophage-mediated clearance in lung is poor. Uptake by epithelial cells and translocation into blood and lymphatic circulation occurs regularly for NPs.

Classes of ENMs

Manufactured nanomaterials cover an enormous range of chemical compositions and geometries, ranging from simple isometric forms (particles), one-dimensional (1D) forms (nanofibers, tubes, rods, wires), and two-dimensional (2D) forms (plate-like or disk-like materials typically referred to as "nanosheets," for very thin and flexible forms, or "nanoplatelets" for thicker stiff materials) as shown in Fig. 29–2. Nanomaterials may be adhered to surfaces of biomedical implants to enhance their function and biocompatibility or incorporated into nanostructured solids or composites to improve strength, conductivity, and durability. Nanomaterials are often integrated into complex, active structures for chemical or biological sensors or other devices.

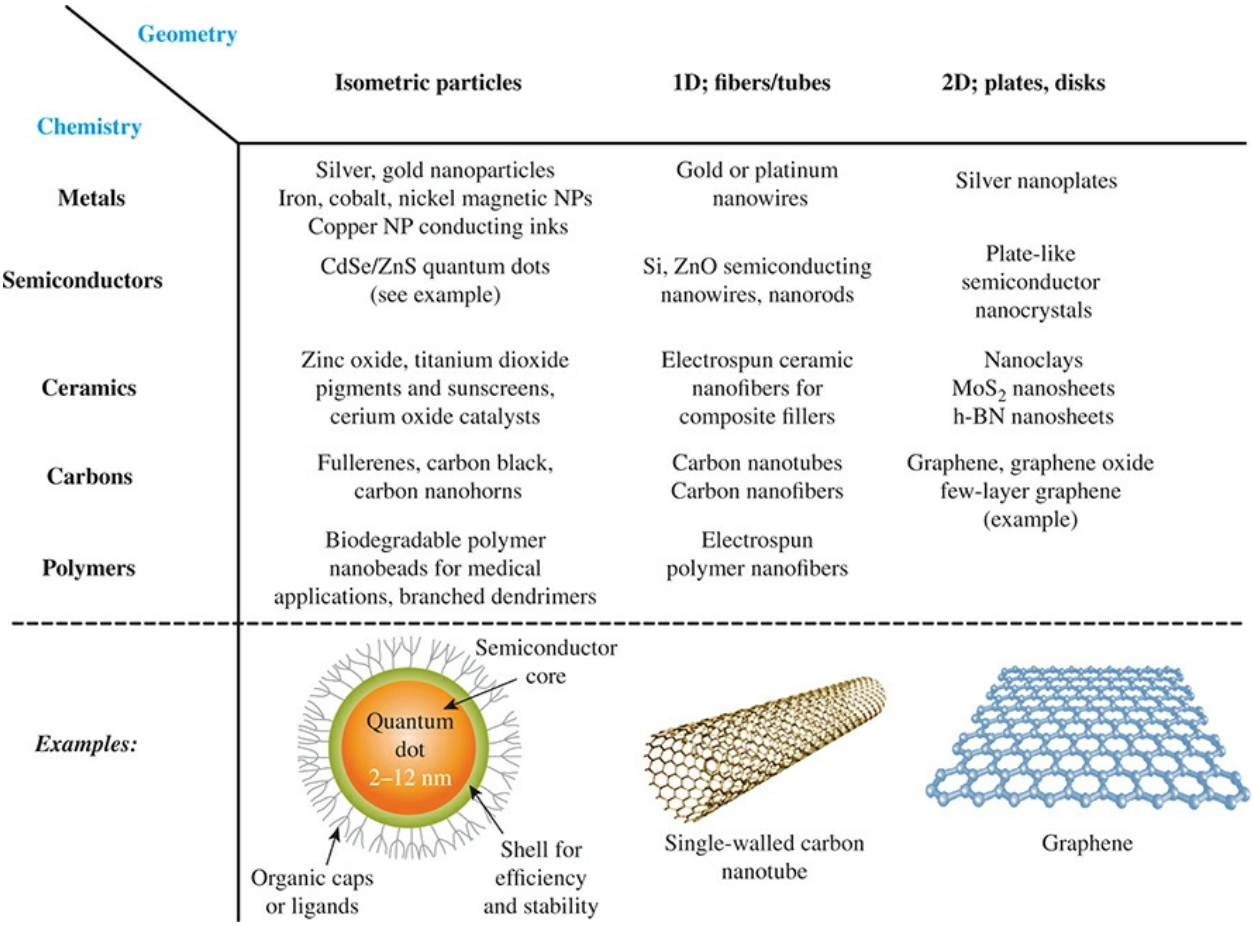

FIGURE 29–2 *Classification of nanomaterials by geometry and chemistry.* The examples in this matrix illustrate the diversity in engineered nanomaterials, a diversity that continues to increase as new nanomaterials are synthesized.

Physicochemical Properties of Nanomaterials Relevant for Toxicity

Figure 29–3 summarizes the nanomaterial properties known or thought to be relevant to biological responses.

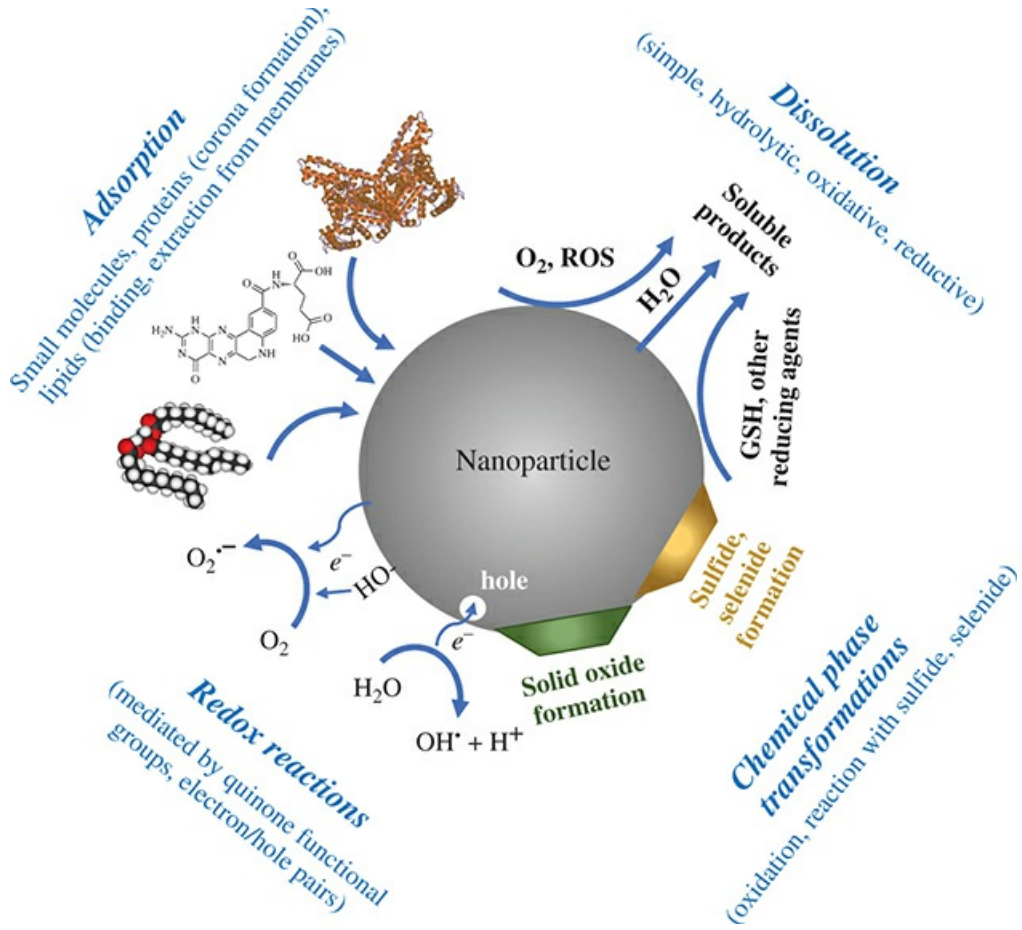

FIGURE 29–3 *Physicochemical and functional NP properties of relevance for toxicology.* (Used with permission from Robert Hurt.)

Particle Size, Distributions, Geometry, and Dimensions—The distribution of sizes and shapes impacts transport and exposure phenomena and can influence cellular uptake. Particle size can change substantially in the environment through dissolution, precipitation, agglomeration, and heteroagglomeration. Agglomeration occurs when two or more similar particles weakly stick to one another through physical interactions. Heteroagglomeration is the process where particles of different compositions form weak associations. For biomedical applications, nanoparticles of narrow size distributions are typically desired for consistent delivery or excretion. Dispersed particles in the circulation less than 5 nm are cleared via renal excretion, particles less than 50 nm undergo hepatobiliary excretion.

Small NPs up to 50 nm in diameter appear to enter cells and subcellular organelles, including mitochondria and the nucleus, by passive diffusion. Single-walled carbon nanotubes (SWCNTs) can directly puncture bacterial cells leading to osmotic lysis and death. SWCNTs that translocate

from the alveoli into the interstitium of the lung promote collagen deposition and interstitial fibrosis. Rigid, long, high-aspect ratio (needle-like) nanomaterials, similar to asbestos fibers, are recognized by macrophages; they undergo incomplete uptake or frustrated phagocytosis if they are longer than the macrophage diameter, resulting in impaired clearance from the lung, lysosomal disruption, and activation of the inflammasome resulting in the release of pro-inflammatory cytokines. High-aspect ratio materials (particulates with significantly greater lengths relative to diameters—e.g., fibers) with 2D structure (nanosheets) also show geometry-dependent cellular interactions.

Surface Charge, Steric Interactions, and Agglomeration—NPs tend to form agglomerates in the dry state, and in many cases in liquid suspension (Fig. 29–4), which leads to strong intermolecular attractive forces. Attractive forces between particles are determined by size, density, chemistry, electronic structure, morphology, and surface charge. In water, van der Waals forces and hydrophobic forces drive the agglomeration of many NP types. To obtain stable dispersions of non-agglomerated nanomaterials, it is typically necessary to add macromolecular coatings that prevent particle–particle attachment through steric hindrance, or to impart an electrical charge on the NP surfaces that lead to particle–particle repulsion. For drug delivery applications, NPs may be coated with biocompatible surfactants such as sodium cholate, or by biocompatible polymers such as phospholipid-polyethylene glycol that stabilize colloidal suspensions of NPs through steric hindrance.

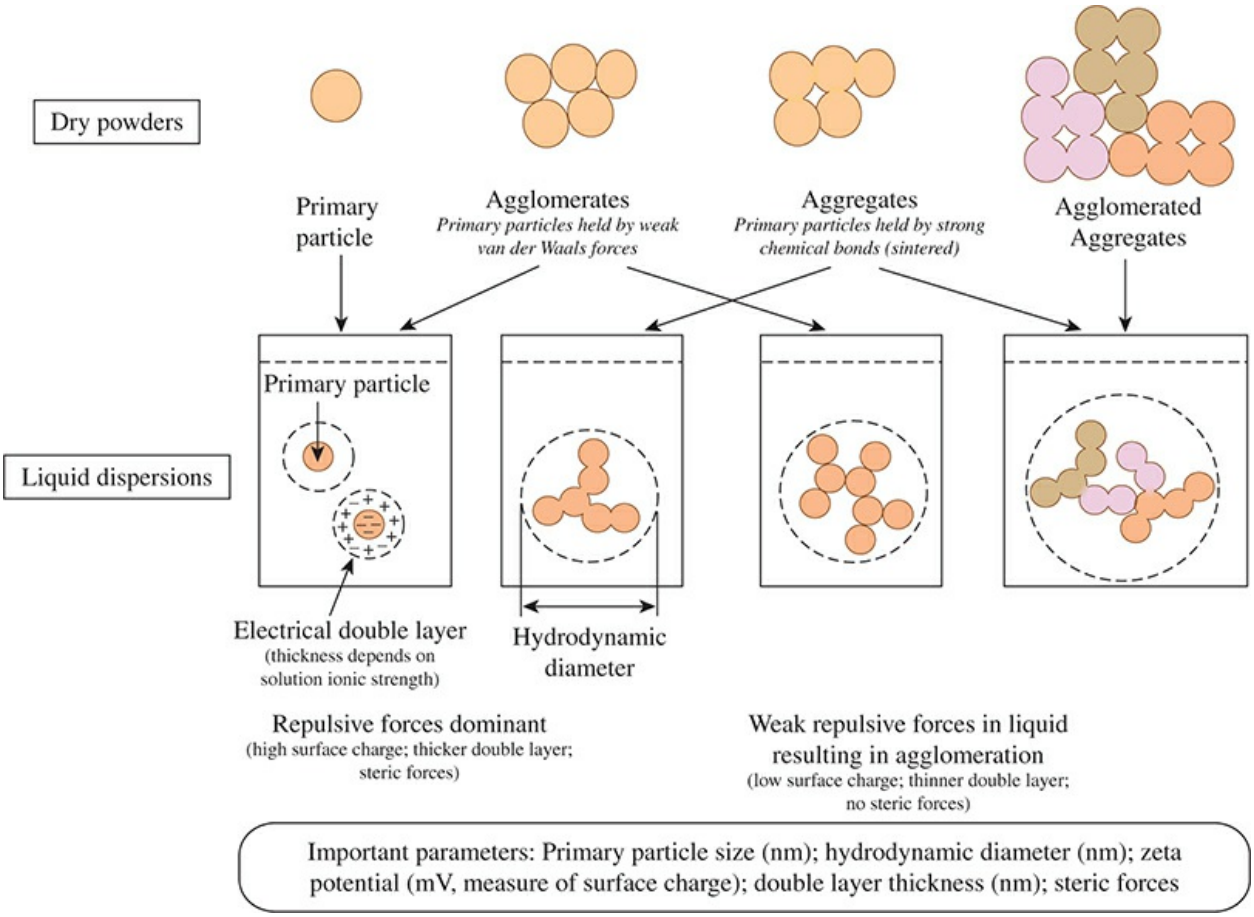

FIGURE 29–4 *Agglomeration and aggregation of nanoparticles in liquids and as dry*

powders. (Reprinted with permission from Jiang J, Oberdörster G, Biswas P. Characterization of size surface charge, and agglomeration state of nanoparticle dispersions for toxicological studies. *J Nanopart Res*. 2009;11:77–89.)

Surface Area and Reactivity—A high surface area of suspended NPs at equivalent mass concentrations is responsible for increased surface reactivity, increased adsorption of chemicals, strong catalytic activity, and enhanced dissolution rates. Determining specific surface reactivity —e.g., ROS generating capacity per unit NP surface area—has been suggested as a measure to group ENMs.

Surface Chemistry, Surface Morphology, and Surface Impurities—High surface area, high surface curvature, and exposed surface atoms or molecules promote increased dissolution and release of ions from metallic or metal oxide NPs relative to bulk particles of the same chemical composition in fluids. Metal ions are toxic to bacteria and aquatic organisms. ZnO NPs in sunscreens absorb ultraviolet (UV) light; however, in water, Zn^{2+} ions are rapidly released and cause acute toxicity. Surface hydrophilicity of charged NPs increases their aqueous suspension, whereas surface hydrophobicity of fullerenes or graphene repels water and enables these hydrophobic nanomaterials to partition into lipid membranes and enter target cells. Surface defects can expose electron donor/acceptor groups that donate an electron to molecular oxygen generating superoxide anions or accept electrons from water producing hydroxyl radicals.

CNTs synthesized in the presence of metal catalysts may contain redox-active metal residues that can undergo redox cycling and iron-catalyzed generation of hydroxyl radicals ($OH^{\bullet}$). The high surface area of ENMs can adsorb organic molecules such as polycyclic aromatic hydrocarbons that are potentially carcinogenic and quinones that are also redox cycling and generate free radicals. Cationic NPs having surface amide groups and cationic dendrimers are especially cytotoxic because they induce membrane damage, especially in lysosomes where the “proton sponge effect” leads to accumulation of water and chloride ions and osmotic rupture.

In summary, surface properties are major determinants of biological reactivity due to (1) high surface area, (2) surface charge, (3) hydrophobicity and partitioning into lipid membranes, (4) dissolution and release of metal ions, and (5) redox activity leading to generation of ROS.

Unique Properties—Ferromagnetic NPs (e.g., iron, nickel, cobalt, and some oxides) less than about 10 nm in diameter respond strongly to an external magnetic field but have no permanent magnetic moment in the absence of the field. This property is exploited for contrast enhancement in diagnostic MRI and for hyperthermia induced by an external magnetic field to kill tumors targeted by magnetic NPs.

Biopersistence—Biopersistence of ENMs is determined by mechanical clearance and dissolution processes; the latter produces biologically active soluble species and can also degrade the particle and eventually clear it from biological tissues. The rate of dissolution of metal oxides is increased by natural organic matter in the aqueous environment; therefore, these NPs have low biodurability and it is predicted that they would not bioaccumulate in the environment.

Carbon nanomaterials are not easily oxidized under most environmental conditions and could bioaccumulate in the environment or be biopersistent in the lungs and other organs following inhalation during occupational exposure or direct injection or implantation in biomedical applications. Biopersistence in the lungs and pleural or peritoneal spaces is an important physicochemical characteristic of asbestos and mineral fibers associated with carcinogenicity.

Chemical Transformation—Most nanomaterials are synthesized with the input of chemical or thermal energy. When transferred into environmental or biological fluid phases, ENMs release this free energy by chemical transformations including dissolution to soluble molecular species, degradation to complex solid debris, or chemical reaction to form new solid phases that include sulfides, selenides, or oxides. Such transformations can dramatically alter nanomaterial properties and require that studies of hazard and risk be extended beyond the pristine particles to include the transformation products. Transformations can occur between the points of synthesis and exposure during nanomaterial processing, handling, storage, and use; upon release into the natural environment; or after contact with biological fluids or tissues causing potential toxic properties of particles to evolve over time.

THE NANOMATERIAL BIOLOGICAL INTERFACE

The high surface area of NPs provides a platform for adsorption of biological molecules including proteins, lipids, and nucleic acids. A “protein corona” is a dynamic interface between the NP and its local environment that governs its initial interaction with target cells. NPs of diverse chemical composition and structure (e.g., iron oxide NPs, polymeric NPs, and CNTs) avidly adsorb albumin, complement, fibrinogen, immunoglobulins, and apolipoproteins. The consequences of protein adsorption to NPs may include denaturation of proteins, resulting in the loss of normal structure and function, or altered enzyme activity, or unfolding that exposes new antigenic determinants.

NPs that are inhaled into the respiratory tract or ingested into the gastrointestinal tract encounter a mucus layer that provides a natural barrier to penetration of particulates and microorganisms. Mucus is a complex glycoprotein composed of hydrophilic, dispersed regions and hydrophobic, globular domains. NPs may adhere to mucins causing enlargement of the pore size with increased susceptibility to penetration of microorganisms. Smaller, charged NPs may be repelled by the hydrophilic domains and will not be able to penetrate the mucus layer. Aquatic organisms and bacterial biofilms are surrounded by similar extracellular barriers that may prevent NP penetration.

Some ENP-types bind nucleic acids and have been proposed as gene delivery devices. Depending on chirality, SWCNTs may show sequence-specific DNA binding. Graphene oxide nanosheets preferentially bind single-stranded RNA or DNA and protect it from enzymatic degradation. Small graphene oxide nanosheets can also intercalate into double-stranded DNA and induce DNA breaks in the presence of Cu^{2+} ions.

TOXICITY MECHANISMS

The mechanistic pathways associated with toxicity may be predictable based on the physicochemical properties of ENMs. Oxidative stress due to direct generation of ROS at the surface of NPs or indirectly by target cells following internalization of NPs is common. The most vulnerable subcellular organelles and physiological functions that can be perturbed by exposure

to ENPs are summarized in Table 29–2. Depending on the dose, duration of exposure, type of NP, and the target cell, the cellular responses may be minimal and reversible, involve activation of adaptive responses, or severe, leading to significant cellular changes culminating in cell death.

TABLE 29–2 In Vitro Mechanisms of Nanoparticle Toxicity

1. Damage to cell wall and plasma membrane
2. Interference with electron transport and aerobic respiration
3. Induction of oxidant stress
4. Activation of cell signaling pathways
5. Perturbed ion homeostasis
6. Release of toxic metal ions from internalized nanoparticles
7. Disruption of lysosomal membrane integrity
8. Incomplete uptake or frustrated phagocytosis
9. Interference with cytoskeletal function
10. DNA and chromosomal damage

The cell wall of bacteria and the plasma membrane of eukaryotic cells are the initial barriers to penetration of NPs into target cells. Carbon nanomaterials are proposed to act as "nanodarts" creating holes in the plasma membrane, resulting in extracellular release of cytoplasmic contents as assessed by efflux of ribosomal RNA and decreased survival.

Many NPs facilitate delivery of imaging agents, genes, proteins, and drugs into mammalian cells: metallic and iron oxide NPs, silica NPs, quantum dots, biocompatible polymers, liposomes, micelles, and dendrimers. NPs can also target specific cell surface receptors triggering internalization by phagocytosis, macropinocytosis, or endocytosis mediated by clathrin, caveolae, or lipid rafts. In order to facilitate gene, protein, or drug delivery, NPs can be engineered to escape from endosomes or lysosomes by coating with pH-sensitive polymers, viral capsids, cations, or biodegradable carriers.

NPs that are recognized by surface receptors may activate cell-type-specific signaling pathways leading to cell proliferation or death by apoptosis, stress-related signaling, or calcium-mediated signal transduction events. Dysregulated intracellular calcium ion homeostasis may be the consequence of influx across a damaged plasma membrane or release of calcium ions from intracellular storage in mitochondria and endoplasmic reticulum. Sustained elevation in intracellular calcium can cause cell death by necrosis.

Small NPs inside mitochondria may disrupt electron transport in the inner mitochondrial membrane and generate excess endogenous ROS, which is a major mechanism leading to cell toxicity and cell death. Endocytosis and phagocytosis of NPs by target cells usually results in fusion with and sequestration in membrane-bound lysosomes. Uptake of quantum dots, CNTs containing metal catalyst residues, or metallic and metal oxide NPs provides a pathway for intracellular delivery of toxic metal ions by a "Trojan horse mechanism." Cellular uptake of

long, rigid, high aspect ratio nanomaterials including CNTs, metallic nanowires, and nanorods is especially hazardous for target cells in the lungs. Macrophages are the initial cells to phagocytize inhaled particulates deposited in the airways or alveoli. Rigid, high aspect ratio ENMs show similar interactions with macrophages as amphibole asbestos fibers. Rigid, elongated nanostructures longer than the diameter of macrophages (~10 μm) show incomplete uptake or phagocytosis with prolonged generation of ROS by the respiratory burst mechanism of phagocytes and extracellular release of damaging lysosomal enzymes, resulting in the release of cathepsins into the cytoplasm and activation of the inflammasome. Cathepsins are proteases that cleave precursors of pro-inflammatory cytokines and initiate inflammatory reactions in the lungs.

Incomplete sequestration of long, rigid, high aspect ratio NPs in lysosomes may also promote their release into the cytoplasm, resulting in physical interference with cytoskeletal function. Cytoskeletal disruption can cause impaired cell motility resulting in injury, inflammation, and fibrosis in lungs as well as impaired bile transport and secretion in hepatocytes. In dividing cells, high aspect ratio NPs may physically interfere with the mitotic apparatus, resulting in chromosomal mis-segregation and polyploidy.

CAVEATS IN NANOTOXICOLOGY ASSAYS

Due to their high surface area and hydrophobicity, NPs can adsorb vital dyes, cell culture micronutrients, or released cytokines. High surface area carbon nanomaterials such as SWCNTs and graphene have the greatest potential to adsorb aromatic amino acids, vitamins, and fluorescent dyes by π–π interactions with their hydrophobic graphenic surfaces. In all toxicological studies, well-characterized positive and negative reference samples should be included over a range of doses and exposure times using standardized methodology. ENMs are highly complex and have unique chemical and physical properties relative to bulk materials of the same chemical composition. Complete materials characterization is required for adequate interpretation of toxicological studies.

SAFETY CONSIDERATIONS IN NANOMATERIAL DESIGN

In principle, it should be possible to engineer NPs with desirable surface properties for commercial or biomedical applications. Capping or coating of NPs using antioxidants may decrease toxicity. Release of toxic metal ions from quantum dots and iron oxide NPs can be minimized using inorganic shells or biocompatible polymers. A long-term goal of mechanistic nanotoxicology is to reveal structure–activity relations that may allow the design of safe nanomaterials through re-engineering or reformulation.

CASE STUDY: DESIGNING SAFER SUNSCREENS

Zinc oxide and TiO_2 NPs are widely used in sunscreens because they show less scattering of visible light and appear transparent, while effectively absorbing UV light in both the UVB and UVA wavelengths. Due to its rapid dissolution in water releasing Zn^{2+} ions, zinc oxide NPs are considered as environmental toxicants.

The potential of zinc oxide and TiO_2 NPs to induce phototoxicity and penetrate the dermis has been a concern for human safety of sunscreens. Skin penetration studies using both ex vivo and in vivo models have shown that these NPs do not penetrate deeper than the outer most layer or stratum corneum of intact skin. The benefits of protection against carcinogenic UV light radiation provided by sunscreens formulated with zinc oxide or TiO_2 NPs outweigh the minimal risks associated with phototoxicity, DNA damage, and skin penetration into shaved or sunburned skin.

MAMMALIAN TOXICOLOGY

Introduction

CNTs exemplify the two opposing faces of nanomaterials. Many highly desirable properties that are suitable for numerous beneficial applications contrast with reports of serious adverse effects in experimental animals. For example, future uses of CNTs in biomedical applications for delivery of drugs, genes, and biosensors or as tissue engineering scaffolds are dampened by reports of inflammatory fibrogenic and even mesotheliogenic effects in laboratory rodents. In vitro studies commonly use very high doses/concentrations in culture media that have no relevance in vivo. Moreover, results of in vitro assays can be misleading due to interference of the nanomaterials to be tested with the testing reagents, adsorption of induced mediators, or interference with optical measurements.

Concepts of Nanotoxicology

Shape, size, and size distributions are important determinants for the deposition efficiency of inhaled materials throughout the respiratory tract (see Fig. 29–4). Uptake into cells is influenced by their surface charge, surface reactivity, the chemistry of surface coatings (functional groups, polymeric coatings), and also surface defects to the material introduced during synthesis or during surface functionalization or processing.

Many ENPs are insoluble in the as-produced form (e.g., metallic Ag^+ and Ni^{2+} metallic NPs), and do not undergo simple dissolution, but can undergo chemical oxidation in solution, in tissue, or in the environment to produce soluble species (e.g., Ag^+ and Ni^{2+}) in a process that gradually degrades and eliminates the particle state. Such NPs are taken up into cells and subsequently dissolve, thereby creating a very high intracellular ionic metal concentration that is cytotoxic. The same ionic concentration outside the cell will elicit no or only a modest response because of selective cell barriers preventing metal ions from entering the cell.

A major challenge for nanotoxicological studies is that many of these properties, once they have been determined for a given ENM, are not stable but can change when prepared for toxicological testing or during storage or testing. Alterations can occur through oxidation or dissolution, or through adsorption of proteins and lipids once they have been taken up into

different compartments of the organism.

Dosemetrics

With NPs, expressing dose–response relationships based on the surface area of the NP is most informative. The concept of particle surface area as dosemetric is biologically plausible because the surface of particles interacts with cell structures, but total surface area may only be a surrogate of the actual biologically relevant surface. There are several surface properties (Fig. 29–4) that affect interaction with cells. Thus, identifying a biologically available surface area will be of great value for defining a proper dosemetric.

The volume of NPs has been suggested as another dosemetric. The “particle overload” hypothesis states: when the volume of phagocytized particles in alveolar macrophages exceeds 6% of the normal macrophage volume, their physiological clearance function becomes impaired; if the phagocytized volume reaches 60%, the clearance function ceases completely.

Particle number is particularly important in characterizing exposure. Exposure concentrations in the air are expected to be very low, which makes it difficult to determine the precise concentration based on measuring mass. However, a low mass of NP consists of a huge number of particles that can reliably be measured in the airborne state using scanning mobility particle sizers or concentration particle counters.

Portals of Entry

The respiratory tract, the gastrointestinal (GI) tract, and skin are the main organs of direct exposure to ENM. For medical application, injection (intravenous, intramuscular, subcutaneous, and other) is an important entry route. Respiratory intake is the most prevalent route for occupational exposures; additives of ENM to food and potential contamination of food from nanoenabled packaging materials result in exposure via GI tract. Skin exposure via cosmetics and skin-care products occurs, although penetration of healthy skin by NP has not been demonstrated.

Dosing of the Respiratory Tract

Dosing of the respiratory tract of laboratory rodents involves administering materials as a bolus in a second or less. However, inhalation is the only physiological method and should be considered the gold standard for exposure to airborne materials. Major differences between bolus-type and inhalation exposures relate to the dose rate, use of anesthesia, and the distribution of administered material within the respiratory tract.

Bolus-type delivery occurs within a fraction of a second, whereas inhalation at realistic concentrations takes hours to months of exposure in order to deposit the same dose in the lung. Treating a dose delivered by bolus to be the same as a dose that has accumulated in the lung over a life-long exposure is scientifically not justifiable. Inundating cells abruptly with an extraordinarily high dose overwhelms the cell’s defense mechanisms and leaves no time for developing adaptive responses. There is a tremendous difference of inducing pulmonary inflammation by either intratracheally instilling (~0.5 second duration) 200 µg of TiO_2 NP versus depositing the same dose by inhalation over a period of 4 hours or 4 days (4 × 4 hours).

The difference in dose rate is four to five orders of magnitude, with a strong inflammatory response at the highest dose rate (instillation) and no response at the lowest dose rate (inhalation).

Adaptive responses are important physiological protective mechanisms, which need to be considered when interpreting results of nanomaterial toxicity testing. However, the organism's capacity to adapt may not only be compromised by very high acute exposures of short duration (high dose and high dose rate) but also be impaired in susceptible parts of the population.

Despite the limitations of bolus-type delivery, they may be viewed as "proof-of-principle" with the findings to be confirmed by subsequent inhalation studies. Although results from bolus-type studies are useful for toxicity ranking against known positive or negative control ENMs, they cannot be used for quantitative risk assessment. The "concept of differential adsorption" states that the physicochemical properties of nanomaterials, (size, surface size and chemistry, charge, surface hydrophobicity, redox activity, etc.) when in contact with media in the different body compartments (respiratory tract lining fluid, gastrointestinal milieu, plasma proteins, other mucosal membranes, and intracellular/extracellular fluid) determine protein and lipid adsorption and desorption patterns and thereby influence biodistribution across barriers and in target tissues and cells (Fig. 29–5).

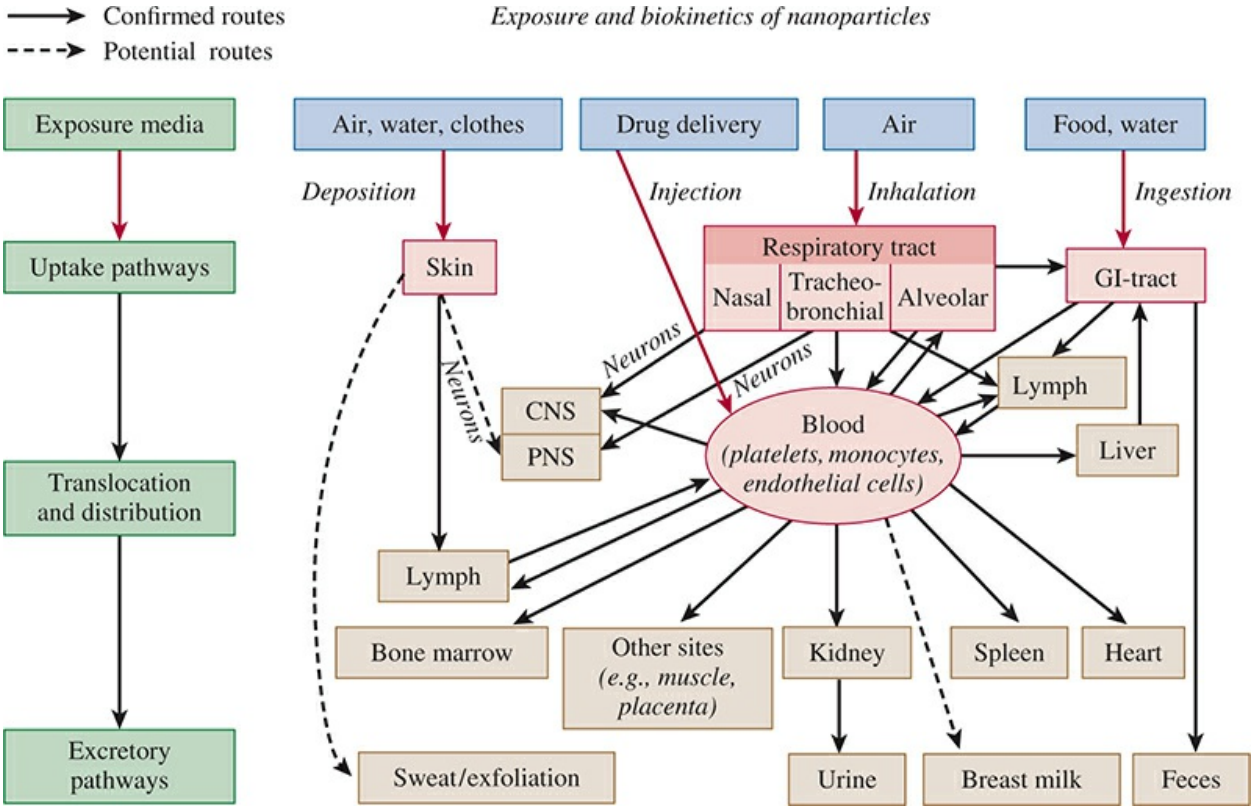

FIGURE 29–5 *Exposure and biokinetics of nanoparticles' routes of exposure and biokinetics (uptake, distribution, elimination) of nanomaterials.* Translocation rates in general are very low (see text). (Reprinted with permission from Oberdörster G, Oberdörster E, Oberdörster J: Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles, Environ Health Perspect 2005 Jul;113(7):823-839.)

Respiratory Tract Deposition

Inhalation of airborne ENMs results in significant deposition in the three compartments of the respiratory tract: the nasopharyngeal region from nose/mouth to the larynx, the tracheobronchial region from larynx to terminal bronchioles, and the alveolar region from the first generation of respiratory bronchioles (bronchioles with some alveoli) to the last generation of alveolar ducts. The deposition efficiency of inhaled particles depends on several particle characteristics, the anatomical structure of the airways, and breathing parameters. Particle size, size distribution, density, and shape are most important for their aerodynamic and thermodynamic diameter, which govern deposition in the respiratory tract by inertial impact, gravitational settling, and displacement by diffusion.

Obvious differences between rats and humans are the maximum size of particles that will reach the alveolar region. In rats, this is about 5 µm aerodynamic size, in humans about 15 µm. Although these sizes are outside the range of single NP, airborne NPs occur for the most part as agglomerates.

Respiratory Tract Clearance and Disposition of NP: Nanomaterials

Once NPs are deposited in the respiratory tract, they will encounter physiological clearance mechanisms. However, there are several differences that separate NPs from larger particles, as indicated in Table 29–2. Alveolar macrophages generally are attracted to deposited particles by chemotactic signals generated at the site of deposition. Nanosized particles may be too small to generate such signals, leading to uptake into the pulmonary interstitium. Translocation into the interstitium and subsequently into blood and lymph circulation distinguishes nanoparticles from microparticles. Figure 29–6 depicts the blood compartment as a plenum from which any tissue/organ can be reached by circulating NPs. However, the amount of NP translocating from the lung to the blood circulation and accumulation in secondary organs is very low. Despite the generally low translocation rates, a continuous exposure may result in significant accumulation in some secondary organs.

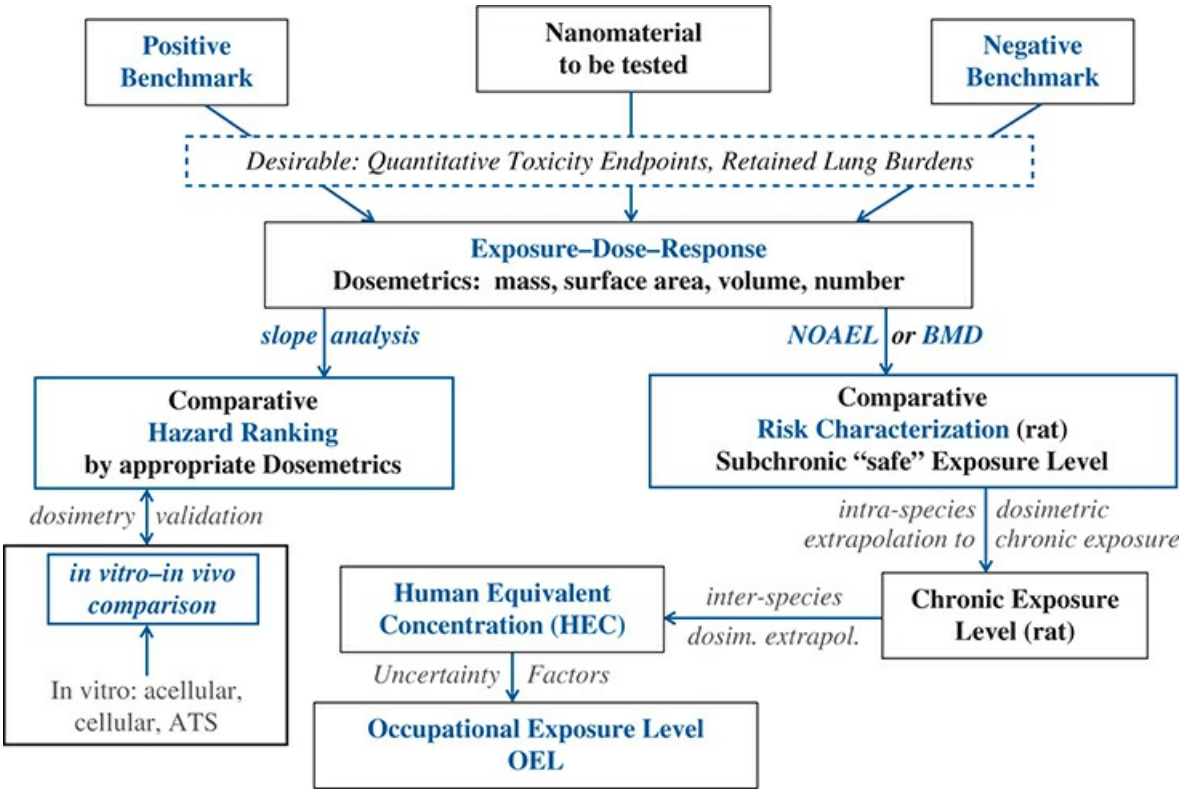

FIGURE 29–6 *Approach for comparative hazard and risk characterization of inhaled nanoparticles based on subchronic (3 months) rat inhalation studies.* NOAEL, no observed adverse effect level; BMD, benchmark dose; OEL, occupational exposure level; ATS, alternative testing strategy. (Reprinted from Oberdörster G, Kuhlbusch TAJ. In vivo effects: Methodologies and biokinetics of inhaled nanomaterials. *NanoImpact*. 2018;10:38–60.)

Nanomaterials and the Brain

In addition to the blood circulation, which does not efficiently contribute to CNS translocation because of the very tight blood-brain barrier, there is uptake into the lymphatic system—again feeding back into the blood circulation—and transport in or next to sensory neurons from the upper respiratory tract. Perineural translocation along the olfactory nerve will deliver NP into the cerebrospinal fluid (CSF). The most efficient pathway of NP translocation to the CNS appears to be via olfactory sensory neurons from the nasal olfactory mucosa directly to the olfactory bulb. Results of epidemiological studies of impaired cognitive function and of neurodegenerative brain pathology associated with exposure to traffic-related particles raised the question as to whether ambient ultrafine particles as constituents of urban air pollution may be etiologically involved.

Elimination of Nanomaterials

Elimination pathways for ENM from the body (Fig. 29–6) include mainly feces and urine. Urinary excretion is restricted to nanostructures <5.5 nm in size for metal-based NP. Circulating fibrous structures of ENM such as large MWCNTs can collect in urine of rats following intravenous application. This phenomenon may be due to a hydrodynamic lining up of nanotubes

so they will pass through glomerular pores. The fecal excretory clearance pathways consist of several inputs: one is mucociliary clearance of deposited particles from the airways into the GI tract and the other is via hepatobiliary clearance of blood-borne ENM via liver and bile into the small intestine.

Another clearance pathway of deposited ENM in the lung involves translocation via interstitium or lymph to the pleura and subsequent elimination via lymphatic openings (stomata) on the parietal pleura to mediastinal lymph nodes from where NP may enter the blood circulation via the thoracic ducts. This pathway is particularly important for ENM fibers because the size of the parietal stomata prevents efficient clearance of structures >10 μm in length. Consequently, the interaction of the retained longer fibers in the pleural cavity with mesothelial cells induces inflammatory and granulomatous responses and in the long-term potentially mesothelioma.

CASE STUDY: MWCNT

Bolus-type Exposures

Bolus-type delivery of CNTs to the respiratory tract of rats and mice revealed induction of dose-dependent significant inflammatory, granulomatous, and fibrogenic responses; MWCNTs can reach subpleural and intrapleural sites. Intraperitoneal injection studies show the potential of CNTs, specifically MWCNTs, to induce severe adverse length-dependent effects at mesothelial sites once they reach the pleural cavity.

Inhalation Studies

The value of short-term inhalation studies for risk characterization can be questioned because such exposures to CNTs at realistic low concentrations are not likely to reveal certain chronic effects if long latency periods are involved. The most meaningful and best justified study for the risk assessment process would be a subchronic multiconcentration (minimum three concentrations) study with sufficient postexposure observation. However, short-term, even one-term, exposure to a relevant concentration is very useful for dosimetric purposes when determining the biodistribution from deposition sites in the lung to secondary organs.

Critical Appraisal of CNT In Vivo Studies

Given the importance of the physicochemical properties of CNTs for inducing adverse effects, it is of utmost importance to determine these properties as they appear in the airborne state at sites of human exposures, at occupational sites, or for the consumer. Adding dispersants for testing purposes will change surface properties; conceptually, inhalation studies in experimental animals for purposes of hazard identification should mimic human exposure conditions regarding airborne size distribution. Of course, differences in respirability between humans and rats/mice must be considered, and adjustments be made without use of surface-altering dispersants.

Appropriately designed multidose subchronic inhalation studies, including a longer recovery period, are essential for deriving no observed adverse effect level (NOAELs); results can be used as basis for deriving occupational exposure levels (OELs) by applying rodent/human dosimetric

adjustments. Using results from bolus-type studies is difficult and raises questions. Mass-based recommended exposure limits may not be sufficiently sensitive for detecting CNTs by air sampling and call for research to determine the most sensitive dosemetrics.

The available in vivo toxicological database of MWCNTs has identified a fibrogenic and carcinogenic effect (hazard identification). SWCNTs also appear to be fibrogenic in the lungs. The paradigm of dose–dimension–durability for fiber carcinogenicity may also apply to CNTs: they are durable, and their dimensions (length, diameter, and agglomeration/entanglement) appear to be important for pulmonary effects. Of course, other properties (chemistry, surface reactivity) may play a role as well.

Biological Degradation of Carbon Nanomaterials— CNTs have been generally regarded as stable nondegradable materials, which has important implications for long-term health effects following inhalation into the lungs. However, SWCNT degradation has been observed in acellular assays that simulate the phagolysosome of macrophages, but only if the tubes have been surface carboxylated, which introduce collateral defects in the side walls. Carboxylated tubes are susceptible to biodegradation by exposure to hydrogen peroxide and horseradish peroxidase or hypochlorite and myeloperoxidase. Graphene oxide is susceptible to oxidative attack by hydrogen peroxide and horseradish peroxidase. These observations may enable design of safer carbon materials that are potentially biodegradable in order to minimize adverse environmental and human health impacts.

TOXICITY TESTING

A conceptual testing paradigm is shown in Fig. 29–6. In order to perform risk assessment or to derive OELs, exposure and hazard data are required. In vitro and in vivo studies will be useful, and results should be derived via well-designed dose–response relationships. Key considerations for designing such studies include careful physicochemical characterization of the ENM to be tested, justification of the method(s) of dosing, selection of target cells, tissues or animal species, and appropriate endpoints.

In vivo animal studies obviously do not generally predict human exposures: the lower loops in Fig. 29–6 refer to dosimetric correlations between in vitro/in vivo and in vivo animals/in vivo humans, going in both directions with the goal to (a) inform the design of in vivo animal studies using available human exposure data and (b) use exposure–dose–response information from animal studies to compare with human data. The upper one-directional animal-to-human loop refers to extrapolating effects and mechanisms from relevant (based on dosimetric relevancy) animal studies to humans, with the goal to be predictive for deriving “safe” exposure levels.

A need for in vitro studies should be stressed as far as uncovering underlying mechanisms of effects are concerned, provided that the caveats pointed out above are considered. In addition, in vitro studies are useful for toxicity ranking of NMs for the purpose of hazard identification. In contrast, the design of in vivo studies allows the full evaluation of exposure–dose–response relationships, which is necessary for the process of risk assessment.

The major exposure route for CNTs is via inhalation at manufacturing sites and during distribution and usage (handling, refilling, disposal); ingestion and dermal exposures also occur but have not received much attention (Fig. 29–6). For medicinal applications, injection is an important route of exposure requiring specific awareness with respect to assuring desired

beneficial (pharmacological) outcomes yet avoiding undesirable (toxicological) responses. For example, the desired pharmacological target organelles for drug delivery by SWCNT are the cell lysosomes, whereas the mitochondria are the target organelles for SWCNT toxicity.

In Vitro Dosimetry

As most ENM toxicity studies are performed using in vitro assays, which are generally short term, dosing-related questions are highly relevant. The dose received by the cell is a function of colloidal dynamics or "particokinetics" in the culture medium governed by diffusion and settling phenomena, which in turn is governed by particle and media properties that include particle size, agglomeration/aggregation state, shape, density, and charge. At equal mass concentrations in the medium, the magnitude of the cellular dose of ENM will differ significantly from those implied by the media concentration.

Models in in vitro dosimetry include using computational fluid dynamics and the distorted grid (DG) model, the multiple path particle dosimetry (MPPD) model, and the in vitro sedimentation, diffusion, and dosimetry (ISDD) model. The value of these models lies in the clear separation between exposure (concentration in the cell medium), the deposited dose on the cell surface, and—depending on cellular uptake—the cellular dose. These doses can be expressed by different metrics: mass, number, or surface area of the nanomaterial.

An alternative in vitro methodology that avoids pretreatment of NP with surfactants or proteins prior to testing is exposure via an air-liquid interface (ALI) system. Respiratory tract epithelial cells are grown on porous culture cell inserts and exposed to NP generated the same way as is done for in vivo inhalation studies. Before exposure, culture medium is withdrawn so that only the basal cell surface is in contact with the medium, and deposition of the aerosolized NP on the apical cell surface simulates realistic in vivo inhalation exposure. Other alternative exposure models for inhalation nanotoxicology include precision-cut rat lung slices and acellular human three-dimensional lung scaffolds. Additional non-animal epithelial barrier models are being developed for evaluation of lung, intestinal, and skin toxicity as static or microfluidic platforms.

Predictive Toxicology

Data about hazard and exposure are key for risk assessment. Critical elements for hazard identification based on toxicity testing of ENM involve detailed information about their physicochemical properties prior to any experiments, the selection of appropriate target cells, validation of in vitro assays in terms of correlation and relevancy to in vivo results, the inclusion of biokinetics in the design of in vivo studies, and the inclusion of realistic doses in the design of dose–response in in vitro and in vivo studies. Biokinetic information is crucial for identifying potential secondary target organs based on significant accumulation of ENM. Real-world exposure scenarios, either measured or estimated, are essential, and exposure–dose–response (in vivo, rodents) and dose–response (in vitro, target cells) relationships can be established to both characterize a hazard and assess a risk (see below). In terms of toxicity ranking, it is desirable to include reference materials in the study design so that a hazard can be expressed relative to a positive (high hazard) or negative (low hazard) control ENM. Mechanistic information discovered through in vitro assays will further aid in the characterization of hazard, provided that

the mechanism is operative at relevant doses/concentrations.

The immediate goal of ENM toxicity testing is hazard characterization and the establishment and validation of respective predictive tests. A distant goal is the incorporation of in silico models. Animal studies are still indispensable for obtaining crucial information about long-term effects of ENM and obtaining results for risk assessment.

Development of a Set of Screening Tools That Reflect Important Characteristics or Toxicity Pathways of the Complex Systems Described Above—To summarize a suggested framework and research strategy, implementation of the following considerations would significantly improve the methodology for development of reliable and validated screening tools:

1. Rigorous physicochemical characterization of nanoparticle types and behavior through the life cycle
2. Dose–response characterization and careful attention to reliable dosimetry at relevant human exposure levels
3. For in vitro studies, selection of relevant cell types and cell models that reflect the route of human exposures
4. Time-course assessments that span acute to chronic exposure durations
5. Application of proper benchmark controls to improve interpretation of toxicological outcomes

DESIGNING ALTERNATIVE IN VITRO METHODS FOR SCREENING PULMONARY TOXICITY OF FINE AND NANOSCALE PARTICLES

The development of reliable and predictive aerosol-based in vitro lung toxicity assays for screening particulate/nanoparticulate materials might (in the future) limit the need for animal testing, particularly during the early phases of hazard evaluations. Additional benefits would include an enhanced capacity to screen several compounds during the early phases of product development using cost-effective and validated in vitro assays. Reliable hazard data developed from these tests could serve as a bridge to the subsequent implementation of longer-term particle inhalation studies, thereby obviating the need for conducting acute (in vivo) toxicity studies, and significantly reducing the numbers of test animals.

The pulmonary microenvironment and the complex interactions in the lung that occur following inhalation and subsequent deposition of particles are difficult to simulate in vitro. Studies generally use only single cell types (e.g., A549 lung epithelial cells—*or* lung or peritoneal macrophages) as simplified models and ignore the complex interactions in the lung microenvironment that occur following exposure to aerosols of particles, bacteria, etc. More complex in vitro systems are being developed, which will facilitate (and hopefully expedite) the transition from the current animal-based inhalation testing system to one that is based primarily on human cell lines and in vitro assays. Such reproducible, accurate, and validated cell-based in vitro screening assays for assessing pulmonary and genetic toxicity will have important benefits

(i.e., screening more compounds in a faster, more reliable, and less expensive manner) and may provide experimental designs to address mechanistic questions.

Many fundamental issues require consideration for optimization when facilitating a transition from an animal inhalation toxicity setup to an in vitro aerosol pulmonary toxicity study. These considerations include, but are not limited to, the following actions:

- Determining the cell types to be used in a co-culture system—to better simulate the lung microenvironment
- Transitioning from primary cell types collected from animals to immortalized cells derived from cell lines and tested for biological functionality
- Determining the number or ratio of lung epithelial cells to alveolar macrophages in a co-culture plate
- Determining the aerosol generation method for particles/nanoparticles and appropriate, reproducible, and quantifiable dosemetrics to be utilized for particle inhalation deposition assessments and for comparisons of results of one study to another
- Demonstrating reproducibility for particle generation studies by measuring particle deposition on filters—choosing appropriate filter types and measurement methods to ensure validation of results
- Deciding on the appropriate endpoints for assessing lung toxicity parameters, which can be benchmarked against in vivo results on the same particle types
- Validating effects, doses, and mechanism with in vivo test
- Time-course protocol to assess the sustainability of any measured response
- Exposure-dose–response assessments
- Utilization of benchmark particulate control test materials in order to develop a frame of reference for better interpretation of results

Read Across—Nanogrouping

As more new nanomaterial types are being produced and entering commerce, the importance of adequately testing, and assessing the hazards and potential risks of these particulates becomes critical. A practical strategy for assessing potential hazards of nanoparticles involves the grouping of similar particle-types, to streamline testing for regulatory purposes. The conceptual design is to group nanomaterials that are similar or complementary in physicochemical characteristics for assessment. These would include the properties and biophysical interactions of nanomaterials, specific types of use and exposure, uptake and kinetics, as well as possible early and apical biological effects.

Transition, Human—Eco-nanotoxicology

The goals of nanotoxicology are to identify and characterize a hazard of ENMs for purposes of risk assessment for humans and the environment. This requires a highly multidisciplinary team approach, covering expertise in toxicology, biology, chemistry, physics, material science, geology, exposure assessment, PBPK and fate and transport modeling, and medicine. It is necessary to develop testing strategies, establish toxicity ranking, determine “safe” exposure levels, and derive preventive exposure guidelines.

ECOTOXICOLOGY OF ENMS

Environmental Uses and Exposures to Nanomaterials

As with many chemicals in the marketplace, a portion of the nanomaterials used in industry and consumer products will enter the greater environment during some part of their life cycle, either through waste during production or through product use. Many identified uses are in cosmetics, clothing (where nanomaterials are often added for antimicrobial and antiodor functions), personal care products, and sporting goods. The most common nanomaterials included in these products are silver and carbon nanomaterials. Other chemicals used in personal care products, cosmetics, and clothing have been found to wash into the wastewater treatment system and end up in the aquatic environment either directly from the treatment facilities or through land applications of biosolids from wastewater treatment plants.

Ecological Risk Assessment of Manufactured Nanomaterials

The goal of this science to date has been to determine how a particulate form of a chemical in a nanosize range influences its toxicity versus its larger bulk counterparts. A further goal is to determine how small changes in surface chemistries, size, shape, and structure affect the interaction with organisms in the environment. The assessment of ecological risk is more complicated than human health assessments, as there are more species involved and higher level impacts on populations, communities of organisms, and ecosystem function to consider.

The basic framework used for ecological risk assessment involves problem formulation, analysis, and risk characterization. Problem formulation involves selecting endpoints of concern, determining impacts on important endpoints (such as a species decline and ecosystem changes), and evaluating the potential for exposure to stressors and their effects at those exposure levels. Nanomaterials may also be transformed within the environment, and therefore the toxicity of the initial nanomaterial may not provide a complete idea of the toxicity over the lifetime of the material.

Toxicity of Manufactured Nanomaterials

Complications of Assays— Some of the major issues in toxicology assays include delivery of nanomaterials in media and approximating environmental conditions, characterizing exposures, maintaining exposures throughout an assay, and determining the state of exposure throughout an assay. Many nanomaterials are not easily dispersible and aggregate substantially when introduced into common exposure media. Researchers have attempted to circumvent this issue by either changing the surface chemistry of the nanomaterial to make it more readily suspendable by adding a hydrophilic functional group or by altering the exposure conditions to include organic material that assists in suspension. Changing the surface chemistry of a nanomaterial can also change its toxicity. The relationship between surface chemistry and toxicity is important to understand because surface functionalization is an essential aspect of nanomaterial formulation for industrial applications. Many coatings can cause toxicity on their own regardless of whether it is attached to the nanomaterial, and can be removed in certain cases by organisms in the test media, or the environment, which dramatically changes the properties of the nanomaterial

through the experiment. Is the toxicity then due to the original particle or now to the altered form of the nanomaterial?

Determining the dose an organism actually encounters requires verifying how much of any nanomaterial actually reaches the organism and is taken up into the organism and its tissues. There are several difficulties in quantifying uptake, including identifying the nanomaterial within the matrix of the organism versus inside the organism. Another issue is calculating the dose as a mass versus surface area. The real adverse impacts of nanomaterials may not be due to ambient environmental concentrations that arise but may be due to some subset of materials that are persistent and biomagnify in the environment.

Ecotoxicity of Nanomaterials

The studies on the toxicity of ENMs to date conclude that toxicity varies with the type of nanomaterial and is not universal across materials. The varying degrees of toxicity depend on the type of nanomaterial, organism studied, and the co-occurrence of other environmental factors such as UV light, organic carbon, or low pH. Most nanomaterials that are needed to kill half of the sample population are in the mg/L range, which is far above the estimates of potential exposures to any nanomaterial.

Silver nanomaterials are some of the most widely used materials and appear to demonstrate the greatest toxicity of materials investigated in the literature. Silver is toxic at μg/L doses to a variety of organisms. Rather than creating a free radical in media, the impacts of metal nanomaterials may be due to metal imbalance in cells after uptake and accumulation, leading to apoptosis and cellular disregulation. Nanosilver and possibly other nanomaterials based on soft metals may also react with environmental sulfides to produce silver sulfide nanomaterials, in which the silver bioavailability and toxicity is much reduced.

Mechanisms of Toxicity

Oxidative stress has been implicated as a major way in which nanomaterials exert toxicity either by generating free radicals within the suspension media or by changing the chemistry of the cells in which they come in contact. Metal oxide nanomaterials have been found to generate oxidative stress with greater toxicity than their bulk counterparts.

The toxicity of nanomaterials to aquatic organisms can be greatly dependent upon the interaction of nanomaterials with the media to which they are introduced. Dissolution of metal oxides and metal nanomaterials is greatly impacted by the characteristics of the media such as pH and salt content. Nanomaterials may also impact the bioavailability and toxicity of other contaminants in the environment.

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QUESTIONS

1. Which of the following is not a nanoparticle?
   - **a.** carbon nanotubes.
   - **b.** bucky-ball.
   - **c.** graphene.
   - **d.** zinc nanorods.
   - **e.** bacteria.

2. Which of the following answers is not true regarding nanoparticles?
   - **a.** NPs can originate from natural sources including forest fires, volcanoes, and viruses.
   - **b.** NPs can originate from unintentional sources including internal combustion engines and electric motors.
   - **c.** NPs can originate from unintentional sources including ferritin and magnetotactic bacteria.
   - **d.** NPs can originate from intentional sources including carbon nanotubes and metal oxide nanoparticles.
   - **e.** NPs can originate from natural, and intentional and unintentional anthropogenic sources.

3. In contrast to larger particles >500 nm, nanoparticles
   - **a.** are highly likely to enter the body by dermal absorption.
   - **b.** are highly likely to enter the body through the respiratory tract.
   - **c.** are unlikely to adsorb to protein or lipid.
   - **d.** are efficiently removed from the lungs via mucociliary transport.
   - **e.** are not likely to undergo uptake and transport in sensory neurons.

4. Which of the following statements is NOT true?
   - **a.** Nanomaterials may be classified by geometry and chemistry.
   - **b.** Engineered nanomaterials include quantum dots, C-nanofiber array, and few-layer graphene.
   - **c.** Agglomerates include primary particles held together by weak van der Waals forces.
   - **d.** Aggregates include primary particles held by strong chemical bonds.
   - **e.** Hydrodynamic diameter is unimportant in particle interactions.

5. Nanoparticles can exert toxicity by all of the following mechanisms except:
   - **a.** damage to DNA and chromosomes.
   - **b.** induction of oxidant stress.

c. interference with biotransformation enzyme activities.
d. activation of signaling pathways.
e. release of toxic metal ions from internalized NPs.

6. Biodistribution of nanoparticles may be influenced by
a. physicochemical properties such as plasma protein and respiratory tract mucus.
b. physicochemical properties such as surface size and chemistry.
c. physicochemical properties such as the gastrointestinal milieu.
d. body compartment media including surface hydrophobicity.
e. body compartment media including size.

7. Assays to determine the toxicity of manufactured nanoparticles suffer from all of the complications below except:
a. the nanomaterial aggregate may no longer be in the nanosize range.
b. aggregates of the nanoparticle may settle out of solution that may affect exposure dose.
c. alterations in surface chemistry to stabilize suspension may evoke other issues in toxicity assessment.
d. coatings of particles may have their own toxicity.
e. uptake of the nanoparticle into an organism is easily determined.

8. The goals of nanotoxicology are
a. to identify and characterize hazards of engineered nanomaterials.
b. to determine “safe” exposure levels.
c. to determine biological and biochemical actions.
d. to determine manufacturing procedures and cost.
e. to determine preventive exposure guidelines.

UNIT 6 ENVIRONMENTAL TOXICOLOGY

CHAPTER 30

Ecotoxicology

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KEY POINTS

- Ecotoxicology is the study of the fate and effects of toxic substances on an ecosystem.
- Chemodynamics is the study of chemical release, distribution, degradation, and fate in the environment.
- A chemical can enter any of the four matrices: the atmosphere by evaporation, the lithosphere by adsorption, the hydrosphere by dissolution, or the biosphere by absorption, inhalation, or ingestion (depending on the species). Once in a matrix, the toxicant can enter another matrix by these methods.
- The *biological availability* (or bioavailability) of a chemical is the portion of the total quantity of chemical present that is potentially available for uptake by organisms.
- Pollution may result in a cascade of events, beginning with effects on homeostasis in individuals and extending through populations, communities, ecosystems, and landscapes.
- Terrestrial toxicology is the science of the exposure to and effects of toxic compounds in terrestrial ecosystems.
- Aquatic toxicology is the study of effects of anthropogenic chemicals on organisms in the aquatic environment.

INTRODUCTION

Ecotoxicology is the science of contaminants in the biosphere and their effects on constituents of the biosphere. It has an overarching goal of explaining and predicting effect or exposure

phenomena at several levels of biological organization (Fig. 30–1).

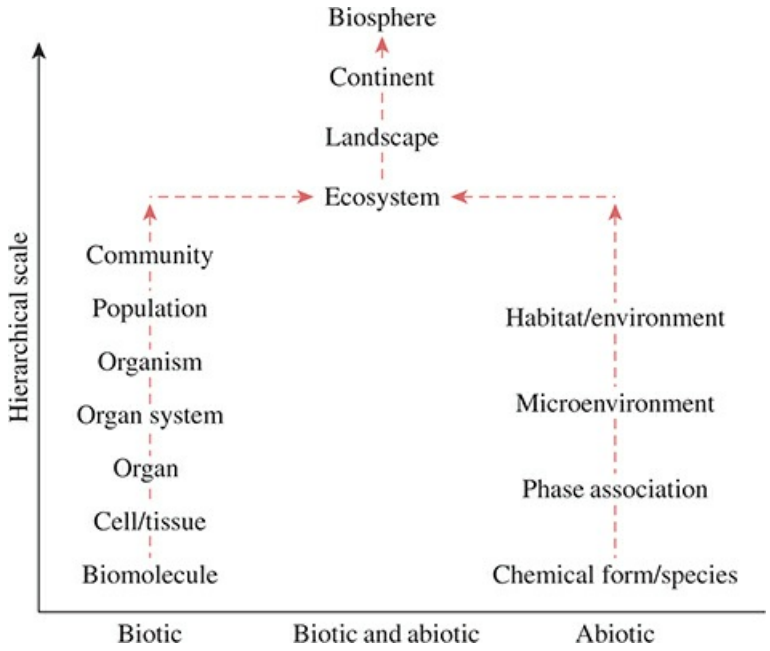

FIGURE 30–1 *Ecological scales relevant to ecotoxicology.* Solely biological scales relevant to ecotoxicology range from the molecular to the community levels: solely abiotic scales range from the chemical to the entire habitat. Biotic and abiotic components are usually combined at levels above the ecological community and habitat. The ecological community and physicochemical habitat combined to form the ecosystem. Ecological systems can be considered at the landscape scale, such as the combination of marine, freshwater, and terrestrial systems at a river's mouth. Recently, the continental and biospheric scales have become relevant as in the cases of ozone depletion, acid precipitation, and global warming.

Relevant effects to nonhuman targets range from biomolecular to global. As the need to predict major effects to populations, communities, ecosystems, and other higher-level entities has become increasingly apparent, more cause-effect models relevant to these higher levels of biological organization are added to the conventional set of toxicology models. Contaminant chemical form, phase association, and movement among components of the biosphere are central issues in ecotoxicology because they determine exposure, bioavailability, and realized dose. Risk to ecological entities is estimated or predicted by combining exposure and effect information. Risk might involve diminished fitness of individuals, increased risk of local population extinction, a drop in species diversity, or reduced nutrient cycling or primary productivity. Because potential ecological endpoints are so diverse, the ecological risk framework tends to be quite flexible (Fig. 30–2).

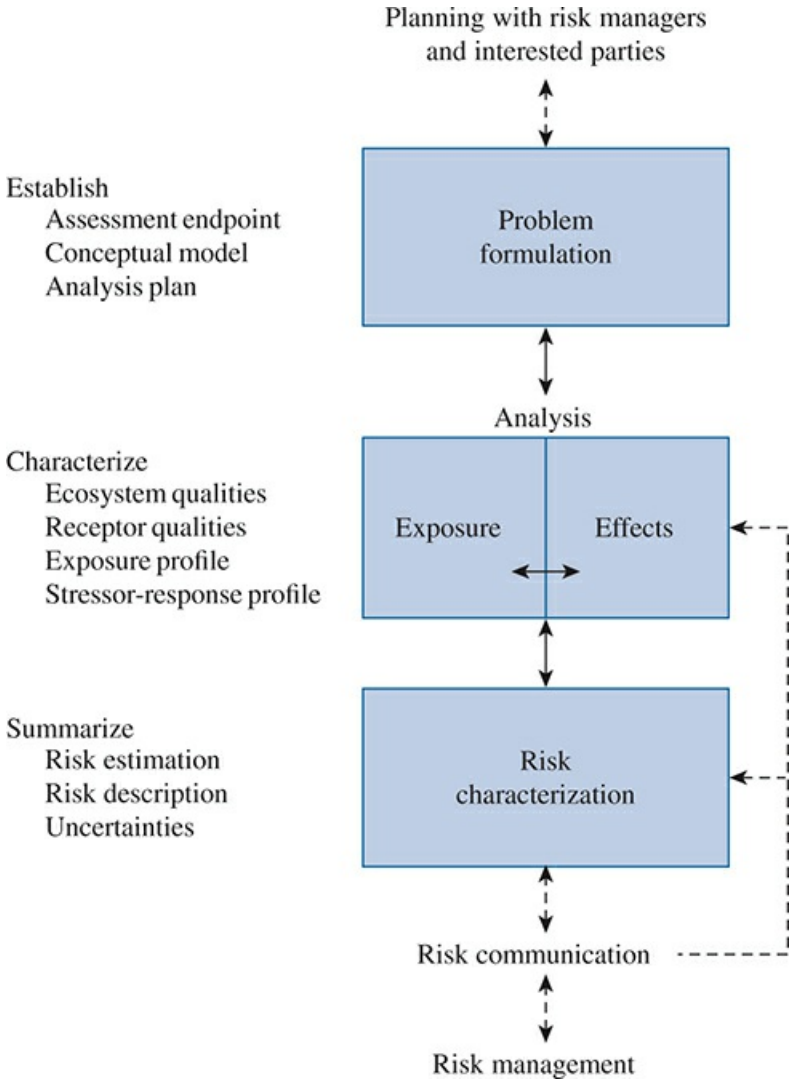

FIGURE 30–2 *The general form of an ecological risk assessment including problem formulation, analysis, and risk characterization stages.* Problem formulation is done in dialog with risk managers and stakeholders and involves a clear statement of the ecological entity to be assessed, a conceptual model for the process, and a plan for conducting the assessment. The analysis stage involves exposure and effects characterizations. Using the context developed during problem formulation and information organized together in the analysis stage, a statement of risk and associated uncertainties are made in the risk characterization stage.

SOME DISTINCT ASPECTS OF EXPOSURE

Predicting exposure and effect is difficult for all relevant ecological entities. Ecotoxicology commonly uses sparse information for a few species to predict effects to many species and their interactions. Relevant exposure routes are the conventional ingestion, inhalation, and dermal absorption. But unique features of exposure pathways must be accommodated for species that ingest a wide range of materials using distinct feeding mechanisms, breathe gaseous or liquid

media using extremely different structures, and come into dermal contact with a variety of gaseous, liquid, and solid media.

Prediction of oral exposure can be compromised because species feed on different materials; however, conventional principles regarding oral bioavailability remain relevant. As an example, some birds are uniquely at high risk of lead poisoning because they ingest and then use lead shot as grit. Shots are ground together in their gizzards under acidic conditions, releasing significant amounts of dissolved lead. The complex sorting of filtered materials on the gills of bivalve molluscs strongly influences the metal content and bioavailability of the material that eventually passes into their guts. Unlike most mammalian species, many invertebrate species are capable of sequestering large amounts of metals in intracellular granules. Incorporation of metals into granules by prey species reduces metal bioavailability to predators. Obviously, a plethora of critical exposure pathways are relevant to ecotoxicology.

Estimation of chemical speciation is central to predicting bioavailability of water-associated contaminants. Speciation can determine the bioavailability of dissolved metals. Movements of nonionic and ionizable organic compounds across the gut or gills are strongly influenced by lipid solubility and the pH-partition theory, respectively. Consequently, determination of a compound's lipophilicity or calculation of pH- and pK_a-dependent ionization facilitates some predictive capability for bioavailability. The free ion activity model (FIAM) and biotic ligand model (BLM) state that uptake and toxicity of cationic trace metals are best predicted from the interaction of the free ion with ligands on biological surface, although exceptions exist.

Bioavailability, bioaccumulation, or exposure concentrations for sediment-associated toxicants require considering chemical speciation and phase partitioning. Metals in sediments are either incorporated into one of many solid phases or dissolved in the interstitial waters surrounding the sediment particles. Bioavailable metals have been estimated by normalizing sediment metal concentrations to easily extracted iron and manganese concentrations because solid iron and manganese oxides sequester metals in poorly bioavailable solid forms.

Ecotoxicologists are also concerned with biomagnification, or the increase in contaminant concentration as it moves through a food web. Biomagnification occurs for many persistent organic pollutants (POPs) and some organometals such as methylmercury. Biomagnification, or environmental amplification, involves sequential steps of solvent switching followed by solvent depletion. Solvent switching might involve partitioning of the compound from water into the lipids in prey tissues. Next, after the predator ingests a prey, digestion of the prey lipids leads to POP partitioning into lipids of the predator tissues. The result of solvent switching and then depletion is a net increase in POP concentration in the predator relative to the prey.

TOXICANT EFFECTS

Ecotoxicological effects can be organized according to biological levels of organization. One may consider effects, in ascending order, at the subcellular (molecular and biochemical), cellular, organismal, population, community, ecosystem, or higher levels of organization. Ecotoxicology deals with, theoretically at least, all species, and in line with other aspects of natural resource management, the primary concern is one of sustainability. Policies and regulations surrounding chemical effects in natural ecosystems are designed to protect ecological features such as population dynamics, community structures, and ecosystem functions.

An important paradigm for elucidating linkages across levels of organization is the adverse outcome pathway (AOP). The AOP conceptually portrays existing knowledge linking a direct molecular initiating effect and an adverse outcome at a biological level of organization relevant to risk assessment. A generalized AOP is presented in Fig. 30–3. With ecotoxicology and ecological risk assessment, the population is the level generally of greatest relevance.

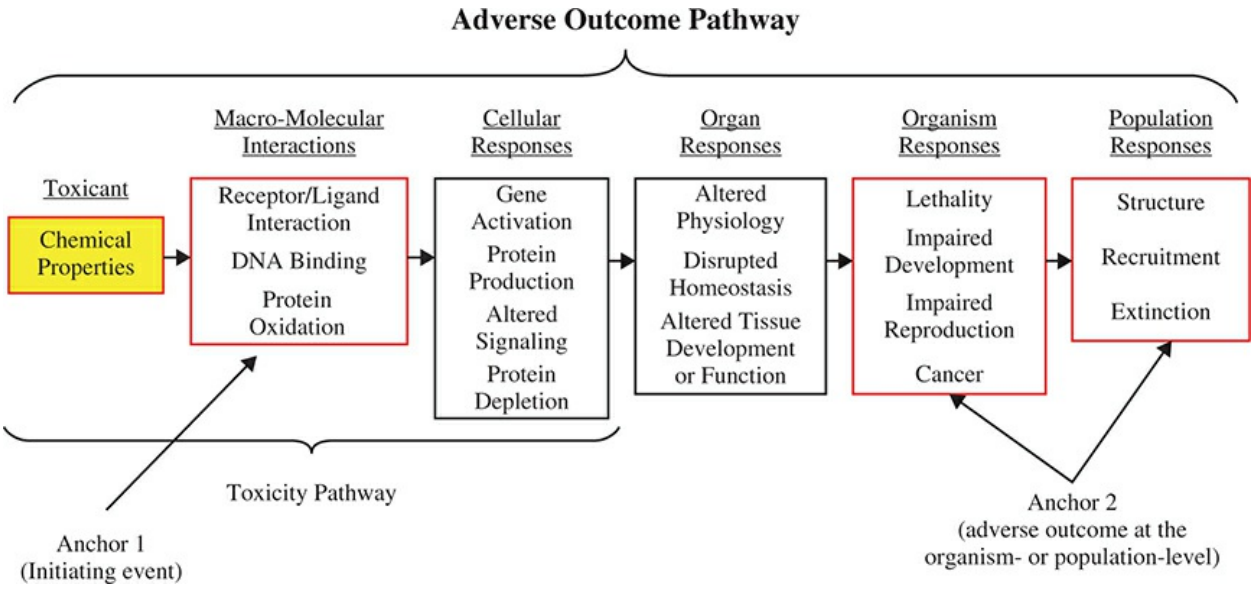

FIGURE 30–3 *An adverse outcome pathway (AOP) is a conceptual framework that portrays existing knowledge concerning the linkage between a direct molecular initiating event and an adverse outcome at a level of biological organization relevant to risk assessment.* (Reprinted from Ankley GT, Bennett RS, Erickson RJ, et al. Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. *Environ Toxicol Chem.* 2010;29:730–741.

Molecular and Biochemical Effects

This lowest level of organization includes fundamental processes associated with the regulation of gene transcription and translation, biotransformation of xenobiotics, and the deleterious biochemical effects of xenobiotics on cellular constituents including proteins, lipids, and DNA.

Gene Expression and Ecotoxicogenomics

Xenobiotics can affect gene transcription through interactions with transcription factors and/or the promoter regions of genes that bind transcription factors in the process of activating transcription. Ligand-activated transcription factors are intracellular receptor proteins that recognize and bind specific compounds, forming a complex that binds to specific promoter regions of genes, thereby activating transcription of mRNAs, and ultimately translation of the associated protein.

Estrogen Receptor— The dominant natural ligand for this nuclear receptor is estradiol (E2). Binding of E2 with ER produces a complex that then binds to estrogen response elements (ERE)

of specific genes that contain one or more EREs, thereby causing gene transcription. Genes regulated in this manner by E2–ER play various important roles in, for example, sexual organ development, behavior, fertility, and bone integrity.

Many chemicals can serve as ligands for ER; in most cases these "xenoestrogens" activate gene transcription acting as receptor agonists. Xenoestrogens include diethylstilbestrol, certain chlorinated hydrocarbon insecticides (e.g., DDT, methoxychlor, and endosulfan), surfactants (nonylphenol), some polychlorinated biphenyls (PCBs), bisphenol A, and ethinyl estradiol, a synthetic estrogen used in birth control pills and observed in municipal effluents and surface waters. Environmental exposures may be sufficient to perturb reproduction or development.

Endocrine disruptions by environmental xenoestrogens appear to be overall stronger for wildlife than for humans, likely due to instances of elevated exposures that are less prone to confounding factors than is typically the case for human exposures. Egg-laying vertebrates provide a unique biomarker of estrogen exposure: vitellogenin (Vtg) is a protein that is normally produced by the liver of females and transported via the bloodstream to the ovary where, as a key component of yolk, it provides nourishment to the developing embryo. Elevated Vtg in males of these species is a useful biomarker of estrogenic chemical exposures.

Aryl Hydrocarbon Receptor—The AhR is a member of the basic helix–loop–helix Per ARNT Sim (bHLH-PAS) family of receptors/transcription factors and is involved in development, as sensors of the internal and external environment in order to maintain homeostasis, and in establishment and maintenance of circadian clocks. Characterized genes that are upregulated by the AhR system in large part code for enzymes involved in the metabolism of lipophilic chemicals, including organic xenobiotics and some endogenous substrates such as steroid hormones. These enzymes include specific cytochrome P450s (mammalian CYP1A1, 1A2, and 1B1, and their counterparts in other vertebrates), a glutathione transferase UDP-glucuronosyltransferase and delete the (UDPGT), an alcohol dehydrogenase and a quinone oxidoreductase.

Some ubiquitous pollutants act as AhR ligands and markedly upregulate gene transcription via the AhR–ARNT signaling pathway. Pollutants that act as ligands for the AhR and upregulate gene expression are the polycyclic aromatic hydrocarbons (PAHs) and the polyhalogenated aromatic hydrocarbons (pHAHs). In general, pHAH-type AhR ligands are more potent AhR ligands and enzyme inducers than PAHs, but due to extensive halogenation, are much poorer substrates for biotransformation.

The inducibility of biotransformation enzymes via the AhR by xenobiotics has been used for biomonitoring, particularly ethoxyresorufin *O*-deethylase (EROD) activity, which is most often quantified in liver tissue of vertebrates. Elevated activities of EROD in various vertebrates have been associated with exposures to PCBs, dioxins, PAHs, and complex mixtures associated with harbor sediments, municipal effluents, paper mill effluents, refinery effluents, and crude oil spills.

Genomics and Ecotoxicogenomics—Advances in "omics"-level technologies for comprehensive interrogation of biological structure, function, and system dynamics continue to revolutionize molecular biology. Species that have been completely or largely sequenced include man, the mouse, rat, cow, dog, chimpanzee, chicken, zebrafish (*Danio rerio*), puffer fish (*Fugu rubripes*), medaka (*Oryzias latipes*), fruit fly (*Drosophila melanogaster*), a sea urchin (*Strongylocentrotus purpuratus*), a soil nematode (*Caenorhabditis elegans*), a yeast

(*Saccharomyces cerevisiae*), and rice (*Oryza sativa*). The number of species with fully sequenced and annotated reference genomes continues to expand. New genomics technologies offer significantly higher resolution as compared to previous sequencing techniques and at lower costs.

Omics have spread into the science and applications of ecotoxicology, collectively termed ecotoxicogenomics, which has great potential for elucidating impacts of chemicals of ecological concern and ultimately for playing an important role in Ecological Risk Assessments (ERAs) and regulatory ecotoxicology. This emerging field can contribute to prioritization of chemicals investigated in ERAs, identification of modes of action of pollutants, identification of particularly sensitive species and populations, and effect prediction at higher levels of organization.

Protein Damage—Inhibitions of acetylcholinesterase (AChE) by certain pesticides and of delta-aminolevulinic acid dehydratase (ALAD) by lead are important research areas. The widely used organophosphate and carbamate classes of insecticides kill by inhibiting AChE, and this mechanism is operative for "nontarget" organisms including invertebrates, wildlife, and humans. Of particular ecological concerns have been ingestions of AChE-inhibiting insecticides with food items or granular formulations (mistaken as seed or grit) by birds and exposures to aquatic animals from agricultural runoff. ALAD, which catalyzes the rate-limiting step of heme synthesis, a key component of cytochromes, hemoglobin, and myoglobin, is very sensitive to inhibition by lead. This sensitivity has been exploited as a biomarker for lead exposure in humans and wildlife. Besides enzyme inhibition, chemicals can damage proteins in other ways, including oxidative damage, and by forming stable adducts like those formed with DNA.

Oxidative Stress—Oxidative stress has been defined as a disturbance in the prooxidant–antioxidant balance in favor of the former, leading to potential damage. Numerous environmental contaminants can act as prooxidants and enhance the production of reactive oxygen species (ROS). Redox cycling chemicals include diphenols and quinones, nitroaromatics and azo compounds, aromatic hydroxylamines, bipyridyliums, and certain metal chelates, particularly of copper and iron.

Another important mechanism particularly significant in aquatic systems is photosensitization. Ultraviolet (UV) radiation (specifically UVB and UVA) can penetrate surface waters to depths dependent on the wavelength of the radiation and the clarity of the water. The UV radiation generates ROS and other free radicals via excitation of photosensitizing chemicals, including common pollutants of aquatic systems.

It is reasonable to assume that oxidative stress comprises an important mechanism accounting in part for the toxicity of diverse pollutants to free-living organisms. Also, oxidative stress is involved in the effects of air pollutants on plants and likely plays a role in forest diebacks observed downwind of industrialized areas.

DNA Damage—Cancer is an important health outcome associated with chemical exposures in wildlife, particularly for bottom-dwelling fish. Ecotoxicology is greatly concerned with multigenerational effects of pollutants on genetic structures and resulting phenotypes of populations and communities. The most widely studied form of damage has been the formation of stable DNA adducts, particularly by PAHs. Complex DNA repair systems have been elucidated in prokaryotic and eukaryotic organisms and these conserved systems are qualitatively similar across diverse phyla.

Cellular, Tissue, and Organ Effects

Cells—Cellular organelles that have received attention as targets in species of ecological interest include mitochondria, lysosomes, and nuclei. Most free-living organisms routinely experience energy deficits. For example, food resources are often highly depleted during the winter for many animals, which adapt by conserving energy (by hibernating or lowering metabolism) or by storing energy beforehand (as the case for many migratory birds). Thus, effects of pollutants on mitochondrial energy metabolism can be particularly important to wildlife. Lysosomes are involved in the degradation of damaged organelles and proteins, and sequester a wide variety of environmental contaminants, including metals, PAHs, and nanoparticles. The accumulation of xenobiotics by lysosomes can elicit membrane damage, which has been used as an early warning measure of pathological chemical effects in both invertebrates and vertebrates.

Chemical effects on nuclei have been examined in ecological contexts. Micronuclei are chromosomal fragments that are not incorporated into the nucleus at cell division, and chemical exposures can markedly increase their frequency. Elevated micronuclei numbers have been observed in fish erythrocytes and in hemocytes in clams from a PCB-polluted harbor. A standardized higher plant (*Tradescantia*) assay for micronuclei has been used for monitoring air pollution.

Histopathology—Detailed microscopic analysis of the structure of cells and tissues can provide important links among chemical exposures, cellular targets and mechanisms, and effects at the organismal level. Moreover, the determination that tissue damage has occurred as demonstrated by histopathological analysis is extremely useful for inferring that a significant deleterious effect has occurred in numerous laboratory and field studies.

Target Organs—An important target organ in ecotoxicology is the respiratory organ of nonmammalian avian species and aquatic vertebrates and many invertebrates. In particular, the gill epithelium is the major site of gas exchange, ionic regulation, acid–base balance, and nitrogenous waste excretions for fish and other aquatic animals. Gills are immersed in a major exposure medium for these animals (surface water), so metabolically active epithelial cells are in direct contact with this medium. They also receive blood supply directly from the heart, through the ventral aorta.

Common structural lesions in gills caused by a diverse array of chemicals include cell death (via necrosis and apoptosis), rupture of the epithelium, hyperplasia and hypertrophy of various cell populations that can lead to lamellar fusion, epithelial swelling, and lifting of the respiratory epithelium from the underlying tissue. Chloride cells have a key role in ionic homeostasis, and metals such as cadmium, copper, lead, silver, and zinc may interfere with their function in ion transport. This may be due to inhibition of ATPase activities and/or increased membrane permeability.

Organismal Effects

Mortality—Chemical pollution of the environment does not in most cases attain levels sufficient to outrightly kill wildlife. Ecotoxicological concerns on the long-term, chronic impacts on organismal variables such as survival, reproduction and development, behavior, and disease susceptibility, and how such impacts translate to impacts at population and higher levels of

organization are paramount. While not a direct toxic chemical effect, hypoxia can be an important cause of fish and invertebrate mortality in aquatic systems; anthropogenic inputs of nutrients associated with sewage or fertilizers that enhance the growth of phytoplankton can cause or exacerbate hypoxia. While direct mortality may not be a commonplace effect of toxic chemicals in natural systems, mortality comprises a major endpoint in toxicity testing.

Reproduction and Development—Impacts on reproduction and development comprise perhaps the greatest concern among potential sublethal effects of xenobiotics on animals inhabiting natural systems. The discovery that some organochlorine insecticides caused eggshell thinning and resulting population crashes of several predatory bird species, and the public's awareness through the publication of Carson's *Silent Spring* in 1962, can be associated with the birth of ecotoxicology.

Chlorinated hydrocarbons have continued to generate concerns, although many (DDT and other insecticides, and PCBs) have had their production and use sharply curtailed. The developmental effects of dioxins (TCDD) and coplanar PCBs on vertebrate development including compromised cardiac development and other effects depend on binding the AhR. Similar concerns have emerged for the developmental effects of PAHs, particularly in fish. Hydrocarbons, in large part PAHs, associated with oil spills, contaminated sediments, paper mill effluents, and creosote used for wood treatment have profound developmental effects in fish embryos. In many cases, the effects observed visually include malformed hearts ("tube heart"), craniofacial deformities, hemorrhaging, and edema of the pericardium and yolk sac, the latter resulting in a distended, faintly blue yolk sac and hence a name given to this syndrome—"blue sac disease." The mechanisms by which PAHs produce this effect are unresolved, and likely include more than a single mechanism—not surprising considering the myriad chemicals comprising hydrocarbon/PAH mixtures in the environment.

Developmental abnormalities arising from toxicant exposure are also of concern for invertebrate species. Antifouling agents in paints applied to ship hulls leach into coastal waters, causing widespread imposex in many marine snails. Acting as endocrine disrupters, organotin compounds cause male characteristics such as a penis or vas deferens to develop in females. Often this imposition of male characteristics on females results in an individual snail's inability to reproduce and eventual collapse of impacted snail populations.

Contaminant effects on development are often difficult to discern in field studies due to the small size of embryos and the fact that early life stages of most organisms are generally more sensitive to xenobiotics than other life stages. Thus, developmental impacts merit careful attention by ecotoxicologists.

Disease Susceptibility—Of great concern are the potential impacts of environmental chemicals on immune systems that render organisms more susceptible to disease. Numerous laboratory studies have demonstrated chemical impacts on immune systems in animals of ecological relevance. These include effects of pesticides on amphibians, PCBs on channel catfish (*Ictalurus punctatus*), heavy metals on rainbow trout, PAHs on bivalves, and flame retardants (polybrominated diphenyl ethers [PBDEs]) on American kestrels (*Falco sparverius*). The potential effects of chemicals on immune function and disease susceptibility in wildlife is a very important subject in ecotoxicology.

Behavior—Subtle effects on behaviors associated with mating and reproduction, foraging, predator–prey interactions, preference/avoidance of contaminated areas, and migration have

potentially important ramifications for population dynamics. In some cases, biochemical mechanisms underlying behavioral effects have been elucidated that may assist with these issues and provide useful biomarkers for behavioral toxicants in field studies.

Chemicals causing behavioral effects in wildlife are often known to be neurotoxicants. Nest attentiveness was reduced in female starlings dosed with the AChE-inhibiting organophosphate insecticide dicrotophos. Behavioral effects of insecticides have also been observed in fish. Noted adverse impacts include olfactory-mediated behaviors such as the alarm response and homing in the Chinook salmon (*Oncorhynchus tshawytscha*) after exposure to the organophosphate diazinon. Similar thresholds were observed for the effects of another organophosphate (chlorpyrifos) on swimming and feeding behaviors of coho salmon (*O. kisutch*). Mercury, particularly as methylmercury, comprises another potent neurotoxicant that has been shown to perturb behavior in wildlife.

Environmental contaminants not generally thought of as neurotoxicants have been shown to perturb behavior; for example, cadmium and copper affect olfactory neurons and associated behaviors (preference/avoidance to chemicals, including pheromones) in several fish species. Copper exposure in zebrafish also led to loss of neurons in the peripheral mechanosensory system ("lateral line"), which could lead to altered behaviors associated with schooling, predator avoidance, and rheotaxis (physical alignment of fish in a current). A loss of retinal ganglion cells in rainbow trout exposed to TCDD led to deficits in visual acuity and prey capture rates. Clearly, numerous mechanisms of chemical toxicity can impact behavior, including direct toxicity to neurons, alterations in hormones that modulate behaviors, and impaired energy metabolism. Impaired behavior may comprise a sublethal impact with substantive ecological consequence.

Cancer—Numerous cases of cancer epizootics in wildlife that are associated with chemical pollution, particularly in specific fish populations, have been reported in North America and northern Europe. Cancer in these animals occurs largely in relatively older age classes and therefore is oftentimes considered a disease unlikely to directly impact population dynamics or other ecological parameters. However, this may not always be the case in species that require many years to attain sexual maturity and/or have low reproductive rates.

A major contributor to this differential cancer susceptibility in wild fish populations is clearly lifestyle; benthic (bottom-dwelling) species such as brown bullhead (*Ameriurus nebulosus*) and white sucker (*Catostomus comersoni*) in freshwater systems, and English sole (*Parophrys vetulus*) and winter flounder (*P. americanus*) in marine systems generally exhibit the highest cancer rates in polluted systems. The bulk of chemicals in these systems associated with cancer epizootics, such as PAHs, PCBs, and other halogenated compounds, reside in sediments; benthic fish live in contact with these sediments and prey in large measure on other benthic organisms. The molecular and biochemical pathways underlying chemical carcinogenesis, such as PAH metabolism, DNA damage, and effects on oncogenes, are qualitatively similar between most fish and mammalian species examined.

It is noteworthy that the great bulk of reports of elevated cancer rates in free-living animals occur in fish. Cancers are rare worldwide in marine mammals. It is likely that elevated exposures play an important role in the relatively high frequency of reports of cancers in benthic fish; relative inherent sensitivities among mammals, birds, reptiles, amphibians, and fish are unclear.

Population

A population is a collection of individuals of the same species that occupy the same space and within which genetic information can be exchanged. Population ecotoxicology covers a wide range of topics with core research themes being (1) epidemiology of chemical-related disease, (2) effects on general population qualities including demographics and persistence, and (3) population genetics.

The level of belief warranted for possible contaminant-related effects in nonhuman populations is assessed by applying routine epidemiological methods. Rules of thumb for gauging the level of belief warranted by evidence that emerged from human epidemiology are also applied in population ecotoxicology (Table 30–1).

TABLE 30–1 A Summary of One Popular Set of Rules of Thumb for Assessing Plausibility of a Causal Association in an Ecological Epidemiology

Rule	Description
1. Strength of association	How strong the association is between the possible cause and the effect, for example, a very large relative risk
2. Consistency of association	How consistently is there an association between the possible cause and the effect, for example, consistent among several studies with different circumstances
3. Predictive performance	How good is the prediction of effect made from the presence/level of the possible cause
4. Monotonic trend	How consistent is the association between possible cause and effect to a monotonic trend (i.e., either a consistent increase or decrease in effect level/prevalence with an increase in exposure)
5. Inconsistent temporal sequence	The effect, or elevated level of effect, occurs before exposure to the hypothesized cause
6. Factual implausibility	The hypothesized association is implausible given existing knowledge
7. Inconsistency with replication	Very poor reproducibility of association during repeated field assessments encompassing different circumstances or repeated formal laboratory testing

NOTE: According to Fox (Practical causal inference for ecoepidemiologists, J Toxicol Environ Health 33:359, 1991), the first four rules are most useful in supporting a causal hypothesis if found to be true (i.e., very strong, consistent, predictive, or monotonic association). The others are most useful for lessening belief in the causal hypothesis if true.

Defining and predicting alterations in population size, dynamics, and demographic composition due to toxicant exposure have always been central in ecotoxicology. Certain species populations fluctuate within a range of densities. These fluctuations are characteristic of the

species strategy for maintaining itself in various types of habitats and toxicant exposure could potentially change this range. Combined with decreases in population densities driven by external forces such as weather events, these toxicant-induced modifications of the average population densities and dynamics can increase the risk of a population's density falling so low that local extinction occurs. Toxicants can change a species population's vital rates, including age- and sex-dependent death, birth, maturation, and migration rates.

Individuals of the same species often are grouped into subpopulations within a habitat and these subpopulations together comprise a metapopulation. Subpopulations in the metapopulation have different levels of exchange and different vital rates that depend on the nature of their habitat. Spatial distances and obstacles or corridors for migration influence migration among patches; habitat quality determines vital rates.

The genetics of exposed populations is studied to understand changes in tolerance to toxicants and to document toxicant influence on field populations, which may have the capacity to become more tolerant of toxicants. Genetic qualities are also used to infer past toxicant influence in an exposed population. Additional evidence demonstrating past toxicant influence on populations can be a change in genetic diversity. A drop in genetic diversity in populations is thought to be an adverse effect because genetic diversity is required in populations to adapt to environmental changes. Toxicants can influence genetic diversity by purely stochastic means.

Community

An ecological community is an interacting assemblage of species populations occupying a defined habitat at a particular time. Populations in a community interact in many ways and, because these many interactions are complex, a community has properties that are not predictable from those of its component populations. Some species have such a crucial role (keystone species) or numerical dominance (dominants) that they are essential to maintaining community structure. Other species contribute to the nature of the community in more subtle ways. Community may also refer to the distribution of species among different functional groups such as decomposers, detritivores, primary producers, primary consumers (herbivores) and secondary consumers (carnivores that consume herbivores). Community qualities like the diversity of species are influenced by contaminants.

Communities take on characteristic structures as predicted by the Law of Frequencies: the number of individual organisms in a community is related by some function to the number of species in the community. Ecotoxicants can alter the resulting community structure in predictable ways by either directly impacting the fitness of individuals in populations that make up the community or altering population interactions.

Common metrics for species richness, diversity, and evenness are used to express changes in biodiversity. Richness is simply the number of species in the sampled community. If a relative number of species in different communities is needed, then the number of species expected in a specified sample size is determined. Rerefaction can estimate species richness from the results of sampling. Evenness is a measure of how equitably the individuals in a community are spread among the species. Finally, diversity (heterogeneity) indices combine the elements of richness and evenness into one number. Generally, but not always, ecotoxicants lower species richness, evenness, and overall diversity. The regulatory premise is that these changes reflect a diminished community.

Structural and functional qualities in communities have been combined to generate

multimetric indices such as the Index of Biotic Integrity (IBI). Ecological insight is used to select and then numerically combine community qualities such as species richness, health of individual animals in a sample, and the number of individuals in a sample belonging to a functional group, such as number of piscivorous fish. The IBI score for a study site is calculated and compared with that expected for an unimpacted site in order to estimate its biological integrity.

Another central theme in community ecotoxicology is toxicant transfer during trophic interactions. Toxicant concentrations can decrease (biodiminution), remain constant, or increase (biomagnification) with each trophic transfer within a food web. Quantifying the trophic position of a species in a community is essential to modeling biomagnification. Most individuals in a community can feed on different species depending on their life stage, seasons, and relative abundances of prey species. These trophic interactions are best described as occurring in a trophic web, not a trophic chain.

Ecosystem to Biosphere

Ecosystems are the functional unit of ecology composed of the ecological community and its abiotic habitat. The ecotoxicologist's interest in ecosystems includes understanding how toxicants diminish an ecosystem's capacity to perform essential functions and to understand toxicant movement enough to assess exposure within different ecosystem components.

Conventional ecosystem studies involve descriptions of contaminant concentrations and movements in easily defined ecosystems such as lakes, forests, or fields. Some toxicants, especially those subject to wide dispersal by air or water, cannot be completely understood in this framework so a landscape scale might be chosen instead. As an example, acid precipitation might be examined in the context of an entire watershed, mountain range, or even a continental region. Other ecotoxicants require a global context to fully understand their movements and accumulation. As an example, hexachlorobenzene concentration in tree bark collected worldwide showed a clear latitudinal gradient.

APPROACHES

Toxicity Tests

Toxicity testing encompassing representative animals and plants at different levels of organization offers a practical approach to characterize chemical effects on biological systems. Toxicity tests do not mimic the complex interactions and variable conditions of natural ecosystems, but address the potential direct effects of toxic substances on individual ecosystem components in a controlled and reproducible manner.

Ecotoxicology tests feature a wide variety of aquatic (including algae, invertebrates, tadpoles, bivalves, shrimp, fish), avian (quail, duck), and terrestrial species (soil microorganisms, crops, honey bees, earthworms, wild mammals). Species are selected based not only on their traditional use as laboratory animals but also on ecological relevance, which further complicates global harmonization of ecological testing. In addition, testing of aquatic species requires water quality monitoring and investigation of the solubility and stability of the test substance under the conditions of testing, along with determination of nominal versus measured concentrations.

Testing can be conducted in aqueous systems without renewal of the test substance (static), renewal at predetermined time intervals (static renewal), or continuous flow of test substance through the test compartment (flow-through).

Acute toxicity testing consists of single species exposed to various concentrations of the test substance. The most common endpoint in acute tests is death, although abnormal behavioral or other gross observations are commonly noted, and nonlethal endpoints occasionally apply (e.g., immobilization for daphnids and shell deposition in oysters). Variations in acute toxicity studies comprise testing of different species (such as fresh vs. saltwater fish, bobwhite quail vs. mallard duck), life stages (embryo, larva, juvenile), environmental influences (e.g., presence of organic material), or sediment exposures. Data from various test concentrations and time points are used to derive concentration–response curves and predicted values such as the LC_{50} (median lethal concentration), EC_{50} (median effective concentration), or IC_{50} (median inhibition concentration). More quantitative values derived from acute tests are the lowest observed effect concentration (LOEC), that is, the lowest concentration where an effect is observed, and the no observed effect concentration (NOEC), the highest concentration resulting in no adverse effects.

Short-term laboratory studies conducted with single species are useful for rapid screening, provide information on thresholds for effects and selective and comparative toxicity, and can be used as range finders to guide subsequent, often more involved, studies. Long-term and reproductive studies evaluate the effects of substances on organisms over extended periods of time and/or sequential generations (chronic toxicity, life cycle, reproduction).

Unique to ecotoxicology are the more elaborate microcosm, mesocosm, and field studies. Microcosms are representative aquatic or terrestrial ecosystems created under laboratory conditions that include several relevant species (such as protozoa, plankton, algae, plants, and invertebrates). Simulated field studies or mesocosms can be created in the laboratory or in the field (e.g., artificial streams, ponds) or consist of enclosures of existing habitats, containing representative soil, water, and biota. Lastly, full-scale field studies (aquatic organisms, terrestrial wildlife, pollinators) evaluate the effects of a substance on wildlife under real-life scenarios of actual use conditions of a product (e.g., pesticide field usage rate), and thus, are more complicated, subject to considerable variability, and require extensive background knowledge of the local population and community dynamics.

Plant studies are a significant component of ecological toxicity testing, particularly for pesticide registration, and involve tiered testing of both target area and nontarget terrestrial and aquatic plants. Target area plants are present where the substance will be routinely used, but which are not anticipated to be affected. Nontarget plants are those outside of the intended use area. Endpoints of phytotoxicity include seedling emergence and growth, vegetative vigor, and rhizobium–legume toxicity, among others. Central to toxicity testing with plants are the substrate and environmental conditions, which greatly influence plant health.

Biomarkers

The term “biomarker” is most often employed to refer to molecular, physiological, and organismal responses to contaminant exposure that can be quantified in organisms inhabiting or captured from natural systems. Biomarkers do not directly provide information concerning impacts on the higher levels of organization that ecotoxicology ultimately endeavors to discern. Nevertheless, biomarkers often provide important ancillary tools for discerning contaminant

exposures and potential impacts of ecological importance. Biomarkers can provide sensitive early warning signals of incipient ecological damage.

Chemical specificity among biomarkers is also highly variable and is imbued with trade-offs. In cases where one has a good idea of the nature of contaminants likely to occur at a site, chemical-specific biomarkers will likely be most informative. If such information is lacking, or mixtures encompassing several classes of chemicals likely occur, nonspecific markers may be superior. Suites of biomarkers prove to be most effective, although the larger the suite, the more time-intensive and costly the analysis will be. Effects of environmental variables such as temperature, time of day or year, salinity and dissolved oxygen, and physiological variables such as sex, age, reproductive status, and nutritional status need to be controlled and accounted for or at least understood. Many biomarkers are invasive and require sacrifice of the organism in order to obtain needed tissues. Biomarkers can provide powerful tools as early warning signals of ecological damage to assist in assessments of environmental contamination and in determining the effectiveness of various environmental management decisions such as cleanups. However, careful case-specific thought must go into the selection of biomarkers, and they rarely are efficacious alone.

Population

Population-level effects are quantified with both field and laboratory approaches. Population density is the most common quality measured in surveys of contaminated habitats. Demographic surveys or experiments can be conducted for exposed populations. Some studies explore age-specific vital rates, but others are designed to explore vital rates for different life ages such as nestling, fledgling, juvenile, and adult. Most result in data sets that can be analyzed profitably using either a simple life table or more involved matrix analysis. The matrix method allows one to describe the population state and to understand the sensitivity of the population to effects occurring to vital rates for various ages or stages. The value of such studies lies in the ability to integrate several effects into a projection of population consequences. Demographic studies are becoming more common in ecotoxicology, especially with species amenable to laboratory manipulation.

Conventional studies of increased tolerance after generations of exposure and molecular genetic surveys of exposed populations are the primary approaches by which genetic consequences are assessed. Increased tolerance is usually detected by subjecting individuals from the chronically exposed population and a naive population to toxicant challenge and formally testing for tolerance differences. Alternatively, a change associated with a tolerance mechanism might be examined in chronically exposed and naive populations.

Community and Ecosystem

Most community and ecosystem effects studies use modified methods developed in community and systems ecology. The approach affording the most control and ability to replicate treatments involves laboratory microcosms. A microcosm is a simplified system that is thought to possess the community or ecosystem qualities of interest. The experimental control and reproducibility associated with microcosms come at the cost of losing ecological realism.

Gaining back some realism by giving up some degree of tractability, outdoor mesocosms are

also applied to community and ecosystem ecotoxicology. Mesocosms (or enclosure) are larger experimental systems, usually constructed outdoors, that also attempt to simulate some aspect of an ecosystem such as community species composition. Aquatic mesocosms can be artificial ponds, streams, or river segments. Terrestrial mesocosms can be pens, enclosures, or large soil plots depending on the effects being quantified.

Field studies explore effects at the community or ecosystem level. The high realism of associated findings from field studies is balanced against the difficulty of achieving true replication and sufficient control of other factors influencing the system's response. Field studies can involve manipulations such as introducing toxicant into replicate water bodies; however, most field studies involve biomonitoring of an existing, notionally impacted, community or ecosystem. Because mesocosm and field studies involve data generation in the presence of many uncontrolled variables and poor replication or pseudoreplication, multivariate statistical techniques for recognizing patterns among locations or through time are commonly applied.

Landscape to Biosphere

Technologies for acquiring, processing, and analyzing large amounts of information have been essential. Archived and new imageries from satellites and high-altitude platforms are now integrated with off-the-shelf geographic information system (GIS) software with affordable computers. Much imagery is gathered with remote sensing technologies that do not require physical contact with the feature being measured. Arrays of sensors are quickly linking to form a readily accessible real-time data stream for the oceans. Remote sensing data from satellites or aircraft provide information for wide spatial areas and the rapidly emerging, ground- or water-based observing system networks have begun to produce extremely rich data streams.

ECOLOGICAL RISK ASSESSMENT

Ecological risk assessment (ERA) applies ecotoxicological knowledge in support of environmental decision making. A widely dispersed ecotoxicant such as acid precipitation or widely used product such as the herbicide, atrazine, might require assessment of risk at a landscape or subcontinental scale. Ecotoxicants requiring a global ERA might include greenhouse gases contributing to global warming, hydrofluorocarbons depleting the ozone layer, and persistent organic pollutants that accumulate to harmful concentrations in polar regions far from their point of release in industrialized latitudes.

Adaptations are based on the context of an ERA. Some ERAs address existing situations. Considerable field information might be available for such a retroactive ERA and epidemiological methods might be applied advantageously. In contrast, predictive ERAs assess possible risk associated with a future or proposed toxicant exposure and might rely more heavily on exposure modeling and laboratory-derived effects data. A life cycle assessment in which "cradle-to-grave" predictions are done for a product that includes all aspects of its raw material extraction, manufacture, distribution, use, and final disposal. Despite adaptations and differing contexts, most ERAs have the same general form (see Fig. 30–2).

Exposure characterization describes or predicts contact between the toxicant and the assessment endpoint. Depending on the ERA, this could involve a simple calculation of average

exposure, or a temporally and spatially explicit description of amounts present in relevant media. Toxicant sources, transport pathways, kinds of contact, and potential co-stressors are also defined.

Ecological effects characterization describes the qualities of any potential effects of concern, describes the connection between the potential effects and the assessment endpoint, and describes how changes in the level of exposure might influence the effects manifesting in the assessment endpoint. Normally, a statement about the strength of evidence associated with the descriptions is presented in the ecological effects characterization. The AOP approach is gaining traction as a powerful approach for ecological (and human health) risk assessments, particularly for effects characterization.

Risk characterization uses the analysis of exposure and ecological effects to address the risk question(s) posed in the problem formulation. This can involve an explicit statement of risk, that is, the probability of a specified intensity of an adverse effect occurring to the assessment endpoint. Often, the information needed to make such an explicit statement is absent and a qualitative statement of the likelihood of an adverse effect is made instead. Regardless of whether a quantitative or qualitative statement of risk is produced, the risk characterization must provide details surrounding the statement, including important uncertainties.

INTERCONNECTIONS BETWEEN ECOSYSTEM INTEGRITY AND HUMAN HEALTH

Consideration of parallelisms and interconnections between human health and ecological integrity, or health, are important. These two fields share common paradigms such as dose–response, toxicokinetics, mechanisms of action, and risk assessment frameworks. By determining how chemicals and other anthropogenic stressors degrade ecosystems can ultimately impact human health and well-being, and vice versa, a holistic understanding of the results of environmental contamination is obtained. A conceptual model attempts to elucidate interconnections linking natural and social systems in a circular manner with continuous feedback. The natural system produces both positive outputs (e.g., natural resources and raw materials) and negative outputs (e.g., hurricanes and disease vectors) to the social system. The culture and institution of the social system transforms the natural system outputs in various ways and subsequently delivers various positive outputs (consumer goods, conservation efforts) and negative outputs (pollution, deforestation) to the natural system. These outputs influence the quantity and quality of life (human and nonhuman) of the natural system, and the circular flow of resources continually creates conditions that influence the well-being of individuals, societies, and ecosystems.

This model formalizes the interconnections between human and ecological health that most of us intuitively sense. Some connections, in the context of environmental pollution, are obvious. Chemical contamination of seafoods valued by humans is one example. Others are less clear but potentially very significant, such as human impacts on aquatic systems that foster the propagation of human disease vectors, or human impacts on global climate that may concomitantly impact humans and ecosystems in varied and complex ways.

The genomics revolution provides powerful methods for evaluating fundamental biological similarities across species, including those employed in biomedical and ecotoxicological research. Research in this area has revealed genetic similarities, or conservation, in many genes and the proteins they code for that are important to organismal adaptations and impacts due to environmental stressors, including chemicals. Certainly many important species differences also exist that contribute to the great complexity of understanding human–ecological interconnections. Cross-fertilizations among biomedical and environmental scientists, as well as social scientists and policy makers, are likely to enhance all areas, and catalyze the integrated protection of human and ecosystem health.

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QUESTIONS

1. What is the mode by which a chemical enters the lithosphere?
   - **a.** evaporation.
   - **b.** adsorption.
   - **c.** dissolution.
   - **d.** absorption.
   - **e.** diffusion.

2. The bioavailability of contaminants in the hydrosphere is directly related to:
   - **a.** chemical concentration.
   - **b.** amount of chemical.
   - **c.** water solubility of chemical.
   - **d.** toxicity of chemical.
   - **e.** molecular size of chemical.

3. All of the following regarding biomarkers are true EXCEPT:
   - **a.** Dermal absorption is considered an external dose.
   - **b.** Biomarkers of susceptibility are useful in extrapolating wildlife diseases to human diseases.
   - **c.** Induction of certain enzymes is an important biomarker.
   - **d.** The biologically effective dose is the amount of internal dose needed to elicit a certain response.
   - **e.** The effects of chemical exposure can be different across species.

4. Which of the following processes is LEAST likely to be affected by endocrine-disrupting

agents?

a. enzyme activity.
b. transcription.
c. hormone secretion.
d. signal transduction.
e. DNA replication.

5. Estrogen exposure has been shown to cause all of the following in wildlife species EXCEPT:

a. sexual imprinting.
b. altered sex hormone levels.
c. immune suppression.
d. gonadal malformations.
e. sex reversal.

6. Which of the following is FALSE regarding terrestrial ecotoxicology?

a. Terrestrial organisms are generally exposed to contaminants via ingestion.
b. Predation is an important confounder of measurements in terrestrial toxicology field studies.
c. Reproductive tests are not important in measuring endpoints in toxicity tests.
d. Enclosure studies are better able to control for environmental factors in field studies.
e. Toxicity tests usually test the effects of an oral chemical dose.

7. An important type(s) of compound that is far more toxic in water than in air is/are:

a. organic compounds.
b. photochemicals.
c. vapors.
d. lipid-soluble xenobiotics.
e. metals.

8. Which of the following are used to record endpoint toxicity of aquatic toxicity tests?

a. LD_{50} and ED_{50}.
b. LC_{50} and EC_{50}.
c. reproductive tests.
d. LD_{50} and LC_{50}.
e. LD_{50} and EC_{50}.

9. Biological availability is:

a. the total amount of chemical within an organism.
b. the concentration of chemical in an environmental reservoir.
c. the threshold concentration of a chemical needed for toxic effect.
d. the concentration of chemical within an organism.
e. the proportion of chemical potentially available for uptake.

10. Chemodynamics does NOT study:
    a. the fate of chemicals in the environment.
    b. the rate at which chemicals are metabolized.
    c. the distribution of chemicals in the environment.
    d. the effects of toxic substances on the environment.
    e. the release of chemicals into the environment.

CHAPTER 31

Air Pollution*

Daniel L. Costa and Terry Gordon

KEY POINTS

- Reducing-type air pollution, characterized by SO_2 and smoke, is capable of producing

deleterious human health effects.

- Photochemical air pollution arises from a series of complex reactions in the troposphere close to the earth's surface and comprises a mixture of ozone, nitric oxides, aldehydes, peroxyacetyl nitrates, and myriad reactive hydrocarbon radicals.
- Indoor air can be even more complex than outdoor air, and outdoor air can permeate the indoor environment in spite of the reduced air exchange in buildings.
- Sick-Building Syndrome may occur in new, poorly ventilated, or recently refurbished office buildings due to the outgasing of combustion products, volatile chemicals, biological materials and vapors, and emissions from furnishings.

AIR POLLUTION IN PERSPECTIVE

Unchecked human progress has led to air pollution catastrophes highlighting the profoundly detrimental impact that reckless prosperity has on the environment. Public outcry has resulted in governmental action to protect air quality and public health. While organically derived fuel is combusted to derive energy, its potential impact on air quality and on public health and the environment will remain. Though great strides have been made in balancing regulation and technology to reduce emissions from stationary and mobile sources, unsatisfactory air quality imposes a risk to public and environmental health throughout the world. Air pollutants can be transported across many miles such that previously pristine, rural areas have measurable pollution above their historic background levels.

Disparities in health across the globe are linked to poverty, access to clean food and water, as well as health care and education, autocratic governments, wars, and all that is associated with these population deficiencies and stressors. Air pollution—both indoor and outdoor—and the breathing of toxic inhalants is among the planet's five top killers. Exposure to carbon and soot from combustion of biomass in cooking and heating in domestic stoves causes up to 8 million deaths per year, primarily in women who are exposed day in and day out over many years, often with their infant children by their sides. Understanding the intersection of technological, socioeconomic, and political challenges will be at the core of any resolution to these issues.

A Brief History of Air Pollution and Its Regulation

For most of history, air pollution has been a problem of microenvironments and domestic congestion. The smoky fires of early cave and hut dwellers choked the air inside their homes, and even when the emissions were vented outdoors, they simply combined with those of the neighbors to settle around the village on damp cold nights. With urbanization and a concomitant decrease in forest wood as a source of fuel to heat and cook, the burning of easily accessible, dirty coal released a sulfurous, sooty smoke. Industrialization with kilns and metal smelters for development of progressive "modern" cities pushed smoke and chemical emissions into the air.

Historically, efforts to regulate air pollution have competed with concerns for national, regional, and industrial economies. In the time of Greece and Rome, individual civil suits could be levied against local polluters, although these were of marginal success. By 1925, air pollution was common to all industrialized nations in the Western hemisphere. Public surveys were

initiated—in Salt Lake City in 1926, New York City in 1937, and Leicester, Great Britain, in 1939—to bring political attention to the problem and promote the implementation of controls. However, it was the cumulative impact of the great air pollution disasters in the Meuse Valley, Belgium, in 1930; Donora, Pennsylvania, in 1948; and the great London fog of 1952 that indicted air pollution as a health risk. In the United States, the Air Pollution Control Act was passed in 1955 (and then the Clean Air Act of 1963, amended in 1967, and the Motor Vehicle Air Pollution Control Act of 1965). The Clean Air Act Amendments (CAAA) of 1970 recognized air pollution as a national issue. The Act charged the newly instituted U.S. Environmental Protection Agency (U.S. EPA) with the responsibility to protect the public from the hazards of polluted outdoor air. Criteria air pollutants (ozone (O_3), sulfur dioxide (SO_2), particulate matter (PM), nitrogen dioxide (NO_2), carbon monoxide (CO), lead (Pb)) were specified as significant health hazards. Today there are 188 hazardous air pollutants (HAPs).

Disasters involving HAPs have occurred and the accidental release of 30 tons of methyl isocyanate vapor into the air of the shanty village of Bhopal, India, on December 3, 1984, killed an estimated 3000 people within hours of the release, with several thousand delayed deaths and 200,000 injured or permanently impaired. The tragedy raised the issue of HAPs to a new level of concern.

The HAPs have since garnered more public and policy attention. There is concern not only for the acute effects of accidental releases of fugitive or secondary chemicals—such as phosgene, benzene, butadiene, and dioxin into the air of populated industrial centers—but also for potential chronic health effects, with cancer often being the focus of attention. While many of these chemicals are now better controlled than in the past, residual risk estimates are yet to be completed for many HAPs.

Internationally, the magnitude and control of air pollution sources vary considerably, especially among developing nations, which often forgo concerns for health and welfare because of cost and the desire to achieve prosperity. While vast improvements are evident as industries are being modernized and emissions controlled, many Asian, African, and South American cities have virtually unchecked air pollution fueled by the desire for economic growth and expansion of low-cost labor. Long-range transport of polluted air masses from one country to another is a global issue for many. Growing prosperity in China has led to enormous growth in coal combustion resulting in transoceanic transport of airborne mercury such that transported mercury now accounts for a majority of mercury deposition in waterways and land across the globe. Similarly, dust or sand storms originating in arid and semi-arid regions of Africa and Asia can also undergo global transport.

TOOLS TO ASSESS RISKS ASSOCIATED WITH AIR POLLUTION

Risk assessment is a formalized process whereby toxicity, exposure, and dose-dependent outcome data can be systematically integrated to estimate risk to a population. Figure 31–1 provides a paradigm incorporating all available data providing evidence of “accountability” of the regulations on public health. The health database for any air pollutant may comprise data from animal toxicology, controlled human studies, and/or epidemiology. Because each of these

research approaches has inherent strengths and limitations, an appropriate risk assessment of an air pollutant requires the careful integration and interpretation of data from all three methods.

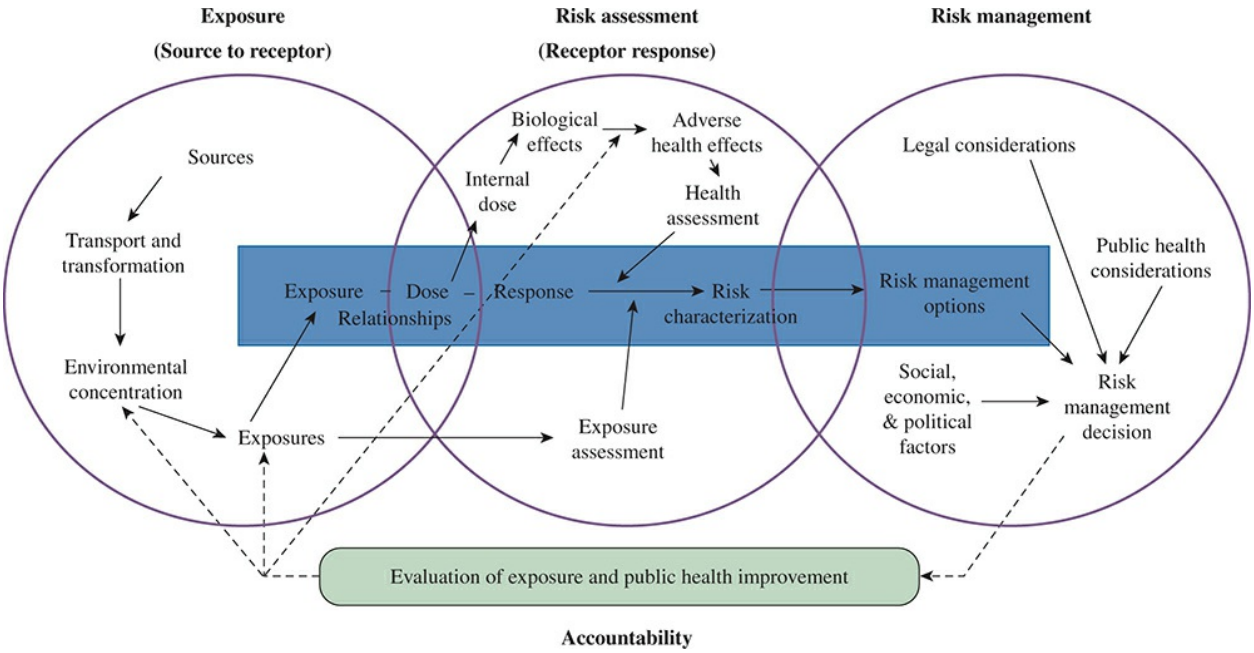


FIGURE 31–1 *NRC risk assessment paradigm.* Components of *risk assessment* within the left circle provide data to development of *risk management* as depicted in the right circle, modified to include an "accountability" component to address air quality management impacts on the process of risk reduction. (Adapted from National Research Council. 2009. Science and Decisions: Advancing Risk Assessment. Washington, DC: The National Academies Press.)

Epidemiological studies reveal associations between exposure to a pollutant(s) and the health effect(s) in the *community* or *population* of interest. Because data are garnered directly under real-world exposure conditions and often involve large numbers of people, the data are of direct utility to regulators assessing pollutant impacts. With proper design and analysis, studies can explore either acute or long-term exposures and theoretically can examine spatial and temporal patterns in mortality and morbidity, both acute and chronic, especially if these responses appear disproportionately in population subsets (i.e., sensitive groups). It is difficult to control confounding personal variables in the population, such as genetic diversity, lifestyle differences, and population mobility. Perhaps most problematic is the lack of adequate exposure data—especially on a personal basis. Studies that involve *controlled human exposures* are very valuable in assessing potential human risk, since they are derived from the species of concern and are rooted in well-established clinical knowledge and experience. Suspected "susceptible or sensitive" individuals representing potential higher-risk groups can also be studied to better understand the breadth of response in the exposed public.

Animal toxicology is used to predict or corroborate, through plausible mechanisms, suspected effects in humans. In the absence of human data, animal toxicology constitutes the essential first step of risk assessment: *hazard identification*. Often required before any controlled human exposure study can be conducted, animal toxicology is particularly useful in elucidating pathogenic mechanisms involved in toxic injury or disease, providing basic knowledge that is critical to extrapolating databases across species, estimating uncertainties, and determining the

relevance of information to humans. Knowledge of the toxic mechanism(s) provides the underpinnings to the "plausibility" of findings in the human context and, under carefully defined and highly controlled circumstances, may allow *quantitative* estimates of risk to human populations. Animal toxicology studies have investigated all of the criteria of air pollutants and many of the HAPs as well. The strength of this discipline is that it can involve methodology that is not practical in human studies and can provide more rapid turnaround of essential toxicity data under diverse exposure concentrations and durations. The minimization of uncontrolled variables (e.g., genetic and environmental) may be the greatest strength of the animal bioassay.

Animal-to-Human Extrapolation: Issues and Mitigating Factors

The clear limitation of animal studies in human risk assessment lies in the unknowns of biology and exposure that weaken the extrapolation of findings in animals to the day-to-day human life scenario. Ideally, a test animal is selected with knowledge that it responds in a manner similar to that of the human (*homology*). *Qualitative* extrapolation of homologous effects is not unusual with many toxic inhalants, but *quantitative* extrapolation is frequently clouded by uncertainties of the relative *sensitivity* of the animal or specific target tissue compared with that of the human. Uncertainties about the target tissue dose also loom large, constituting the first obstacle to quantitative extrapolation. However, it must be appreciated that mechanisms may well differ at different dose levels and some responses may be misleading if assessed only at the higher dose levels. However, animal studies have provided the largest database on a wide range of air toxicants and have proven utility in predicting adverse human responses to chemicals.

An essential part of response extrapolation from species to species is knowledge of the relative dosimetry of the pollutant along the respiratory tract. Significant advances in studies of the distribution of gaseous and particulate pollutants have been made with empirical and mathematical models, the latter of which incorporate parameters of respiratory anatomy and physiology, fluid dynamics, and physical chemistry into predictions of deposition and retention. Empirical models combined with theoretical models aid in relating animal toxicity data to humans and help refine the study of injury mechanisms due to better estimates of the target dose.

OVERARCHING CONCEPTS

What Is an Adverse Health Effect?

When relating a health effect to an air pollutant, a response must be appreciated at two levels—that of the individual person and that of the population. Clearly, an effect upon an individual can be beyond an acceptable limit potentially putting that person's overall health in jeopardy; but this response may be lost in an index reflecting a population-based response. The risk to a population reflects the averaging of individual responses or risks and may be measured as a shift in the normal distribution of some index of response for that population. Hence, on average, the entire population may be judged to be at some enhanced risk. These two forms of risk are clearly related, but most often in practice, the population risk is considered most appropriate from a public health perspective. Generally, it is also most credibly quantifiable.

Defining an air pollutant effect as "adverse" within the range of effects that may result from exposure is not always straightforward. Clearly, in humans, some effects would pass uncontested

as adverse, for example, death, acute life-threatening dysfunction or disease, irreversible impairments, and pain. In animal models, pathology has traditionally been the hallmark of an adverse effect. In either humans or animals, however, other effects that reflect minor and temporary dysfunctions or discomfort could be argued as not warranting significant or costly concern, especially if the effects are minor and transient with no long-term untoward outcomes. This vein of thought would simply attribute these effects to be within normal physiological ranges and are readily compensated within functional or biochemical reserve. Thus, if one is to assess the impacts of air pollution on health, it is desirable that some objective criteria define what is indeed adverse based on the nature and magnitude of the effect under evaluation. Distinguishing an air pollution effect from other adverse stimuli or disease processes can be complex and fraught with confounding factors, such as smoking and negative lifestyle factors.

Susceptibility

A common thread through these subject areas is the influential role of susceptibility, which can take the form of hyperresponsiveness or loss of reserve. A minor reversible effect in most individuals may be a dysfunction that cannot be reversed or compensated in certain individuals (Fig. 31–2), such as cardiopulmonary-compromised individuals who function with little or no reserve. The same sensitivity in measurement that serves to predict an adverse effect must be separated from signals that are essential for homeostasis and the maintenance of life. Clearly, dissecting and defining these phenomena will have implications not only for assessing clinical adversity but also for predictive toxicology.

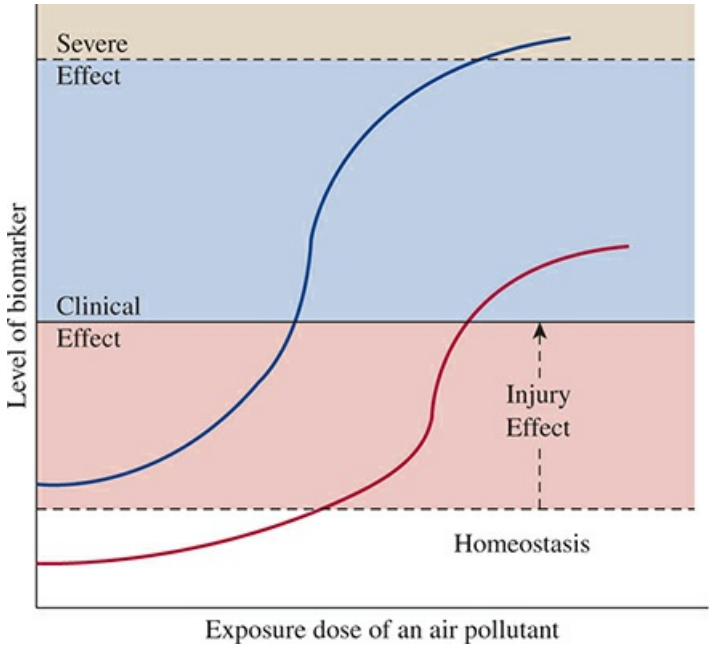


FIGURE 31–2 *Schematic illustration of the elements of the dose–response to an air pollutant(s) of a susceptible (blue curve) versus a healthy (red curve) individual.* The hypothetical susceptible individual may be more sensitive or may have a *loss of reserve*, either of which results in an *inability to maintain* homeostasis. The *leftward shift or increased slope* in the dose–response curve suggests an increase in responsiveness. Either situation may

contribute to sensitivity and the likelihood of enhanced progression from subtle to severe outcomes.

There is no widely accepted definition for a "susceptible" individual and quite frequently the term is used interchangeably with "vulnerable." Some utilize "vulnerability" to refer to extrinsic (i.e., nonbiological) factors (e.g., increased exposure to ambient air pollutants because one's school is located adjacent to a high traffic volume roadway), whereas "susceptibility" refers to intrinsic biological factors such as genetics, age, or preexisting disease. Regardless of the precise definition, a susceptible subpopulation encompasses both intrinsic and extrinsic factors that increase the response to air pollution (Table 31–1).

TABLE 31–1 Factors That Influence the Response of Individuals or Subpopulations to Ambient Air Pollutants

Susceptibility Factors	Vulnerability/Exposure Factors
Preexisting cardiopulmonary disease	Proximity to point source
Genetic factors	Proximity to high traffic volume roadway
Age	Occupation
Gender	Activity level
Race/Ethnicity	Use of air conditioning/building leakiness
Obesity	In utero exposure
Pregnancy	Geographic location (e.g., East vs. West coast of the United States)
Diabetes	Lower social economic status

Data from Integrated Science Assessment (ISA) For Sulfur Oxides – Health Criteria (Final Report, Sep 2008). USA, Washington, U.S. Environmental Protection Agency, EPA/600/R-08/047F, 2008.).

The existence of sensitive individuals and groups is well accepted among those who conduct air pollution health assessments, but relatively little is actually known about the host traits that render certain individuals sensitive to inhaled particles and gases. Some definable subgroups that are considered inherently more susceptible or vulnerable to air pollution include children, the elderly, and those with a preexisting disease (e.g., asthma, cardiovascular disease, and lung disease). The importance of susceptibility in air pollutant responses is gaining more and more attention, as test subject responses that were once considered "outliers" in empirical or observational studies may well be evidence of unusual responsiveness.

In some cases, susceptibility may simply reflect differences in dosimetry. Children spend more time outdoors than adults, are more active, and have basal ventilation rates that exceed

adults on a volume-to-body weight ratio, and therefore may experience overall greater dose to the lungs. Adult humans and animals with obstructive airway disease, for example, may have "hotspots" of particle deposition in the airways that exceed normal local tissue doses manyfold. Also, the loss of functional reserve or compensation due to age or disease may alter a response threshold or impair recovery and the re-establishment of homeostasis.

Coordinated studies in animals and human subjects are proving to be useful to investigate specific questions such as the roles of pre-existing disease, diet (e.g., antioxidant content), exercise (as it relates to dosimetry), age, gender, and race. The goal of such susceptibility studies is to elucidate patterns, common factors, or pathways that may inform potential intervention or mitigation strategies as well as information to reduce the uncertainties regarding risk factors.

The use of CRISPR/Cas9 technology to target and generate specific mutations in the mouse genome has significantly reduced the time and costs associated with the development of genetically modified mice. These engineered animal models add to the availability of natural mutants that have been inbred historically to "fix" a desired genotype with a specific phenotype expression. Current technology can also target specific genes for isolated expression in the lung (e.g., linked to surfactant protein C), and in some cases controlled by genes which an investigator can switch on or off using a pharmacologic or chemical pre-challenge. These advances allow the dissection of underlying mechanisms under controlled scenarios and avoid the problem of having a gene inappropriately active or inactive through all life stages or throughout all body tissues.

EXPOSURE

Air Pollution: Sources and Personal Exposure

Six major air pollutants (PM, O_3, NO*x*, SO_2, CO, and Pb) are considered ubiquitous to industrialized communities and are thought to carry the greatest risk to human and environmental health. Except for O_3, these pollutants are emitted by anthropogenic combustion processes along with myriad special chemicals (mostly volatile organic compounds [VOCs]) considered under the category of HAPs. There are many natural sources of air pollutants as well (e.g., volcanoes, wildfires, windblown dust, and natural biogenic vapors), but it is the anthropogenic sources that emit pollutants that concentrate where people live that raise concerns about their potential health impacts. These factors do not dismiss the significance of potential risks posed by the natural emissions but put focus on the potential for human exposure and risk.

Indoor versus Outdoor—People in most industrialized nations spend in excess of 80% of their time indoors while at work, school, and home or between these places in an automobile. Perhaps the most significant risk factor in indoor air for children is the presence of asthma. With an asthma prevalence of nearly 10% in children, the risk of exposure to pollutants and allergens in concentrated form is particularly great.

As to the issue of total exposure, children and outdoor workers are thought to be more likely to encounter outdoor air pollution at its worst; in fact, the relatively high physical activity levels of these subgroups lead to larger doses of any given pollutant being delivered to the lungs. Defining personal exposure can be extremely difficult, as personal monitoring is tedious, expensive, and can sometimes be confounded by other contributions to the indicator being monitored. Hence, exposure measures are typically drawn from ambient measurements or

derived from models developed from studies of groups of people carefully characterized across personal exposure modifiers—exercise, personal lifestyles, etc.

Indoor air at times can be more complex than outdoor air, a point raised in challenging the legitimacy of studies that rely solely on outdoor monitoring data. The national monitoring network for the criteria pollutants has been shown to reflect human exposure reasonably well for some pollutants, especially those that are chemically nonreactive. Indeed, outdoor air permeates the indoor environment despite the reduced air exchange in most buildings. However, many variables determine how well components of outdoor air infiltrate. The complexity of these multiple sources underscores the importance of appreciating the total exposure scenario if we are to understand the nature of air pollution and its potential effects on human health (Fig. 31–3).

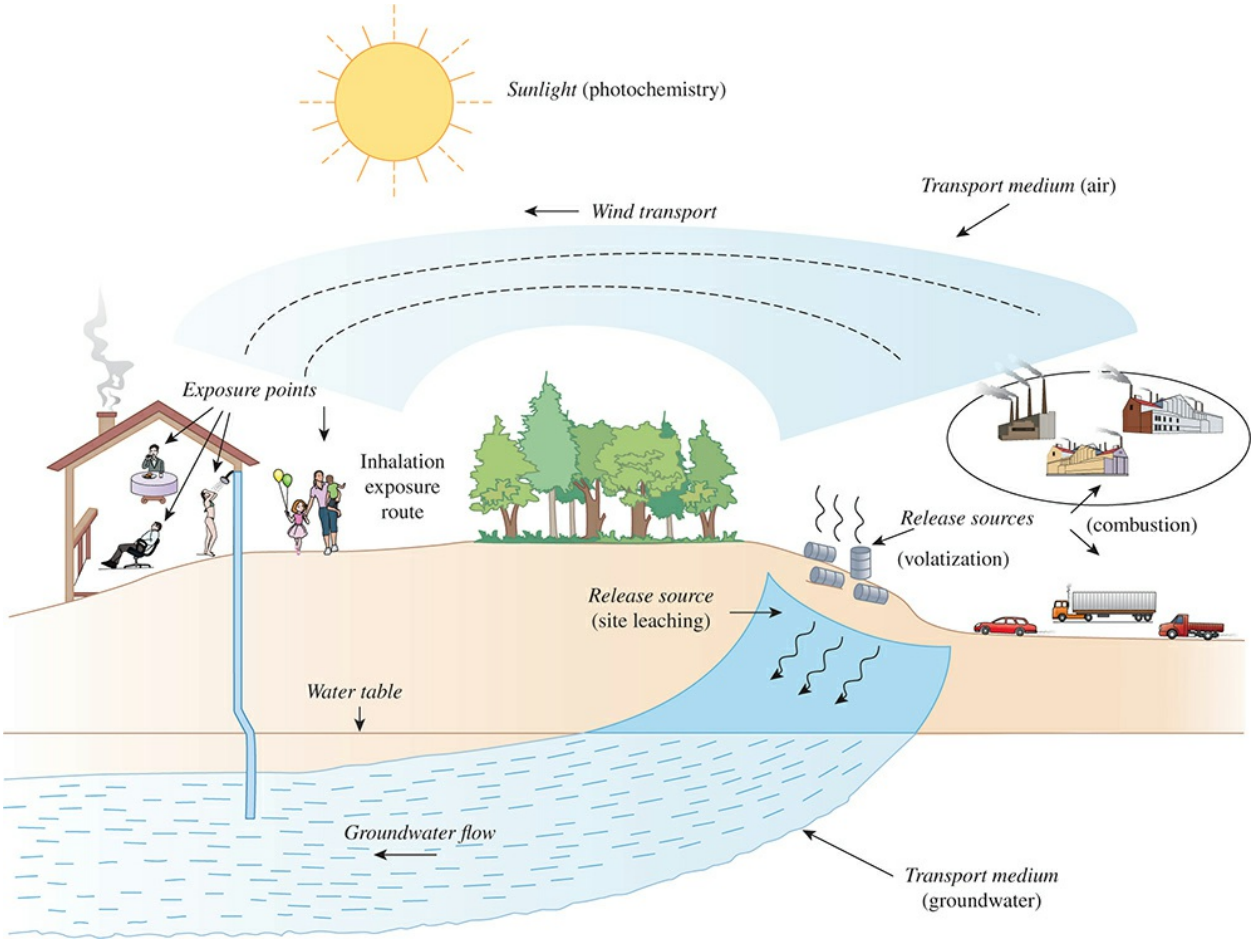


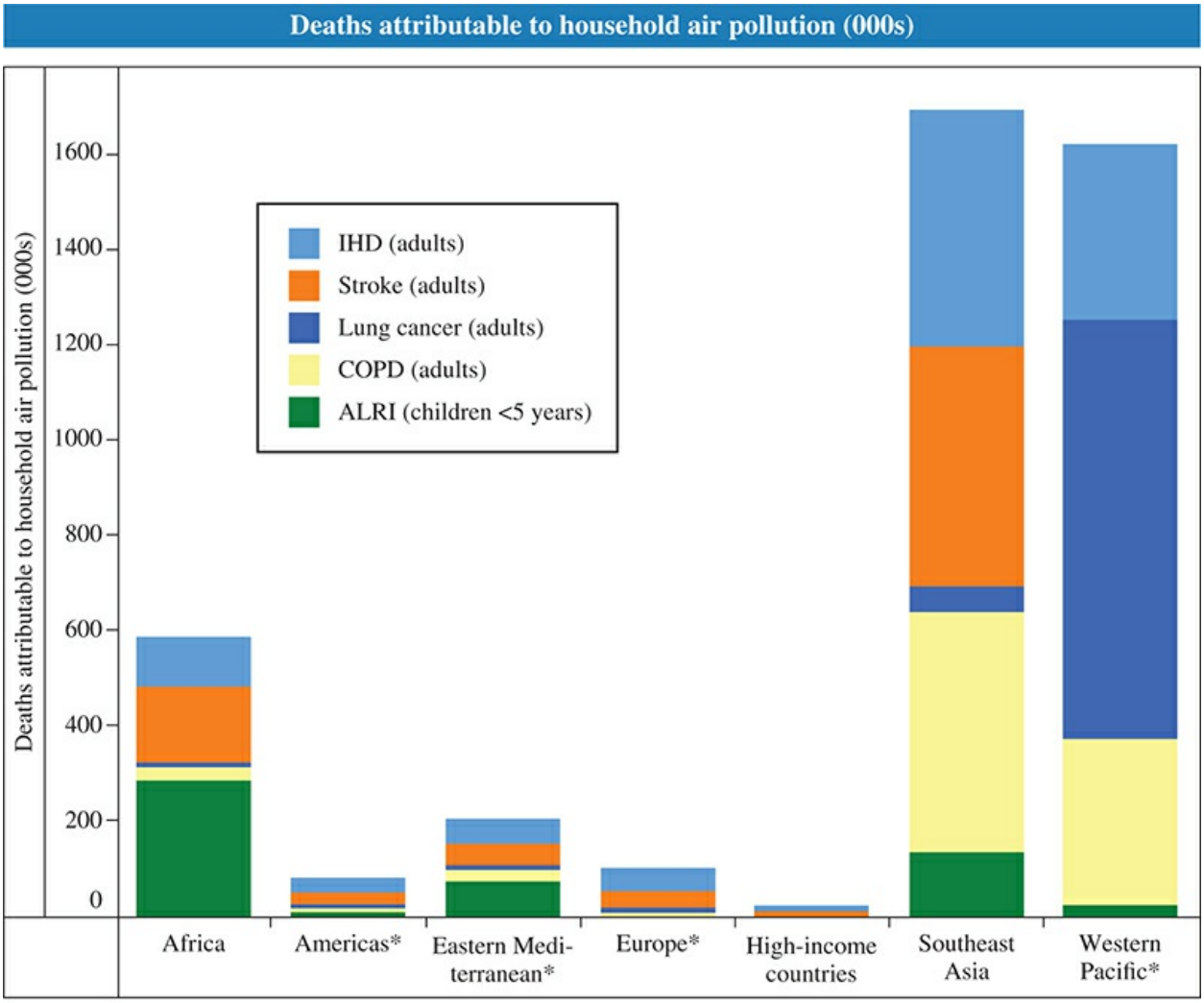
FIGURE 31–3 *Illustration of contributors to the total personal exposure paradigm showing how these indoor and outdoor factors interact.*

Two broadly defined illnesses are largely unique to the indoor building environment. The first is "sick-building syndrome," which is a collection of ailments defined by a set of persistent symptoms enduring at least 2 weeks and appears to occur in at least 20% of those exposed. Frequently, this syndrome occurs in new, poorly ventilated, or recently refurbished office buildings. The suspected causes include combustion products, cleaning chemicals, biological emissions from mold, and vapor emissions from furnishings. The perception of irritancy to the eyes, nose, and throat, headaches, nasal congestion, difficulty breathing, etc. can become

intolerable with repeated exposures.

The second syndrome (building-related illnesses) is a group of illnesses that consists of well-documented conditions with defined diagnostic criteria and generally recognizable etiology. These illnesses typically call for conventional medical treatment strategies, since simply exiting the building where the illness was contracted may not readily reverse the symptoms. Several biocontaminant-related illnesses (e.g., Legionnaires disease, hypersensitivity pneumonitis, humidifier fever) fall into this group, as do allergies to animal dander, dust mites, and cockroaches. Medical treatment and mitigation of exposure (source elimination or personal protection) are generally needed to abate symptoms. Pollutants include CO, NO_2, and many VOCs (passively emitted from new furniture or rugs, or from mold in the ventilation system), such as trichloroethylene (a VOC common to the indoor air arising from chlorinated water or dry-cleaned clothes). Complex indoor environments comprising of chemicals and biologicals (dust mites, fungi, molds, etc.) may also lead to unexpected interactions that remain virtually unstudied and thus under-appreciated in the assessment of indoor pollution.

Indoor Air in the Developing World—Pollution of the indoor environment in the developing world is a major issue. Superimposed on the infiltration of ambient air pollutants are indoor emissions from cooking practices, the cultural use of incense, tobacco, and various other substances, like perfumes. Unvented or poorly vented cookstoves which burn biomass are used much as they have been for centuries. Figure 31–4 indicates deaths attributable to household air pollution around the world. Chronic lung diseases, such as bronchitis, emphysema, and cancer, are major killers of exposed women, while children suffer from bronchitis and various other infectious lung diseases.



* Low- and middle-income countries.

FIGURE 31–4 *Illustration of contribution of burning solid fuels to global mortality by region.* The figure shows considerable region-by-region differences in total deaths that can be attributed to combustion of solid fossil fuels such as wood and other biomass materials. ALRI, acute lower respiratory infection; COPD, chronic obstructive pulmonary disease; IHD, ischemic heart disease. (Data from the World Health Organization.)

Evolving Profile of Outdoor Air Pollution—Classically, ambient air pollution was distinguished based on the chemical redox nature of its primary components SO*x* and NO*x*. Reducing-type air pollution occurred during winter periods of oil and coal combustion used for heating and power coupled with meteorological inversions, while the oxidant atmospheres occurred during the warmer months of spring and summer, when sunlight is most intense and can catalyze reactions among the constituents of auto exhaust. Atmospheric conditions today seem to promote transport across broad areas affecting background levels. Urban air pollution is a worldwide problem: the yearly mortality for people exposed to O_3 is estimated at 700,000, whereas PM-related mortality is higher at 3.1 million per year.

EPIDEMIOLOGICAL EVIDENCE OF HEALTH EFFECTS

Outdoor Air Pollution

Acute and Episodic Exposures— Several air pollution incidents have been documented where pollutant concentrations rose to levels that are clearly hazardous to human health. Where a single chemical has been accidentally released, establishing the relationship between cause and ill effect is straightforward. However, most air pollution situations involve complex atmospheres, and establishing a specific cause other than the air pollution incident itself can be difficult. “Natural” events like vegetation/forest fires have significant impacts on wide regional air quality and public health. Air pollution toxicology has focused more and more on O_3 where clinical and animal exposure studies easily could measure impacts on lung function. Indeed, many health effects could be ascribed to oxidant/irritant properties of O_3.

A series of epidemiological studies showed an association between PM mass concentration and daily mortality. Studies utilizing time-series analyses that could blunt the impact of weather, smoking, and other variables that might obscure patterns in health variables linked to the air monitoring data showed significant and consistent associations between health outcomes of ambient PM at levels previously thought to be safe. Time-series analyses are based on Poisson regression modeling to distinguish changes in daily death counts (or hospital admissions) associated with short-term changes in air pollution. The statistical methodology applied in these time-series studies could detect short-term trends and minimize the effects of other pollutants and potential confounders with longer time constants.

PM stands as the preeminent air pollutant. The major health outcome revealed in the study of PM has been the involvement of the cardiovascular system as a prime target for adverse impact. While the heart, as part of the cardiopulmonary system, has always held an indirect role in health impacts or disease from air pollution, both epidemiological and toxicological studies now point to major cardiac involvement in PM-associated mortality. Not surprisingly, effects are most apparent in subpopulations already compromised by cardiopulmonary and perhaps vascular diseases (e.g., diabetes). Several pathways have been proposed that attempt to link exposure and cardiac effects (Fig. 31–5).

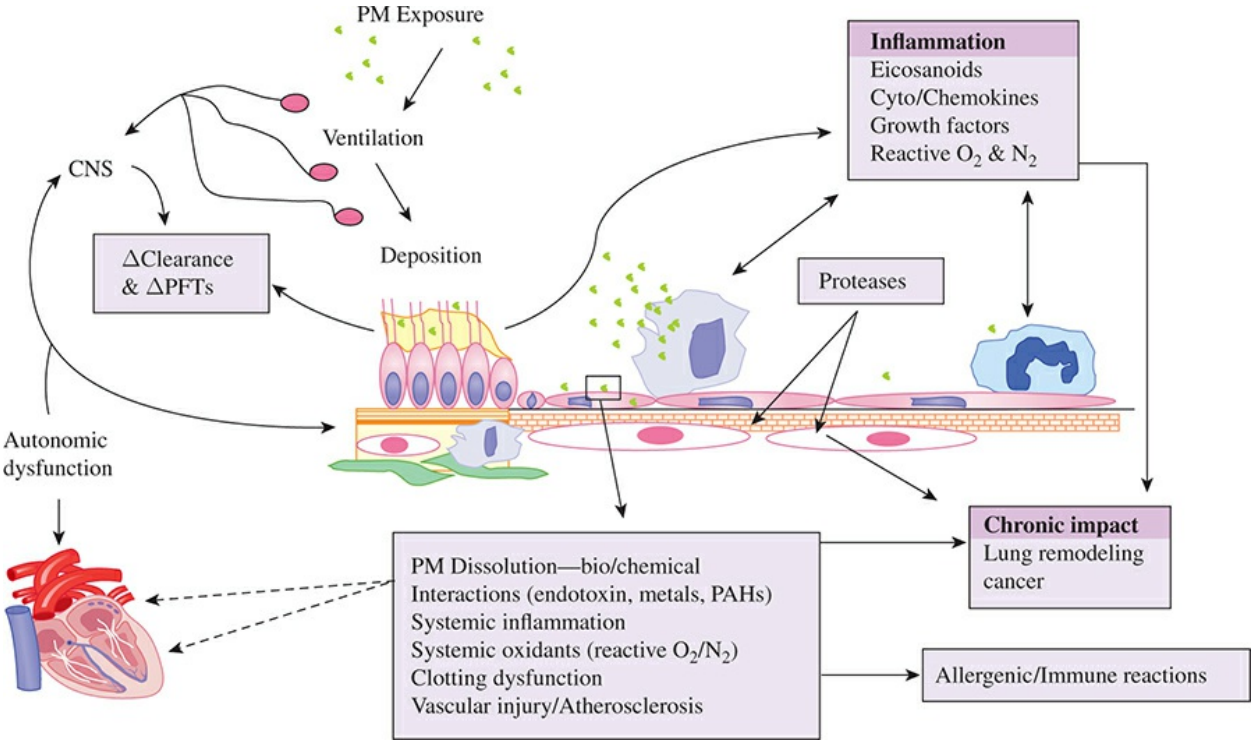


FIGURE 31–5 *Schematic of the multiple mechanisms thought to function in cardiopulmonary response(s) to air pollutants—derived from current hypothesized mechanisms for particulate matter.*

The actual "biochemical lesion" caused by PM is generally thought to involve oxidant mechanisms (generation of reactive oxygen and perhaps nitrogen species) by constituents or attributes (e.g., reactive surface area) of the particles at the cell or molecular level.

Long-Term Exposures—Epidemiological studies of the chronic effects of air pollution are difficult to conduct by the very nature of the goal: outcomes associated with long-term exposures. Looking back in time, retrospective, cross-sectional studies frequently were confounded with unknown variables and inadequate historical exposure data, such as cigarette smoking. Without extensive information on both active and passive smoking, the ability to discern the impact of an air pollution disease outcome such as chronic bronchitis and emphysema would be greatly impaired. Prospective studies have the advantage of more precise control of confounding variables, such as the tracking of urinary cotinine as an index of tobacco smoke exposure, but they can be very expensive and require substantial time and dedication on both the part of the investigators and the study population. Depending on the study size and design, exposure assessments can be complex, and the loss of subjects due to dropout is sometimes unpredictable.

Both retrospective and prospective epidemiological studies have suggested a positive association between urban pollution and progressive pulmonary impairments. These updated analyses, which included gaseous co-pollutant and new fine-particle measurements, and more comprehensive personal information on the enrollees, confirmed the linkage of health effects, including an increased risk of lung cancer, with $PM_{2.5}$ exposures. These findings reinforce the concerns for potential chronic health impacts of PM and the heightened risk of premature death

from lifelong air pollution exposure. Atherosclerosis and other cardiovascular disease outcomes that might be considered underlying causes of cardiac mortality were significantly associated with long-term exposure to ambient PM.

POLLUTANTS OF OUTDOOR AMBIENT AIR

Classic Reducing-Type Air Pollution

High concentrations of the reducing-type air pollution, characterized by SO_2 and smoke, can produce dramatic human health effects. Empirical studies in human subjects and animals have long stressed the irritancy of SO_2 and its reaction products in these incidents, while the full potential for interactions among co-pollutants in the smoky, sulfurous soup has a mixed record of replication in the human exposure laboratory. It is an irritant gas that has a toxicology of its own and, through atmospheric reactions, can transform photochemically into sulfites or sulfates as a secondarily irritant particle.

Sulfur Dioxide

***General Toxicology*—**Sulfur dioxide is a water-soluble irritant gas that is absorbed predominantly in the upper airways. It is a sensory irritant that can stimulate bronchoconstriction and mucus secretion in several species, including humans. Relatively high exposure concentrations of SO_2 cause (more than 250 ppm) airway cellular injury and subsequent proliferation of mucus-secreting goblet cells. Even at concentrations <0.1 ppm, such as might be encountered near an industrial source, long-term residents may experience a higher incidence of bronchitis with chronic exposure. An increase in the airflow during deep rapid breathing augments deeper penetration of the gas into the lung. Once deposited along the airway, SO_2 dissolves into surface lining fluid as sulfite or bisulfite and is readily distributed throughout the body and cleared by the kidneys.

***Pulmonary Function Effects*—** Inhaled SO_2 induces mild bronchoconstriction, which is reflected as a measurable increase in airflow resistance due to narrowing of the airways. Concentration-related increases in resistance have been observed in guinea pigs, dogs, cats, and humans. Airflow resistance increased more when the gas was introduced through a tracheal cannula than via the nose, since nasal scrubbing of the water-soluble gas was bypassed.

Sulfuric Acid and Related Sulfates—Conversion of SO_2 to sulfate is favored in the environment with ammonia neutralization to ammonium sulfate $[(NH_4)_2SO_4]$ or ammonium bisulfate $[NH_4HSO_4]$. During oil and coal combustion or metal ore smelting, sulfuric acid condenses downstream of combustion processes with available metal ions and water vapor or in smoke plumes to form submicron sulfuric acid fume and sulfated fly ash. Photochemical reactions also promote acid sulfate formation via both metal-dependent and independent mechanisms. Fine-particle sulfates may exist as sulfuric acid (the primary source of free H^+). Partially or fully neutralized forms of sulfate predominate due to the abundance of atmospheric ammonia. Fine PM sulfates are transported long distances and contribute to regional summer haze, pose a health hazard to groups such as asthmatics, and stress the general environment as

acid rain.

General Toxicology—Sulfuric acid irritates by virtue of its ability to protonate (H^+) receptor ligands and other biomolecules. This action can either directly damage membranes or activate sensory reflexes that initiate inflammation. Ammonia, which exists in free air at about 25 ppb and in much higher concentrations within the mammalian naso-oropharynx (in the human up to 350 ppm), is capable of neutralizing most of the inhaled irritant acidic sulfates. Unlike O_3, inhaled sulfuric acid does not stimulate a classic neutrophilic lung inflammation. Rather, a disturbed eicosanoid homeostasis results in macrophage dysfunction and altered host defense.

Pulmonary Function Effects—Sulfuric acid, like SO_2, produces an increase in flow resistance in guinea pigs due to reflex airway narrowing, or bronchoconstriction. Because of the predominantly neutralized form of sulfate in the atmosphere, sulfates are not thought to be primary or singular bad-actors in air pollution incidents unless at very high concentrations.

Effects on Mucociliary Clearance and Macrophage Function— Sulfuric acid does alter the clearance of particles from the lung. Collectively, there seems to be coherence in the data to rank sulfate irritancy: sulfuric acid > ammonium bisulfate > ammonium sulfate. Acidity [H^+] appears to be the primary driver on most respiratory effects.

Chronic Effects—As a fine aerosol, sulfuric acid deposits deeper along the respiratory tract, and its high specific acidity imparts greater effect on phagocytes and epithelial cells. The primary concern regarding chronic inhalation of acidic aerosols is the potential for bronchitis; this has been a problem in occupational settings in which employees are exposed to sulfuric acid mists (e.g., battery plants). Studies in several animal models suggest that the airways of exposed animals become progressively more sensitive to challenge with acetylcholine, show a progressive decrease in diameter, and experience an increase in the number of secretory cells, especially in the smaller airways. While highly variable, the possibility that chronic irritancy by inhaled sulfuric acid may elicit bronchitis-like disease in susceptible individuals (perhaps over a lifetime or in children) appears to be reasonable.

Particulate Matter

PM was referred to as “soot” and it consisted of incompletely burned carbonaceous materials, acid sulfates, various metals, and silicates associated with the solid nature of the fuel. Improvements in combustion methods simultaneously reduced the size of emitted particles. A side benefit of the smaller particles was the reduction in light diffraction through the emissions and hence a less visible plume. Oxidized sulfur becomes sulfate and many of the metals appear as oxides.

PM in the atmosphere can be solid, liquid, or a combination of both with a mélange of variably combusted or photochemically derived organic compounds coexistent with inorganic species and biological materials or associated compounds. The compositional matrix of PM can vary significantly due to varied emission sources and secondary transformations in the atmosphere, which are weather mediated. Particles of larger size (PM_{10}) tend to have more local sources, and the larger particles tend to settle from the air due to gravity. Particles in the range of 10 to 2.5 μm ($PM_{10–2.5}$—coarse PM) are inhalable by humans but tend to deposit in the upper

airways due to their mass. In urban settings throughout the world, there is considerable spatial and temporal heterogeneity of coarse PM while $PM_{2.5}$ appears somewhat more homogenous throughout a regional air shed. The size designation of fine and coarse PM is based on their relative respirability—those in the range of PM_{10} deposit in the nasal cavity and larger thoracic airways while the $PM_{2.5}$ deposit in the small airways and gas exchange areas of the lung.

Metals—There have been many conventional acute and chronic rodent inhalation studies conducted with specific metal compounds, often as oxides, chlorides, or sulfates. The effects of metals delivered by inhalation may differ from their impacts when administered by other routes and as such inhalation exposures perhaps best relate to occupational exposures. Metals may arise from natural as well as anthropogenic activities, and as a result metals are a common constituent in ambient PM. Coarse $PM_{10–2.5}$ arises largely from natural sources and thus has prominent earthen metals such as iron, sodium, silica, and magnesium—usually in geologically stable oxide forms. On the other hand, fine ($PM_{2.5}$) and ultrafine ($PM_{0.1}$) are richer in combustion-derived metals, often reflecting the fuel source and nature of combustion. For example, diesel fuel may have vanadium, nickel, and perhaps zinc and iron, while coal may have zinc and selenium. Their chemical forms vary from water-soluble metal salts and sulfates to oxide and phosphate forms. High temperatures and efficient combustion frequently take metals to their smallest size and most oxidized form—the oxides. Metals are also emitted from vehicles burning fuels to which metal compounds were added (e.g., lead, manganese, and platinum) or as engine wear and catalyst by-products. Similarly, metals may also be derived from brake (copper, iron), tire (zinc), and road (earthen silicates or dust re-dispersion) wear.

Metal compounds can be separated nominally as those that are essentially water-insoluble (e.g., metal oxides and hydroxides such as those that might be released from high-temperature combustion sources or derived from the geocrustal matrix) and those that are soluble or somewhat soluble in water (often chlorides or sulfates such as those that might form under acidic conditions in a smoke plume or leach from acid-hydrated silicate particles in the atmosphere). Solubility appears to play a role in the toxicity of many inhaled ambient-PM–associated metals by enhancing metal bioavailability (e.g., nickel from nickel chloride versus nickel oxide), but insolubility also can be a critical factor in determining toxicity by increasing pulmonary residence time within the lung (e.g., insoluble cadmium oxide versus soluble cadmium chloride). Moreover, some metals can promote electron transfer to form reactive oxidants.

Gas–Particle Interactions—Gas–particle interactions can involve multiple components of the particles, gases/vapors, and sunlight and lead to toxicity of either the particle or the gas. Complex chemistry occurs within the effluent of the combustion source. Similar interactions may result from gaseous pollutants that impair the clearance of particles from the lung or otherwise alter their metabolism. Studies focusing on irritancy and infectivity raise the prospect that realistic exposure scenarios of gaseous and particulate pollutants can interact through either chemical or physiologic mechanisms to enhance health risks of complex polluted atmospheres.

Ultrafine Carbonaceous Matter—Ultrafine ambient carbon particles are emitted during combustion and can mix with less well combusted carbonaceous material or may occur in a fairly pure form from high-temperature pyrolysis. The size of these particles allows them to slip (Brownian motion) between gas molecules moving primarily by diffusion. Agglomeration among themselves, on surfaces, or with larger particles in the air is their primary mode of

dissipation. When concentrations exceed about a million particles per cubic centimeter, they rapidly agglomerate with each other forming larger clumps or chains of ultrafine particles. These agglomerates are typified by diesel exhaust particles in the air. As an air pollutant, elemental carbon particles generally do not exist as singlets except near their emission points. Fine PM consists in part of agglomerates of these and other carbonaceous organic material. Some organic materials that exist in the vapor form may also condense on ultrafine black carbon (e.g., diesel PM). Estimates of the carbonaceous (including organic) content of ambient fine PM vary considerably but are nominally considered to be about 10% to 60% of the total PM mass depending on the urban or regional area.

Diesel exhaust particles vary widely in the ratio of organic and elemental carbonaceous materials, which may influence their inflammatory and carcinogenic potential. Whole diesel exhaust also contains significant amounts of gaseous pollutants: NO*x*, CO, and SO_x as well as various VOCs and carbonyl irritants. Exposure to diluted diesel exhaust in humans reveals that the exhaust mix is inflammogenic in the lung and to a degree cytotoxic to airway cells. The use of diesel particles alone in toxicology studies does not seem to display similar toxicity to the total exhaust, thus underscoring the importance of interactions among air pollutants as a critical consideration in air pollution toxicity.

Ultrafine particles in the near-traffic environment exist in extremely high numbers. The predominance of cardiovascular associations gives some credence to a hypothesis that ultrafine particles somehow enter the systemic circulation and/or trigger systemic inflammation that links to the cardiovascular effects. The ultrafine mode of black carbon has been suggested to be more toxic than fine-mode $PM_{2.5}$, perhaps due to enhanced surface reactivity, co-pollutant carrying capacity, or deeper penetration of ultrafine particles from the lung into the circulation. Recent commercial introduction of “engineered” nanoparticles brings many of the same concerns as ultrafines by virtue of their similar sizes. Additionally, being “engineered” particles, they may possess design features that “natural” combustion ultrafine (or nano) particles do not.

Chronic Effects and Cancer—In 2013, the WHO’s International Agency for Research on Cancer (IARC) concluded that air pollution causes lung cancer and that the component of air pollution most likely responsible was PM due to the complexity of their carcinogenic components, including PAHs. Cigarette smoking (also replete with PAHs) remains a confirmed cause of lung cancer and clearly associated with other systemic cancers. Yet, it has been difficult to separate PM cancer from those cancers that appear linked to the many hazardous air pollutants (HAPs) that occur in urban air and are also thought to be carcinogenic. Only about 10% of the more than 2800 compounds that have been identified in the air have been assayed for carcinogenic potency. Volatile and related halogenated organic and nitrogen-containing compounds account for most of the compounds that have been studied with animal and genotoxicity bioassays. Most of these compounds are derived from combustion sources ranging from tobacco to power plants to incinerators to motor vehicles. The profile of outdoor carcinogens contrasts with that of indoor air, where the sources are thought to derive largely from environmental tobacco smoke and radon, with some contribution from off-gassed organics. Thus, human exposure to airborne toxicants is highly complex compositionally as well as in its temporal and spatial heterogeneity.

The lung cancer risk of any individual is a complex function of the carcinogenic nature of the substance, the amount, and nature of material deposited in the lungs, which is itself a function of the concentration in the ambient air, the physical and chemical properties of the inhalant that

may determine deposition efficiency, innate repair mechanisms, and the cumulative volume of air inhaled. The majority of lung cancer risk from ambient air pollution lies within the PM fraction, including the many polycyclic organic chemicals and the less volatile nitroaromatics. These persistent organics associate with the PM matrix and thus could have a prolonged residence time at deposition sites within the respiratory tract. Genetic bioassays have revealed the potent mutagenicity, and presumably carcinogenicity, of various chemical fractions of ambient aerosols. Some compounds require metabolic transformation to activate their potency while others may be detoxified by their metabolism. Some VOCs that are inhaled have target tissues away from the lung—in the case of benzene, bone marrow leukemia.

The cells lining the respiratory tract turn over relatively quickly as they interface directly with the ambient environment with every breath. Their DNA is vulnerable to carcinogenic or oxidant-induced replication errors from inhaled substances that, when fixed as mutations, could give rise to tumors. Co-pollutants, such as irritant gases, that initiate inflammation may promote this carcinogenic activity by damaging cells and enhancing their turnover.

Photochemical Air Pollution

Photochemical air pollution (notably O_3) arises secondarily from a series of complex reactions in the troposphere activated by the ultraviolet (UV) spectrum of sunlight. In addition to O_3, it comprises a mixture of nitric oxides (NO*x*), aldehydes, peroxyacetyl nitrates (PAN), and myriad aromatics, alkenes, reactive radicals, and intermediates. If SO_2 is present, sulfate particles may also be formed and, collectively, they yield "summer haze." Likewise, the complex chemistry can generate organic PM, nitric acid vapor, and various condensates.

Of the photochemical air pollutant gases, O_3 is by far the toxicant of greatest concern. It is highly reactive and more toxic than NO*x*, and because its generation is fueled through cyclic hydrocarbon radicals, it reaches greater concentrations than the hydrocarbon radical intermediates. Although O_3 is of toxicological importance in the troposphere, in the stratosphere it plays a critical protective role. About 10 to 50 km above the Earth's surface, UV light directly splits molecular O_2 into atomic $O^{\bullet}$, which then combines with O_2 to form O_3. The O_3 also dissociates back but much more slowly. The result is an accumulation of O_3 to several ppm within a relatively thin strip of the stratosphere forming an effective "permanent" barrier by absorbing the short-wavelength UV in the chemical process. This barrier had in recent years been threatened by various anthropogenic emissions (Cl_2 gas and certain chlorofluorocarbons) that enhance O_3 degradation (creation of an "O_3-hole"), but recent restrictions in the use of these degrading chemicals seem to have been effective in reversing this process. Recently, concern is mounting that the hole may be growing again due to the release of other halogenated gases. The benefits of this stratospheric O_3 are the reduction of excess UV light infiltration to the Earth's surface and reduced skin cancer risk.

The impact of O_3 in the troposphere, where its accumulation serves no known purpose, poses a threat to the respiratory tract when inhaled. Near the Earth's surface, NO_2 arising from combustion processes efficiently absorbs the longer wavelength UV light spectrum, from which a free O atom is cleaved, initiating the following simplified series of reactions:

$$NO_2 + h\nu(\text{UV light}) \rightarrow O^{\bullet} + NO^{\bullet}. \quad (31\text{-}1)$$

$$O^{\bullet} + O_2 \rightarrow O_3. \quad (31\text{-}2)$$

$$O_3 + NO^{\bullet} \rightarrow NO_2. \quad (31\text{-}3)$$

This process is inherently cyclic, with NO_2 regenerated by the reaction of the $NO^{\bullet}$ and O_3. In the absence of unsaturated hydrocarbons (olefins and substituted aromatics) arising from fuel vaporization or combustion, as well as biogenic terpenes, this series of reactions would approach a steady state with little buildup of O_3. The free electrons of the double bonds of unsaturated hydrocarbons are attacked by free atomic $O^{\bullet}$, resulting in oxidized compounds and radicals that react further with $NO^{\bullet}$ to produce more NO_2. Thus, the balance of the reactions sequence shown in Eqs. (31-1) to (31-3) is tipped to the right, leading to buildup of O_3. This reaction is particularly favored when the Sun's intensity is greatest at midday, utilizing the NO_2 provided by morning rush-hour traffic. Carbonyl compounds (especially short-chained aldehydes) are also by-products of these reactions. Formaldehyde and acrolein account for about 50% and 5%, respectively, of the total aldehyde content in urban atmospheres. Peroxyacetyl nitrate (CH_3COONO_2), often referred to as PAN, and its homologs also arise in urban air, most likely from the reaction of the peroxyacyl radicals with NO_2.

Short-Term Exposures to Smog

Concerns that the complex atmosphere might be even more hazardous than O_3 led to studies with actual (outdoor-derived) or synthetic (photolyzed laboratory–prepared) smog to assess the potency of the ambient-like pollution mixtures. When human subjects were exposed to real-world photochemical air pollution (Los Angeles ambient air pumped into a laboratory exposure chamber), they experienced changes in lung function similar to those described in controlled clinical studies of O_3 alone (i.e., reduction in spirometric lung volumes), thus supporting the view that O_3 was the pollutant of primary concern. Acute animal studies using synthetic atmospheres (usually irradiated auto exhaust) provided supportive evidence indicating deep lung damage, primarily within the small airway and proximal alveolar epithelium. As a result, O_3 was the focus in many of early air pollution studies despite a modicum of evidence that co-pollutants might also be involved in eliciting the effects observed with smog.

Chronic Exposures to Smog

Epidemiological studies in human populations and empirical studies in animals have attempted to link degenerative lung diseases with chronic exposure to photochemical air pollution. Cross-sectional and prospective field studies have suggested an accelerated loss of lung function in adults living in areas of high pollution; however, there were problems with confounding factors (meteorology, poor exposure assessment, and confounding variables).

Studies conducted in children living in modern-day Mexico City, which has oxidant and PM levels, have found severe epithelial damage and metaplasia in the nasal epithelium as well as permanent remodeling of the nasal epithelium. When children migrated into Mexico City from cleaner, nonurban regions, even more severe damage was observed, suggesting that the tissue remodeling in permanent residents imparted some degree of adaptation. Because the children were of middle-class origin, socio-economic variables were less likely confounders.

Ozone

General Toxicology—Ozone is the primary oxidant of concern in photochemical smog because of its inherent bioreactivity and its concentration relative to other reactive species. Unlike SO_2 and PM reductions that have largely been done at their source, mitigation strategies for O_3 have been somewhat less successful despite significant reductions in automobile emissions. Reductions in O_3 have been partially offset by population growth, which brings additional vehicles and vehicle miles driven. With suburban sprawl and the downwind transport of air masses from populated areas to more rural environments, the geographic distribution of those exposed has also expanded, as has the temporal profile of individual exposure.

Ozone, at concentrations that occur in urban areas, induces in humans and experimental animals morphologic, functional, immunologic, and biochemical alterations mostly in the lungs. Because of its low water solubility, a substantial portion of inhaled O_3 penetrates deep into the lung, but its reactivity is such that about 17% and 40% are scrubbed by the nasopharynx of resting rats and humans, respectively. Nevertheless, regardless of species, the region of the lung that is predicted to have the greatest O_3 deposition (dose per surface area) is the centriacinar region, from the terminal bronchioles to the alveolar ducts. Because O_3 penetration increases with increased tidal volume and flow rate, exercise increases the dose to the target area. Thus, it is important to consider the role of exercise-associated dosimetry in a study of O_3 or any inhalant before making cross-study comparisons, especially if that comparison is across species.

As a powerful oxidant, O_3 seeks to extract electrons from other molecules. The surface fluid lining the respiratory tract and the cell membranes that underlie the lining fluid contain a significant quantity of polyunsaturated fatty acids, either free or as part of the lipoprotein structures of the cell. The double bonds within these fatty acids have a labile, unpaired electron that is easily attacked by O_3 to form ozonides that progress through a less stable zwitterion or trioxolane (depending on the presence of water); these ultimately recombine or decompose to lipohydroperoxides, aldehydes, and hydrogen peroxide. These pathways are thought to initiate propagation of lipid radicals and autooxidation of cell membranes and macromolecules.

Evidence of free radical–related damage in vivo includes detection of exhaled breath pentane and ethane and tissue measurements of diene conjugates. Damage to the air–blood interface disrupts its barrier function and promotes inflammation. Inflammatory cytokines (e.g., interleukins 6, 8, and others, and TNF) are released from epithelial cells and macrophages that mediate early responses and initiate repair. This inflammatory process is generally transient, but it may also interact with neural irritant responses to affect lung function acutely.

Pulmonary Function Effects—Exposure to O_3 produces a variety of pulmonary function changes. Although the human studies almost always involve exercise, their deficit in lung

function occurs more rapidly and is more dramatic than animals that have been tested similarly. Even in controlled human studies involving O_3, considerable interindividual differences in response have been observed that are linked to individual physical and biological features as well as possibly genetic factors. It is not entirely clear what mechanisms underlie the altered lung function (in terms of changes in FEV_1) produced by O_3. Chest pain/discomfort on deep breathing is thought to contribute to O_3-induced decreases in an effort-dependent lung function such as FEV_1. These are thought to be vagally mediated and involve C-fiber stimulation; the responses can be abrogated by analgesics, such as ibuprofen and opiates, which also reduce pain and inflammation. Thus, pain reflexes involving C-fiber networks are thought to be important in the reduction in forced expiratory volumes.

Airway responsiveness to specific (e.g., allergen) and nonspecific (e.g., cold air and inhaled methacholine) bronchoconstriction is another commonly used test of the pulmonary response to inhaled pollutants such as O_3. These types of tests are very important because airway hyperresponsiveness is a central feature of asthma and asthmatics are a sizeable subpopulation (7% to 9% of the total population in the United States and 1% to 18% worldwide) that may be particularly sensitive to the adverse respiratory effects of inhaled pollutants. Hyperreactive airways may be a marker of predisposition to other pollutants or aeroallergens.

Inflammation of the Lung and Host Defense—The mechanism by which O_3 produces decrements in pulmonary function appears to be largely neurologic, but the cross-links with airway inflammation are unclear. O_3-induced lung dysfunction does not appear to be enhanced in asthmatics whose lungs are generally in a state of inflammation, yet asthmatics are more sensitive to the bronchoconstrictive effects of both SO_2 and sulfuric acid aerosols. It may be that inflammation sensitizes nerve endings to external irritants. In animal models, age and obesity may factor into the pulmonary response to O_3. Both human and animal studies have demonstrated that sensitivity to O_3 appears to have a genetic component as well. O_3-induced pulmonary neutrophilia, airway hyperresponsiveness, and permeability are significantly affected by SNPs in a single gene linked to the Toll-like receptor 4 (Tlr4) locus that has been associated with endotoxin sensitivity. The role of genetic polymorphisms in the pulmonary response to O_3 is complicated, however, and many other pathways including TNF, IL-1, Nrf-2, and NF-κB can modulate the response to O_3 in animal models.

Polymorphisms in glutathione-*S*-transferase Mu 1 (GSTM1), catalase, and myeloperoxidase genes have been associated with enhanced response to ambient ozone. Genetic polymorphisms in other genes, including NAD quinone oxidoreductase (NQO), GSTP1, heme oxygenase-1, and TNF, are also involved.

Chronic Effects—Morphometric studies of the centriacinar region of rat lungs exposed for 12 hours per day for 6 weeks to 0.12 or 0.25 ppm O_3 show hyperplasia and hypertrophy of type 1 alveolar cells coupled with damage and alterations in ciliated and Clara cell populations in upstream small airways. The impact of O_3 in the distal lung may be cumulative and perhaps more importantly may be without threshold. When returned to clean air, most of this epithelial pathology regressed, but there was nonetheless evidence of residual interstitial remodeling beneath the epithelium in the alveolar-duct region. Cellular changes throughout the respiratory tract were noted in infant and adult nonhuman primates exposed chronically to O_3.

Paradoxically, O_3 can induce tolerance to itself and is a curious phenomenon in light of pathologies seen in episodic and chronic exposures. Classic O_3 tolerance takes the form of protection against a high or even lethal dose in rodents that had a pre-exposure to a very low initial challenge(s) several days before. This term, *tolerance*, is sometimes used to describe "adaptation" or acclimatization with repeated exposures to near-ambient levels of O_3. Adaptation to O_3 begins during and immediately after the initial exposure and is fully expressed in 2 to 4 days. This adaptive phenomenon of diminished lung function and correlative inflammatory endpoints has been substantiated in humans. The linkages between acute, adaptive, and long-term processes remain unclear, because both pathology and lung dysfunction develop over longer periods of exposure in animals. The precise mechanism for O_3 adaptation is not known and several theories abound, including changes in cell profiles, lung surface fluids, and induced antioxidants.

Ozone Interactions with Co-pollutants—The effects of O_3 may be altered by co-pollutants in the mixture or may otherwise interact to yield a novel composite outcome. An approach simplifying the complexity of smog, yet addressing the issue of pollutant interactions, involves exposures of animals or humans to assembled synthetic mixtures of pollutants that occur together in ambient air. The most frequent combinations involve interactions of O_3 and NO_2 or O_3 and PM (e.g., ambient, sulfuric acid, or diesel particles). Not surprisingly, study designs involving temporal or spatial exposures add a level of complexity in interpretation that may or may not support either augmentation or antagonism of lung function impairment, lung pathology, or a host of other indices of injury. This complexity emphasizes the careful consideration of the myriad factors that might affect outcomes.

The interpretability of any multicomponent study is dependent on its statistical design and the ability to either separate or determine the nature of the interacting variables. The bottom line is that interactions are highly dependent on the conditions of exposure, endpoints selected for study, and a host of other factors. As the number of interacting variables increases, so does the difficulty in interpretation. It is indeed the complex mixture that we wish to evaluate, and creative toxicological approaches to understanding mixture responses will demand new systems perspectives if real progress is to be achieved.

Nitrogen Dioxide

General Toxicology—Nitrogen dioxide, like O_3, is a deep lung irritant that can produce pulmonary edema if it is inhaled at high concentrations. Potential life-threatening exposure is a real-world problem for farmers, as near-lethal high levels of NO_2 can be liberated from fermenting fresh silage. Being heavier than air, a generated NO_2 and CO_2 displace air and oxygen at the base of silo and diffuse into closed spaces where workers can inadvertently get exposed to very high concentrations perhaps with depleted oxygen. Typically, shortness of breath rapidly ensues with exposures nearing 75 to 100 ppm NO_2, with delayed edema and symptoms of pulmonary damage. Not surprisingly, the symptoms are collectively termed "silo-filler's disease." NO_2 is also an important indoor pollutant, especially in homes with unventilated gas stoves or kerosene heaters or in countries with the unvented burning of biomass fuels.

The distal lung lesions produced by acute NO_2 are similar among species. Theoretical

dosimetry studies indicate that NO_2 is deposited along the length of the respiratory tree, with preferential deposition in the distal airways. Damage is most apparent in the terminal bronchioles. At high concentrations, the alveolar ducts and alveoli are also affected, with type 1 cells again showing their sensitivity to oxidant challenge.

Pulmonary Function Effects—Exposure of normal human subjects to concentrations of less than or equal to 4 ppm NO_2 for up to 3 hours produces no consistent effects on spirometry. Whether asthmatics have a particular sensitivity to NO_2 is controversial. Several factors appear to be involved (e.g., exercise, inherent sensitivity of the asthmatic subject, and exposure method). Meta-analyses, which have incorporated the findings of many studies to achieve a weight-of-evidence perspective, support a heightened effect of NO_2 on asthmatics, but it's not clear if the observed small but statistically significant changes in airway hyperresponsiveness in asthmatics exposed to NO_2 below 0.6 ppm are adverse.

Inflammation of the Lung and Host Defense—Unlike O_3, NO_2 does not induce significant neutrophilic inflammation in humans at exposure concentrations encountered in the ambient outdoor environment. There is some evidence for bronchial inflammation after 4 to 6 hours at 2.0 ppm, which approximates the highest transient peak indoor levels of this oxidant. Exposures at 2.0 to 5.0 ppm have been shown to affect T lymphocytes, particularly $CD8^+$ cells and natural killer cells, that function in host defenses against viruses. Although these concentrations may be high, epidemiological studies variably show effects of NO_2 on respiratory infection rates in children, especially in indoor environments.

Other Oxidants

Peroxyacetyl nitrate (PAN) is thought to be responsible for much of the eye-stinging activity of smog. It is more soluble and reactive than O_3, and hence rapidly decomposes in mucous membranes. The cornea is a sensitive target and is prominent in the burning/stinging discomfort often associated with oxidant smogs.

Carbon Monoxide

Carbon monoxide is classed toxicologically as a chemical asphyxiant because its toxic action stems from its formation of carboxyhemoglobin, preventing oxygenation of the blood for systemic transport. Motor vehicles account for two-thirds of urban CO, depending on the location in a community, traffic density, vehicle types and age, and the urban structure. It must be kept in mind that there are many sources of CO—anywhere combustion is ongoing. Both main and side-stream tobacco smoke, faulty home heating systems, and mobile auxiliary heating sources emit CO when used inappropriately (i.e., unventilated) can create life-threatening circumstances. No overt clinical human health effects have been demonstrated for COHb levels below 2%, while levels above 40% cause fatal asphyxiation.

Hazardous Air Pollutants

HAPs represent an inclusive classification for air pollutants of anthropogenic origin that are generally of measurable quantity in the air. The diverse nature of even 30 of the 187 HAPs (Table 31–2) complicates a general discussion of their toxicology because the group includes various classes of organic chemicals (by structure, e.g., acrolein, benzene), minerals (e.g., asbestos), polycyclic hydrocarbon particulate material (e.g., benzo(*a*)pyrene), and various metals and metal compounds (e.g., mercury and beryllium compounds) and pesticides (e.g., carbaryl and parathion).

TABLE 31–2 List of 30 Urban Air Toxics ("Dirty 30")

Acetaldehyde	Dioxin	Mercury Compounds
Acrolein	Propylene dichloride	Methylene chloride (dichloromethane)
Acrylonitrile	1,3-Dichloropropene	Nickel compounds
Arsenic compounds	Ethylene dichloride (1,2-dichloroethane)	Polychlorinated biphenyls (PCBs)
Benzene	Ethylene oxide	Polycyclic organic matter (POM)
Beryllium compounds	Formaldehyde	Quinoline
1,3-Butadiene	Hexachlorobenzene	1,1,2,2-Tetrachloroethane
Cadmium compounds	Hydrazine	Tetrachloroethylene (perchloroethylene)
Chloroform	Lead compounds	Trichloroethylene
Chromium compounds	Manganese compounds	Vinyl chloride

Reprinted from U.S. Environmental Protection Agency (EPA). Urban Air Toxic Pollutants. Available at: https://www.epa.gov/urban-air-toxics/urban-air-toxic-pollutants.

Accidental and "Fence-Line" Exposures

The relationship between the effects associated with an accidental release of a large quantity of a volatile chemical into the air from a point source such as a chemical plant and the effects associated with a chronic low-level exposure over many years or a lifetime is not clear. Regarding cancer risk, exposures must be minimized if not eliminated if cancer risk is to be kept as close to zero as possible. With noncancer risks, the roles of nonspecific or specific host defenses, thresholds of response, and repair and recovery after exposure complicate the assessment of risk. In large part, the issue here relates to a cumulative index, often a $C \times T$.

Methyl isocyanate provides a contrast between the catastrophic effects of a large accidental release versus long-term effects linked to cyclic or continuous fugitive releases of small amounts of chemical vapor. The reactive nature of methyl isocyanate with aqueous environments is of such magnitude that upon inspiration, almost immediate mucous tissue corrosion can be perceived. The vapor undergoes hydrolysis within the mucous lining of the airways to generate hydrocyanic acid, which destroys the airway epithelium and causes acute bronchoconstriction and edema. The damage is immediately life-threatening at concentrations above 50 ppm; at 10 ppm, it is damaging in minutes. Even months after exposure, the airway and lung damage remain. There is also cardiac involvement secondary to the damage to the pulmonary parenchyma and arterial bed (i.e., pulmonary hypertension and right-sided heart hypertrophy).

In the United States, methyl isocyanate has been measured in the ambient air in Katawba Valley, Texas, as a result of small but virtually continual fugitive releases of the vapor into the

community air (“fence-line”) from an adjoining region with several chemical plants. There is concern that low-level exposure over many years may have more diffuse, chronic effects. Residents complain of odors and a higher frequency of respiratory disorders, but clear evidence of injury or disease is lacking. Fence-line exposures to a variety of industrial chemicals remain a significant potential risk for many communities—often low-income or worker communities near one or more industrial sources. Silent continuous or periodic releases from pipelines or transfer points within facilities can disperse through communities unbeknownst to anyone.

Phosgene is a common intermediate reactant used in the chemical industry, particularly in pesticide formulation, that is subject to accidental or low-level fugitive releases. Because of its direct pulmonary reactivity, it lends itself to use as a model pulmonary toxicant for studies addressing $C \times T$ relationships that can inform assessment of risk under varied exposure patterns. These studies suggest that there may be a threshold below which compensatory and other bodily defenses (e.g., antioxidants) may be able to cope perhaps with long-term low-level tolerance, although there is evidence of mild interstitial fibrosis.

Aldehydes

Two aldehydes that capture attention for their toxicity/irritancy and their ubiquity are formaldehyde (HCHO) and acrolein ($H_2C{=}CHCHO$). As carbonyl compounds, they are short-chained (2 to 4 C) and are generated as photo-oxidation products of unsaturated hydrocarbons in the outdoor air and are common intermediates in chemical reactions that can arise in the indoor environment. Formaldehyde accounts for about 50% of the estimated total aldehydes in polluted air, while acrolein, the more irritating of the two, accounts for about 5% of the total. Acetaldehyde (C_3HCHO) and other longer-chain aldehydes make up the remainder, but they are not as intrinsically irritating, exist at low concentrations, and have less solubility in airway fluids.

Formaldehyde

Formaldehyde is very water-soluble and is absorbed in mucous membranes in the nose, upper respiratory tract, and eyes. The concentration–response curve for formaldehyde is steep: 0.5 to 1 ppm yields a detectable odor, 2 to 3 ppm produces mild irritation, and 4 to 5 ppm is intolerable to most people. Formaldehyde is thought to act via sensory C-fibers that signal locally as well as through the trigeminal nerve to reflexively induce bronchoconstriction through the vagus nerve.

Two aspects of formaldehyde toxicology highlight concerns. One is its near ubiquitous presence in indoor atmospheres as an off-gassed product of construction materials such as plywood, furniture, or improperly polymerized urea–formaldehyde foam insulation. Also, there is epidemiological evidence that links formaldehyde to asthma and an increase in lower respiratory tract infections in children. Formaldehyde is a probable human carcinogen, based on evidence of nasopharyngeal cancer and possibly leukemia in animal studies.

Acrolein

Acrolein is more reactive than formaldehyde. It penetrates a bit deeper into the airways and may cause more cellular damage. Concentrations below 1 ppm cause irritation of the eyes and the mucous membranes of the respiratory tract. The mechanism of increased resistance appears to be

mediated through both a local C-fiber and centrally mediated cholinergic reflexes. Acrolein produces a wide range of adverse pulmonary effects including lung cancer that may be linked mechanistically to *p53* (a tumor suppressor gene) DNA adducts and mutations, as well as cardiovascular effects.

THE MULTIPOLLUTANT REALITY OF AIR POLLUTION

The pollutant profiles of any community's atmosphere vary considerably in space and time. These pollutants are emitted locally and are mixed with pollutants transported over varied distances while undergoing photochemical transformation. The reductionist approach of evaluating nominally one pollutant at a time has been very successful in providing data to target pollutants of primary concern and improving public health. But, there are likely chemical and physiological interactions between and among pollutants that are of public health consequence. Indeed, interactions are more likely to be apparent as exposure concentrations decrease, and while we may be concerned about synergistic interactions as found with some particles and SO_2, it may be that antagonisms also occur among pollutants that are prevalent. As the science and the statistical methods to look at interactions have improved, there is renewed interest in multipollutant toxicology.

CONCLUSION

The breadth and complexity of the problem of air pollution—from the development of credible databases to utilizing these data sources to inform regulatory action and decision making—have been the theme throughout the chapter. The classical and still most prominent air pollutants provide a foundation for understanding and appreciating the nuances and dissecting outcomes upon which defensible air pollution policy and decision making can be applied to public health. The key role of the toxicologist is to ask questions to provide understanding while developing sensitive and relevant assay methods to reveal the causality information needed to support decision making hand-in-hand with epidemiology.

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QUESTION

1. Which of the following compounds is NOT an oxidant-type air pollutant?
   a. NO_2.
   b. SO_2.
   c. O_3.
   d. radical hydrocarbons.
   e. aldehydes.

2. Which of the following pollutants contributes most to nontobacco-smoking lung cancer?
   a. asbestos.
   b. vinyl chloride.
   c. benzene.
   d. products of incomplete combustion.
   e. formaldehyde.

3. Inhalants, such as NO_2 and trichloroethylene, can increase proliferation of opportunistic pathogens in the lungs by:
   a. destroying goblet cells in the respiratory tract.
   b. damaging the alveolar septa.
   c. inactivating cilia in the respiratory tract.
   d. killing alveolar macrophages.
   e. dampening the immune system.

4. Which of the following is NOT a characteristic of SO_2 toxicology?
   a. SO_2 is a major reducing-type air pollutant.
   b. Increased airflow rate increases the amount of SO_2 inhaled.
   c. SO_2 inhalation causes vasoconstriction and increased blood pressure.
   d. SO_2 is predominately absorbed in the conducting airways.
   e. SO_2 inhalation increases mucus secretion in humans.

5. Which of the following would be MOST likely to occur on sulfuric acid exposure?
   a. vasoconstriction.
   b. decreased mucus secretion.
   c. an anti-inflammatory response.
   d. vasodilation.
   e. bronchoconstriction.

6. All of the following statements regarding particulate matter are true EXCEPT:
   a. Metals are most commonly released into the environment during coal and oil combustion.
   b. The interaction of gases and particles in the atmosphere can create a more toxic product than the gas or particle alone.
   c. Solubility does not play a role in the bioavailability of a metal.

d. The earth's crust is an important source of atmospheric magnesium.
e. Diesel exhaust contains reducing- and oxidant-type air pollutants.

7. Which of the following statements is NOT true?
   a. Ozone (O_3) combines with a nitric oxide radical to form NO_2.
   b. O_2 combines with an oxygen radical to form ozone.
   c. O_3 can cause damage to the respiratory tract.
   d. Accumulation of O_3 in the stratosphere is important for protection against UV radiation.
   e. Cl_2 gas is known to cause O_2 degradation.

8. Which of the following is NOT a likely symptom of NO_2 exposure?
   a. increased secretion by club cells.
   b. pulmonary edema.
   c. shortness of breath.
   d. loss of ciliated cells in bronchioles.
   e. decreased immune response.

9. Which of the following statements regarding aldehyde exposure is FALSE?
   a. The major aldehyde pollutants are formaldehyde and acrolein.
   b. Formaldehyde is found in tobacco smoke, but acrolein is not.
   c. Acrolein causes increased pulmonary flow resistance.
   d. Formaldehyde exposure induces bronchoconstriction.
   e. The water solubility of formaldehyde increases its nasopharyngeal absorption.

10. Carbon monoxide (CO) exerts its toxic effects via its interaction with which of the following?
    a. DNA polymerase.
    b. actin.
    c. kinesin.
    d. hemoglobin.
    e. microtubules.

* This chapter has been reviewed by the U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and the policies of the Agency.

UNIT 7 APPLICATIONS OF TOXICOLOGY

CHAPTER 32

Analytical and Forensic Toxicology

Bruce A. Goldberger, Dayong Lee, and Diana G. Wilkins

KEY POINTS

- Analytic toxicology involves the application of the tools of analytic chemistry to the qualitative and/or quantitative estimation of chemicals that may exert adverse effects on living organisms.
- Forensic toxicology involves the use of toxicology for the purposes of the law; by far the

most common application is to identify any chemical that may serve as a causative agent in inflicting death or injury on humans or in causing damage to property.

- The toxicological investigation of a poison death involves (1) obtaining the case history in as much detail as possible and gathering suitable specimens, (2) conducting suitable toxicological analyses based on the available specimens, and (3) the interpretation of the analytic findings.
- The toxicologist as an expert witness may provide two objectives: testimony and opinion. Objective testimony usually involves a description of analytic methods and findings. When a toxicologist testifies as to the interpretation of analytic results, that toxicologist is offering an "opinion."

> *What is there that is not poison? All things are poison and nothing without poison. Solely the dose determines that a thing is not a poison.*
>
> *—Paracelsus*

Analytical toxicology has its roots in forensic applications, and it involves the application of the tools of analytical chemistry to the qualitative and/or quantitative estimation of chemicals that may exert effects on living organisms. Forensic toxicology involves the use of toxicology for the purposes of the law. The most common application is to identify any chemical that may serve as a causative agent in inflicting injury or death on humans, or in causing damage to property. There is no substitute for the unequivocal identification of a specific chemical substance that is demonstrated to be present in tissues from the victim at a sufficient concentration to explain the injury with certainty.

To aid in deciding whether adverse effects of xenobiotics contribute to death, injury, or other harm to persons or property, great efforts are made to initiate and implement analytical procedures in a forensically credible manner. Examples include measurement of ethanol in blood or breath, testing urine for the presence of drugs or their metabolites, diagnosis and treatment of health problems induced by chemical substances, therapeutic drug monitoring, detection of drugs and other chemicals used for the purpose of performance-enhancement, monitoring worker exposure to toxic hazards and determination of toxicants and potential metabolites in the environment.

ANALYTICAL TOXICOLOGY

When the nature of a suspected poison is unknown, a systematic, standardized approach must be used to identify the presence of most common toxic substances. An approach that was first suggested by Chapuis in 1873 in *Elements de Toxicologie* is based on the origin or nature of the toxic agent. Such a system can be characterized as follows:

1. Gases—Gases are most simply measured by means of gas chromatography.
2. Volatile substances—These are generally liquids of various chemical types that vaporize at ambient temperatures. Gas chromatography is the simplest approach for simultaneous separation and quantitation.
3. Corrosive agents—These include mineral acids and bases. Many corrosives consist of ions

that are normal tissue constituents. Clinical chemical techniques can be applied to detect these ions when they are in great excess over normal concentrations.

4. Metals—Metals are encountered frequently as occupational and environmental hazards. Separation procedures involve destruction of the organic matrix by chemical or thermal oxidation. Selected analytical methods must be capable of determining the speciation and relative amount of each form present to interpret the degree of toxicity.
5. Anions and nonmetals—These present an analytical challenge as these agents are rarely encountered in an uncombined form.
6. Nonvolatile organic substances—This group includes drugs, pesticides, natural products, pollutants, and industrial compounds. These substances are solids or liquids with high boiling points. Separation procedures generally rely on differential extractions of biological fluids and tissues (Fig. 32–1). These extractions often are inefficient, and recovery of the analyte from the matrix may be poor. Immunoassay may permit avoidance of extractions and facilitate quantification.

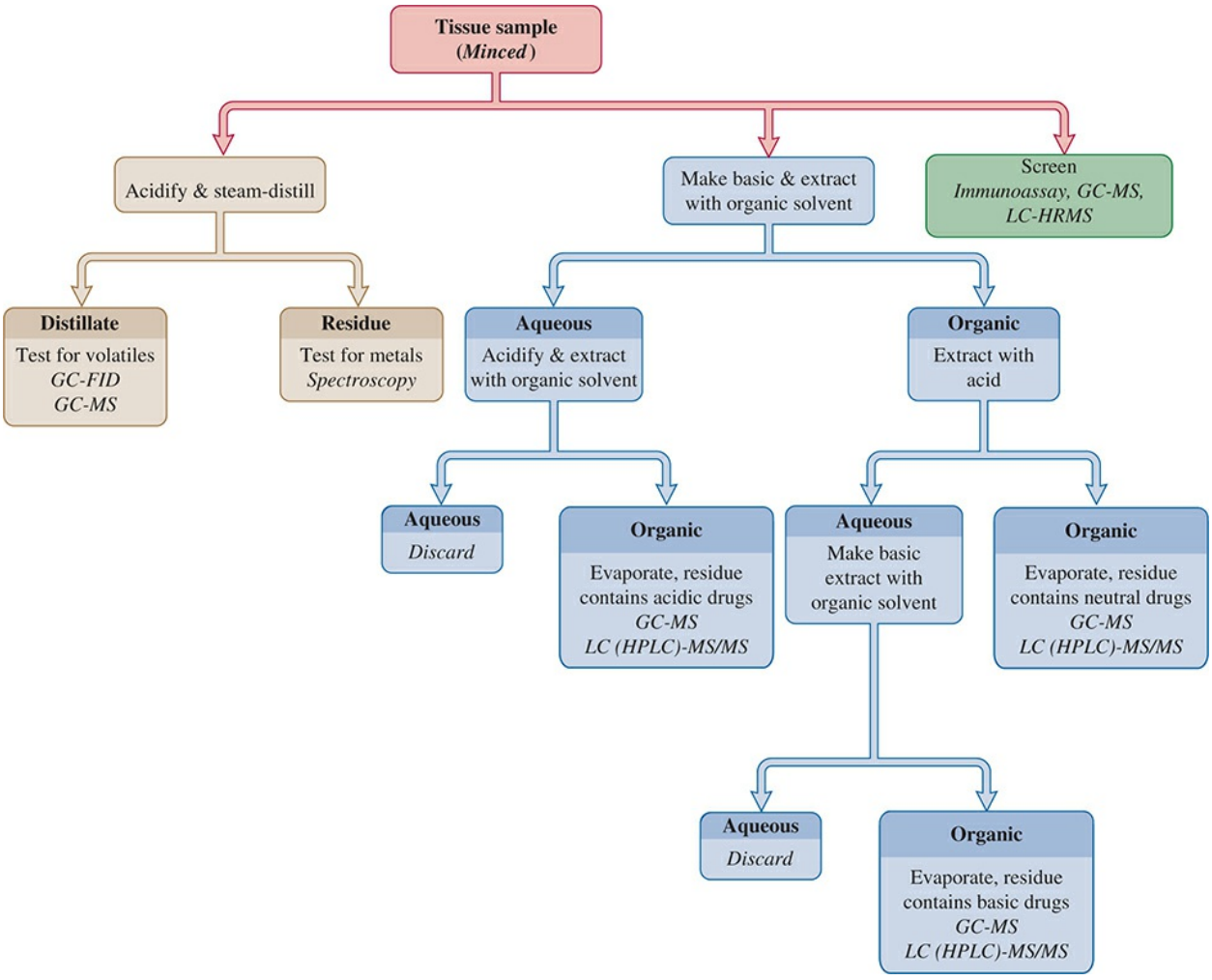

FIGURE 32–1 *A scheme of separation for poisons from tissues.*

ROLE IN GENERAL TOXICOLOGY

It is universally acknowledged that the chemical under study must be either pure or the nature of any contaminant well-characterized, to enable interpretation of the experimental results with validity. Chemicals may degrade when in contact with air, by exposure to ultraviolet or other radiation, by interaction with constituents of the vehicle or dosing solution, and by other means. Developing an analytical procedure by which these changes can be recognized and corrected is essential in achieving consistent and reliable results over the course of a study.

Finally, analytical methods are necessary to determine the bioavailability of a compound that is under study. Some substances with low water solubility are difficult to introduce into an animal, and various vehicles may be investigated. A comparison of the blood concentrations for the compound under study provides a simple means of comparing the effectiveness of vehicles.

ROLE IN FORENSIC TOXICOLOGY

The duties of a forensic toxicologist in postmortem investigations include the qualitative and quantitative analysis of drugs or poisons in biological specimens collected at autopsy and the interpretation of the analytical findings with respect to the physiological and behavioral effects of the detected chemicals in the deceased at the time of injury and/or death. The cause of death in cases of poisoning cannot be proved beyond contention without toxicological analysis that confirms the presence of the toxicant in either body fluids or tissues of the deceased.

The results of postmortem toxicological testing provide valuable epidemiological and statistical data. Forensic toxicologists are often among the first to alert the medical community to new epidemics of substance abuse and the dangers of abusing over-the-counter drugs. Similarly, they often determine the chemical identity and toxicity of novel analogs of psychoactive agents that are subject to abuse, including “designer drugs,” such as china white (methylfentanyl), ecstasy (methylenedioxymethamphetamine), GHB (gamma-hydroxybutyric acid), 4-methylmethcathinone (mephedrone), methylone, 3,4-methylenedioxypyrovalerone, 5,6-methylenedioxy-2-aminoindane (MDAI), and synthetic cannabinoids K2 and Spice.

TOXICOLOGICAL INVESTIGATION OF A POISON DEATH

The toxicological investigation of a poison death may be divided into three steps: (1) obtaining the case history and suitable specimens, (2) the toxicological analyses, and (3) the interpretation of the analytical findings.

Case History and Specimens

Today, thousands of compounds are readily available that are lethal if ingested, injected, or inhaled. Usually, a limited amount of specimen is available on which to perform analyses;

therefore, it is imperative that, before the analyses are initiated, as much information as possible concerning the facts of the case be collected. The age, sex, weight, medical history, and occupation of the decedent as well as any treatment administered before death, the gross autopsy findings, the drugs available to the decedent, and the interval between the onset of symptoms and death should be noted. In a typical year, a postmortem toxicology laboratory will perform analyses for such diverse poisons as over-the-counter medications (e.g., analgesics and antihistamines), prescription drugs (e.g., benzodiazepines and opioids), drugs of abuse (e.g., cocaine, marijuana, and methamphetamine), and gases (e.g., inhalants and carbon monoxide).

Specimens of many different body fluids and organs are necessary, as drugs and poisons display varying affinities for body tissues. Therefore, detection of a poison is more likely in a tissue in which it accumulates. Fluids and tissues should be collected before embalming, as this process will dilute or chemically alter the poisons present, rendering their detection difficult or impossible. Although forensic toxicology laboratories typically receive blood, urine, liver tissue, and/or stomach contents for identification of xenobiotics, they have been increasingly called upon to meet the analytical challenges of many alternative types of samples. Nontraditional matrices, such as bone marrow, hair, and nails, among others, may be submitted to the laboratory. For example, on occasion, toxicological analysis is requested for cases of burned, exhumed, putrefied, and skeletal remains. The vitreous humor of the eye is isolated and sequestered from putrefaction, charring, and trauma; thus, it is a useful specimen for the detection of most drugs, anions, and even volatile poisons such as alcohols, ketones, and glycols. Hair analysis has been used to measure individual exposure to heavy metals, such as arsenic, mercury, and lead, as well as many drugs of abuse and other pharmaceuticals, pesticides, and plastics. Nails, another keratinized matrix, have also been used to determine exposure to selected xenobiotics in both antemortem and postmortem cases. Finally, in severely decomposed bodies, the absence of blood and/or the scarcity of solid tissues suitable for analysis have led to the collection and testing of maggots (fly larvae) feeding on the body. The fundamental premise underlying maggot analysis is that if drugs or intoxicants are detected, they could only have originated from the decedent’s tissues on which the larvae were feeding.

Toxicological Analysis

Before the analysis begins, several factors must be considered, including the amount of specimen available, the nature of the poison sought, and the possible biotransformation of the poison. In cases involving oral administration of the poison, the gastrointestinal (GI) contents are analyzed first because large amounts of residual unabsorbed poison may be present. The urine may be analyzed next, as the kidney is the major organ of excretion for most poisons and high concentrations of toxicants and/or their metabolites often are present in urine. After absorption from the GI tract, drugs or poisons are carried to the liver before entering the general systemic circulation; therefore, the first analysis of an internal organ is conducted on the liver.

A thorough knowledge of drug biotransformation is often essential before an analysis is performed. The parent compound and any major pharmacologically active metabolites should be isolated and identified. Besides GC- and LC-MS targeted analysis, clinical and forensic toxicologists increasingly utilize high-resolution MS technologies including time-of-flight (TOF) and Orbitrap mass analyzers. This analytical advance offers retrospective data interrogation and versatile screening methods where new analytes can be rapidly included in the test panel. The more recent generation of MS technologies interfaces quadrupole mass analyzer with TOF

allowing higher resolution and greater mass accuracy for simultaneous screening and quantitative analyses of over a hundred drugs and metabolites. Such analytical development is imperative to address the growing complexity and transience of the recreational drug market.

The analysis may be complicated by the normal chemical changes that occur during the decomposition of a cadaver. The natural enzymatic and nonenzymatic processes of decomposition and microbial metabolism may destroy a poison that was present at death or produce substances or compounds with chemical and physical properties similar to those of commonly encountered poisons. Cadaveric alkaloids, which produce color reactions like morphine, result from the bacterial decarboxylation of the amino acids ornithine and lysine, producing putrescine and cadaverine, respectively. During decomposition, phenylalanine is converted to phenylethylamine, which has chemical and physical properties very similar to those of amphetamine. The hydrolysis, oxidation, or reduction of proteins, nucleic acids, and lipids may generate numerous compounds, such as hydroxylated aliphatic and aromatic carboxylic acids, pyridine and piperidine derivatives, and aromatic heterocyclics such as tryptamine and norharmane. All these substances may interfere with the isolation, identification, and quantitation of the toxicants being sought. However, many poisons—such as arsenic, barbiturates, mercury, and strychnine—are extremely stable and may be detectable many years after death.

Forensic toxicology laboratories analyze specimens by using a variety of analytical procedures. Initially, nonspecific tests designed to determine the presence or absence of a class or group of analytes may be performed directly on the specimens. Examples of tests used to rapidly screen urine are the FPN (ferric chloride, perchloric, and nitric acid) color test for phenothiazine drugs and immunoassays for the detection of amphetamines, benzodiazepines, and opiate derivatives, among others. Positive results obtained with these tests must be confirmed by a second analytical procedure that identifies the particular drug. Whenever possible, the most specific test for the compound of interest should be performed.

Analytical methods must be of sufficient rigor to provide reliable qualitative and quantitative data. For quantitative analysis, the accuracy, precision, linearity, and specificity of the procedure must also be established. Linearity should be determined by using multiple drug-fortified calibrators whose concentrations bracket the anticipated concentrations in the biological specimen. Precision, which statistically demonstrates the variance in the value obtained, is determined by replicate analyses of a specimen of a known concentration. Additional assay parameters, such as analyte stability and recovery from the biological matrix, for example, can also be determined.

Interpretation of Analytical Results

Once the analysis of the specimens is complete, the toxicologist must interpret the findings regarding the physiological or behavioral effects of the toxicants on the decedent at the concentrations found. Specific questions may be answered, such as the route of administration, the dose administered, and whether the concentration of the toxicant present was sufficient to cause death or alter the decedent’s actions enough to cause his or her death.

In determining the route of administration, the toxicologist notes the results of the analysis of the various specimens. Generally, the highest concentrations of a poison are found at the site of administration. Therefore, the presence of large amounts of drugs and/or poisons in the GI tract and liver indicates oral ingestion, while higher concentrations in the lungs than in other visceral organs can indicate inhalation or intravenous injection.

It is necessary to demonstrate that absorption of the toxicant has occurred and that it has been transported by the general circulation to the target organ in order to exert its lethal effect. This is established by blood and tissue analyses. An exception to the rule is provided by strong, corrosive chemicals, such as sulfuric acid, lye, and phenol, which exert their deleterious effects by directly digesting tissue, causing hemorrhage and shock. Urine results establish only that the poison was present in the body at some time before death.

The physiological effects of most drugs and poisons generally correlate with their concentrations in blood plasma and serum. The survival time between the administration of a poison and death may be sufficiently long to permit biotransformation and excretion of the agent. Blood values may appear to be nontoxic or consistent with therapeutic administration. Death from hepatic failure after an acetaminophen overdose usually occurs at least 3 to 4 days after ingestion. Postmortem acetaminophen concentrations in blood may be consistent with the ingestion of therapeutic doses. Therefore, fatal acetaminophen overdose is determined by case history, central lobular necrosis of the liver, and, if available, analysis of serum specimens collected from the decedent when he or she was admitted to the emergency department.

An extension of forensic toxicology is the analysis of impurities of illicit drug synthesis in biological specimens. Many drugs of abuse, particularly methamphetamine, are illicitly manufactured in clandestine laboratories, and side reactions or incomplete conversion of the reactants yield an impure mixture of methamphetamine and synthetic impurities. These impurities can be characteristic of a particular synthetic method, and their detection in biological specimens can indicate the use of an illicitly produced drug that is not a legal pharmaceutical product, can suggest the synthetic method that was used to produce the drug, can point to a possible common source of illicit production, and can provide a link between manufacturers, dealers, and users.

Many tissues are available for the purpose of identifying xenobiotics in postmortem specimens. The advent of improved analytical techniques with greater sensitivity and selectivity, including GC-MS/MS and LC-MS/MS, has expanded the array of biological specimens in which substances can be detected. These specimens include oral fluid (saliva), sweat, meconium, amniotic fluid, breast milk, and semen among others. In oral fluid, for example, the pharmacokinetics of many drugs and metabolites is closely aligned to that of blood pharmacokinetics, and thus can serve as an alternative matrix for illicit and therapeutic drug detection, as well as detection of exposure to environmental toxicants and pesticides.

CRIMINAL POISONING OF THE LIVING

Over the past few decades, forensic toxicologists have become more involved in the analysis of specimens obtained from living victims of criminal poisonings. Generally, this increase in testing is a result of two types of cases: (1) administration of drugs to incapacitate victims of kidnapping, robbery, or sexual assault and (2) poisoning as a form of child abuse.

While alcohol is still often a primary factor in cases of alleged sexual assault, common drugs of abuse or other psychoactive drugs are often involved (Table 32–1). Of particular concern are the many potent inductive agents medically administered prior to general anesthesia. Many of these drugs, such as benzodiazepines and phenothiazines, are available today through illicit sources or legal purchase in foreign countries. When administered surreptitiously, they cause sedation and incapacitate the victim while also producing amnesia in the victim as to the events

while drugged, without causing severe central nervous system depression. These cases often present a difficult analytical challenge to the toxicologist. Usually, the victim does not bring forth an allegation of assault until 24 hours to several days after the attack. Thus, the intoxicating drug may have been largely eliminated or extensively metabolized such that extremely low concentrations of drug or metabolites are present in the victim's blood, urine, and/or hair specimens.

TABLE 32–1 Distribution of Drugs of Abuse Encountered Urine Specimens in 1179 Cases of Alleged Sexual Assault*

Rank	Drug/Drug Group	Incidence
1	No drugs found	468
2	Ethanol	451
3	Cannabinoids	218
4	Benzoylecgonine (cocaine metabolite)	97
5	Benzodiazepines	97
6	Amphetamines	51
7	Gamma-hydroxybutyrate (GHB)	48
8	Opiates	25
9	Propoxyphene	17
10	Barbiturates	12

*Thirty-five percent of the drug-positive specimens were positive for more than one drug.
Data from ElSohly MA, Salamone SJ. Prevalence of drugs used in cases of alleged sexual assault. J Anal Toxicol. 1999;23(3):141–146.

Poisoning as a form of child abuse involves the deliberate administration of toxic or injurious substances to a child, usually by a parent or other caregiver. The victims of such poisonings range in age from a few months to the teens. Common agents used to intentionally poison children have included syrup of ipecac, table salt, laxatives, diuretics, antidepressants, sedative-hypnotics, and narcotics. As in the case of sexual assault, sophisticated MS testing methods may be required to detect such chemicals as emetine and cephaeline, the emetic alkaloids in syrup of ipecac.

FORENSIC DRUG TESTING

Concerns regarding the potentially adverse consequences of substance abuse for the individual, the workplace, and society have led to widespread urine analysis for controlled or illicit drugs. Currently, such testing is conducted routinely by the military services, regulated transportation and nuclear industries, many federal and state agencies, public utilities, federal and state criminal justice systems, and numerous private businesses and industries. Significant ethical and legal ramifications are associated with such testing. Those having positive test results may not receive employment, be dismissed from a job, be court-martialed, or suffer a damaged reputation.

Forensic drug testing differs from other areas of forensic toxicology in which urine or an alternative specimen is the only specimen analyzed and testing is performed for a limited number of drugs and metabolites. While urine remains the most prevalent biological matrix for forensic drug testing, the popularity of oral fluid as an alternative matrix is increasing because the collection is more convenient and less invasive. Under the federal certification program, analyses are performed for a limited number of classes or drugs of abuse. Initial testing is performed by immunoassays on rapid, high-throughput chemistry analyzers. A confirmation analysis in Health and Human Services-certified laboratories is performed by GC-MS and LC-MS/MS.

Many individuals who are subject to regulated urine testing have devised techniques to mask their drug use either by physiological means such as the ingestion of diuretics or by attempting to adulterate the specimen directly with bleach, vinegar, or other products that interfere with the initial immunoassay tests. Thus, specimens are routinely tested for adulteration by checking urinary pH, creatinine, and specific gravity, nitrates, chromates, and noting any unusual color or smell. A mini-industry has developed to sell various products that are alleged to “beat the drug test” by interfering with either the initial or confirmatory drug test. Most chemical adulterants can be detected in urine by specific colorimetric tests that can be readily adapted to high-volume auto-analyzers. Thus, laboratories routinely test not only for drugs of abuse but also for a wide variety of chemical adulterants. In most instances, a positive test result for adulteration has a consequence similar to a positive drug test.

HUMAN PERFORMANCE TESTING

Forensic toxicology activities also include the determination of the presence of ethanol and other drugs and chemicals in blood, breath, or other specimens and the evaluation of their role in modifying human performance and behavior. A common application of human performance testing is to determine impairment while driving under the influence of ethanol or drugs.

There has been growing concern about the deleterious effects of drugs other than ethanol on driving performance. Several studies have demonstrated a relatively high occurrence of drugs in impaired or fatally injured drivers. These studies tend to report that the highest drug-use accident rates are associated with the use of such illicit or controlled drugs as cocaine, benzodiazepines, marijuana, and phencyclidine.

COURTROOM TESTIMONY

The forensic toxicologist often is called upon to testify in legal proceedings as an expert witness. A court recognizes a witness as an expert if that witness possesses knowledge or experience in a

subject that is beyond the range of ordinary or common knowledge or observation. An expert witness may provide two types of testimony: objective testimony and opinion. Objective testimony by a toxicologist usually involves a description of his or her analytical methods and findings. When a toxicologist testifies as to the interpretation of his or her analytical results or those of others, that toxicologist is offering an opinion. In qualifying someone as an expert witness, the court considers the witness's education, on-the-job training, work experience, teaching or academic appointments, and professional memberships and publications as well as the acceptance of the witness as an expert by other courts.

Whether a toxicologist appears in criminal or civil court, workers' compensation, or parole hearings, the procedure for testifying is the same: direct examination, cross-examination, and redirect examination. Regardless of which side has called the toxicologist to court, the toxicologist should testify with scientific objectivity. An expert witness is called to provide informed assistance to the jury. The jury, not the expert witness, determines the guilt or innocence of the defendant.

ROLE IN CLINICAL TOXICOLOGY

Analytical toxicology in a clinical setting can aid in the diagnosis and treatment of toxic incidents, as well as in monitoring the effectiveness of treatment regimens. It is useful to clearly identify the nature of the toxic exposure and measure the amount of the toxic substance that has been absorbed. Frequently, this information, together with the clinical state of the patient, permits a clinician to relate the signs and symptoms observed to the anticipated effects of the toxic agent. This may permit a clinical judgment as to whether the treatment must be vigorous and aggressive or whether simple observation and symptomatic treatment of the patient are sufficient.

A cardinal rule in the treatment of poisoning cases is to remove any unabsorbed material, limit the absorption of additional poison, and hasten its elimination. Although the instrumentation and the methodology used in a clinical toxicology laboratory are similar to those utilized by a forensic toxicologist, a major difference between these two applications is responsiveness. In emergency toxicology testing, results must be communicated to the clinician within hours to be meaningful for therapy. Primary examples of the usefulness of emergency toxicology testing are the rapid quantitative determination of acetaminophen, salicylate, alcohols, and glycol serum concentrations in instances of suspected overdose.

Ethanol is the most common chemical encountered in emergency toxicology. Although relatively few fatal intoxications occur with ethanol alone, serum values are important in the assessment of behavioral, physiological, and neurological function, particularly in trauma cases where the patient is unable to communicate and surgery with the administration of anesthetic or analgesic drugs is indicated. Intoxications from accidental or deliberate ingestion of other alcohols or glycols—such as methanol from windshield deicer or paint thinner, isopropanol from rubbing alcohol, and ethylene glycol from antifreeze—are often encountered in emergency departments. Following ingestion of methanol or ethylene glycol, patients often present with similar neurological symptoms and severe metabolic acidosis due to the formation of toxic aldehyde and acid metabolites. A rapid quantitative serum determination for these intoxicants will indicate the severity of intoxication and help direct the therapy.

ROLE IN THERAPEUTIC MONITORING

Historically, the administration of drugs for long-term therapy was based largely on experience. A dosage amount that was selected and administered at appropriate intervals based on what the clinician had learned was generally tolerated by most patients. If the drug seemed ineffective, the dose was increased; if toxicity developed, the dose was decreased, or the frequency of dosing was altered. At times, a different dosage form might be substituted. Establishing an effective dosage regimen was particularly difficult in children and the elderly.

The factors responsible for individual variability in responses to drug therapy include the rate and extent of drug absorption, distribution, and binding in body tissues and fluids, rate of metabolism and excretion, pathological conditions, and interaction with other drugs. Monitoring of the plasma or serum concentration at regular intervals will detect deviations from the average serum concentration, which, in turn, may suggest that one or more of these variables need to be identified and corrected. Drugs that are commonly monitored during therapy are presented in Table 32–2.

TABLE 32–2 Drugs Commonly Indicated for Therapeutic Monitoring

Antiarrhythmics
Digoxin
Digitoxin
Lidocaine
Procainamide and *N*-acetylprocainamide
Quinidine
Antibiotics
Amikacin
Chloramphenicol
Gentamicin
Tobramycin
Vancomycin
Anticancer
Methotrexate
Anticonvulsants
Carbamazepine
Gabapentin
Lamotrigine
Levetiracetam
Phenobarbital
Phenytoin
Primidone
Topiramate
Valproic acid
Zonisamide
Antidepressants
Amitriptyline and nortriptyline
Doxepin and nordoxepin
Fluoxetine and norfluoxetine
Impramine and desipramine
Paroxetine
Sertraline and desmethylsertraline
Antipsychotics
Clozapine
Pimozide
Bronchodilators
Caffeine
Theophylline
Immunosuppressants
Azathioprine
Cyclosporine
Mycophenolic acid
Sirolimus
Tacrolimus
Mood stabilizing
Lithium

CONCLUSION

The analytical techniques employed by forensic toxicologists have continued to expand in complexity and improve in reliability and sensitivity. Many new analytical tools have been applied to toxicological problems in almost all areas of the field, and the technology continues to open new areas of research. Forensic toxicologists continue to be concerned about conducting unequivocal identification of toxic substances in such a manner that the results can withstand a legal challenge. The issues of substance abuse, designer drugs, increased potency of therapeutic agents, widespread concern about pollution, and the safety and health of workers present challenges to the analyst's knowledge, skills, and abilities. As these challenges are met, analytical toxicologists will continue to play a substantial role in the expansion of the discipline of toxicology.

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QUESTIONS

1. Which of the following is most commonly used as a drug of sexual assault?
   - **a.** narcotics.
   - **b.** amphetamines.
   - **c.** benzodiazepines.
   - **d.** ethanol.
   - **e.** antidepressants.

2. All of the following statements regarding analytic and forensic toxicology are true EXCEPT:
   - **a.** Analytic toxicology uses analytic chemistry to characterize a chemical's adverse effect on an organism.
   - **b.** Medical examiners and coroners are most important in determining cause of death.
   - **c.** Tissues and body fluids are vital in forensic toxicology.
   - **d.** Forensic toxicology is used for purposes of the law.
   - **e.** Chapuis first characterized a system for classifying toxic agents.

3. Which of the following criteria is NOT routinely used to check for adulteration of drug urine analysis?
   - **a.** urea.
   - **b.** pH.
   - **c.** color.
   - **d.** specific gravity.

e. creatinine.

4. Which blood alcohol concentration (BAC) is most commonly used as the statutory definition of DUI?
   a. 0.04.
   b. 0.06.
   c. 0.08.
   d. 0.12.
   e. 0.16.

5. Which of the following drugs is NOT properly matched with its most common analytic method?
   a. benzodiazepines—GC/MS.
   b. ibuprofen—TLC/HPLC.
   c. amphetamines—immunoassays.
   d. barbiturates—GC/immunoassays.
   e. ethanol—immunoassays.

6. For which of the following drugs is serum NOT used during toxicology testing?
   a. ethanol.
   b. cocaine.
   c. aspirin.
   d. barbiturates.
   e. ibuprofen.

7. Which of the following is LEAST important in determining variability in response to drug therapy?
   a. drug interactions.
   b. distribution in body tissue.
   c. body mass index.
   d. pathological conditions.
   e. rate of metabolism.

8. Which of the following statements is FALSE regarding steady state?
   a. Steady-state concentrations are proportional to the dose/dosage interval.
   b. Steady state is attained after approximately four half-lives.
   c. The steady-state concentrations are proportional to F/Cl.
   d. Monitoring of steady-state drug concentration assumes that an effective concentration is present.
   e. Fluctuations in concentration are increased by slow drug absorption.

9. Which of the following is an indirect method of measuring a chemical or its metabolite?
   a. blood test.
   b. hair sample.

c. urinalysis.
d. hemoglobin adduct detection.
e. breath analysis.

10. Which of the following statements regarding analytic/forensic toxicology is TRUE?
a. Antidepressants are commonly used to incapacitate victims.
b. It is easy to test for and prove that marijuana is a factor in an automobile accident.
c. Heroin is the drug most commonly encountered in emergency toxicology.
d. Toxicologists can play an important role in courtroom testimonies.
e. Ethanol intoxication often results in death.

CHAPTER 33

Clinical Toxicology[1]

Louis R. Cantilena Jr.

KEY POINTS

- Clinical toxicology encompasses the expertise in the specialties of medical toxicology, applied toxicology, and clinical poison information.
- Important components of the initial clinical encounter with a poisoned patient include stabilization of the patient, clinical evaluation (history, physical, laboratory, and radiology), prevention of further poison absorption, enhancement of poison elimination, administration of antidote, and supportive care with clinical follow-up.

HISTORY OF CLINICAL TOXICOLOGY

Historical Aspects of the Treatment of Poisoning

The history of poisons and poisoners dates to ancient times. The use of toxic smoke can be traced to as early as 2000 bc in ancient India, and formulas for creating poisonous and noxious vapors have also been found from 1000 bc in Chinese writings. Documentation regarding the use of antidotes can be found in the Odyssey and Shastras from approximately 600 bc. From 129 to 200 ad, Galen wrote *De Antidotis I*, *De Antidotis II*, and *De Theriaca ad Pisonem* that described the development of a universal antidote known as alexipharmic or theriac by King Mithridates VI of Pontus who lived from 132 to 63 bc. The antidote reportedly contained 36 or more ingredients that were ingested every day, conferring protection against a broad spectrum of poisons, such as venomous stings and bites from vipers, spiders, and scorpions. Refinement of theriac (antidote) formulations continued for nearly 2000 years, however, William Heberden wrote *Antitheriaka: An Essay on Mithridatium and Theriaca* in 1745 questioning the effectiveness of these products.

The use of oral charcoal can be dated to early Greek and Roman civilization when wood charcoal was used for the treatment of maladies such as anthrax and epilepsy. By the 1960s, the use of activated charcoal became routinely recommended for the treatment of patients poisoned with substances thought to be adsorbed by charcoal.

Modern Development of Effective Antidotes for Poisoning

The search for safe and effective antidotes has been challenging. It was not until the mid-twentieth century that a scientific and systematic approach to drug development became possible and, in fact, became a requirement by newly created regulatory agencies in charge of controlling the sale and distribution of medicinal products.

The therapeutic area of poison treatments and prophylaxis presents several challenges including the inability to perform controlled human studies to evaluate efficacy, due to the small numbers of poisoned patients available for enrollment in therapeutic trials and the often-sporadic occurrence of specific poisonings. In 2002, the Food and Drug Administration (FDA) enacted the Animal Efficacy Rule, which allows FDA to approve products for serious or life-threatening conditions caused by exposure to lethal biological, chemical, radiological, or nuclear substances

without human efficacy testing.

INTRODUCTION OF THE POISON CONTROL CENTER

Advances in clinical toxicology and improvements in the quality of care of the poisoned patient have included poison control centers (PCCs). Healthcare professionals who work in these centers share information through publications and national conferences that ultimately result in systematic enhancements in the treatment of the poisoned patient. Poison centers are generally staffed by a medical director (medical toxicologist), administrator or managing director, specialists in poison information, and educators for poison prevention programs. Poison centers provide direct information to patients with expert recommendations for needed treatment, critical diagnostic and treatment information for healthcare professionals, education for healthcare personnel, and poison prevention activities through public education. In addition, PCCs have demonstrated that they can provide data that can lead to the recognition of improper use of medications, emergence of new illicit drug exposures or practice, prescription product diversion, and product tampering events.

CLINICAL STRATEGY FOR THE TREATMENT OF THE POISONED PATIENT

The following general steps represent important elements of the initial clinical encounter for a poisoned patient:

1. Clinical stabilization of the patient
2. Clinical evaluation (history, physical, laboratory, and radiology)
3. Prevention of further toxicant absorption
4. Enhancement of toxicant elimination
5. Administration of antidote (if available)
6. Supportive care, close monitoring, and clinical follow-up

Clinical Stabilization

Assessment of the patient’s airway (ability to move air into and out of the lungs), breathing (the presence of spontaneous respirations), and circulation (adequate blood pressure and perfusion of vital organs) is the initial step of emergency treatment. Some drugs, such as a benzodiazepine, can cause significant sedation early after exposure but often have a comparatively mild clinical course, whereas other chemicals, such as camphor, show little clinical effects initially but can produce a fatal outcome. Some chemicals and drugs can cause seizures as part of their toxic-effect profile. Control of drug/toxicant-induced seizures can be an important component of the initial stabilization of the poisoned patient. On occasion, especially in critically ill patients, treatment interventions are initiated before a patient is completely stable.

Clinical History in the Poisoned Patient

The primary goal of obtaining the medical history in poisoned patients is to determine the substance to which the patient was exposed and the extent and timing of exposure. Unfortunately, the clinical history from the initial clinical encounter with the poisoned patient is sometimes not available because either the patient is unresponsive or the history provided is unreliable. The history may be undependable due to the inability of the patient to recall pertinent facts relating to the ingestion or exposure, or in the setting of an attempted suicide or a patient who has taken illegal substances, the patient often is not willing to provide an accurate history. Additional resources sometimes employed to obtain an informative clinical history include interviewing family members and emergency medical technicians who were at the scene, searching an electronic medical record, or contacting a pharmacist who can sometimes provide a listing of prescriptions recently filled or an employer who can provide a list of chemicals found in the work environment for an occupational exposure.

When estimating the extent of exposure to the poison, one should maximize the estimated dose received. That is, one should assume that the entire contents of the prescription bottle were ingested, that the entire bottle of liquid was consumed, most of the body surface area was exposed to a topical chemical, or that the highest possible concentration of airborne contaminant was present for a patient poisoned by inhalation. With an appropriate estimate of the dose, the toxicologist can refer to various information resources to determine what the range of expected clinical effects might be from the estimated exposure. The estimation of expected clinical effects helps to effectively triage the poisoned patient.

Estimating the timing of the exposure to the poison is frequently the most difficult aspect of the clinical history when treating the poisoned patient. Often the toxicologist must turn detective to determine the most likely window of time that the exposure occurred. When the history is unobtainable or unreliable, the treating clinical toxicologist is left in the setting of empirical treatment of an “unknown ingestion” poisoning.

Physical Examination

A thorough examination of the patient is required to assess the patient’s condition, determine the patient’s mental status, and, if altered, determine possible nontoxicology explanations for the abnormal mental status such as trauma or central nervous system infection. In addition, findings from the physical examination allow one to categorize the patient’s physical examination parameters into broad clinical presentation classes referred to as toxic syndrome, or toxidrome, which is a constellation of clinical signs and symptoms that, when taken together, are likely associated with exposure from certain classes of toxic chemicals. Table 33–1 lists the clinical features of these major toxic syndromes.

TABLE 33–1 Clinical Features of Toxic Syndromes

	Blood Pressure	Pulse	Temperature	Pupils	Lungs	Abdomen	Neurological
Sympathomimetic	Incr.	Incr.	Slight Incr.	Mydriasis	NC	NC	Hyperalert, incr. reflexes
Anticholinergic	Slight incr. or NC	Incr.	Incr.	Mydriasis	NC	Decr. bowel sounds	Altered mental status
Cholinergic	Slight decr. or NC	Decr.	NC	Miosis	Incr. bronchial sounds	Incr. bowel sounds	Altered mental status
Opioid	Decr.	Decr.	Decr.	Miosis	NC or rales (late)	Decr. bowel sounds	Decr. level of consciousness
Ethanol-sedative hypnotics	Decr.	Decr.	Decr.	Variable	NC	Decr. bowel sounds	Agitation; decr. level of consciousness
Withdrawal from ethanol-sedative hypnotics	Incr.	Incr.	Incr.	Mydriasis	NC	Incr. bowel sounds	Agitation; hallucinations; seizures
Withdrawal from opioids	Incr.	Incr.	Variable	Mydriasis	NC	Incr. bowel sounds; vomiting	Anxious, piloerection

Categorization of the patient's presentation into a toxic syndrome permits initiation of rationale treatment based on the most likely category of toxicant responsible without knowing the specific chemical causing the poisoning. Occasionally, a characteristic odor detected on the poisoned patient's breath or clothing may point toward exposure or poisoning by a specific chemical (Table 33–2).

TABLE 33–2 Characteristic Odors Associated with Poisonings

Odor	Potential Poison
Apple blossoms	Chloroacetophenone (tear gas)
Bitter almonds	Cyanide
Carrots	Cicutotoxin (water hemlock)
Eggs	Hydrogen sulfide, mercaptans
Garlic	As, organophosphates, DMSO, thallium
Mothballs	Naphthalene, camphor
Pear-like	Paraldehyde, chloral hydrate
Rotten eggs	Hydrogen sulfide, mercaptans, *N*-acetylcysteine
Vinyl	Ethchlorvynol
Wintergreen	Methylsalicylate

Abbreviation: DMSO, dimethyl sulfoxide.

Follow-up clinical examinations can help gauge the progression of the clinical course of poisoning as well as determine the effectiveness of treatment interventions and assess the need for additional treatment procedures.

Laboratory Evaluation

A common misconception concerning the initial treatment of poisoned patients is that a definitive diagnosis of the poison responsible for the patient's clinical presentation is made based upon clinical laboratory testing in real time. Table 33–3 lists drugs or other chemical tests that are typically available for STAT measurement in a medical center-type hospital facility. As one can see, the number of chemicals for which quantitative detection is possible in a rapid turnaround timeline is extremely limited, compared to the large number of possible poisons. This further emphasizes the importance of recognizing clinical syndromes for poisoning and for the clinical toxicologist to initiate general treatment and supportive care for the patient with poisoning from an unknown substance.

TABLE 33–3 List of Tests That Are Commonly Measured in a Hospital Setting on a STAT Basis

Acetaminophen	Osmolality
Carbamazepine	Phenobarbital
Carboxyhemoglobin	Phenytoin
Digoxin	Procainamide/NAPA
Ethanol	Quinidine
Gentamicin	Salicylates
Iron	Theophylline
Lithium	Tobramycin
Magnesium	Valproic acid
Methemoglobin	Vancomycin

Abbreviation: NAPA, N-acetylprocainamide.

For the relatively few chemicals that can be quantified on a rapid turnaround basis in an emergency department (ED) setting, the quantitative measurement can often provide both prognostic and therapeutic guidance. Predictive relationships of drug plasma concentration and clinical outcome and/or suggested concentrations that require therapeutic interventions are available for several other drugs including lithium, digoxin, iron, phenobarbital, and theophylline. "Action levels" or toxic threshold values for the measured plasma concentrations of various drugs or other chemicals represent mean concentrations of the respective substance that have been shown to produce a significant harmful effect during a retrospective analysis of

clinical case series. However, the pharmaco- (or toxico-) dynamic variability for a given toxicant or for a combination of toxicants is significant.

Because of the limited clinical availability of rapidly available "diagnostic" laboratory tests for poisons (Table 33–3), toxicologists can sometimes gain important insight into what potential drugs were ingested by performing simple calculations based on routine clinical laboratory data. The anion gap (AG) and the osmol gap calculations are used as diagnostic aids when the clinical history suggests poisoning and the patient's condition is consistent with exposure to chemicals known to cause elevations of these parameters. Although both the AG and the osmol gap can provide very useful information from readily available clinical chemistry measurements, these determinations must be interpreted cautiously.

The AG is calculated as the difference between the serum Na ion concentration and the sum of the serum Cl and HCO_3 ion concentrations. A normal AG is <12. When there is laboratory evidence of metabolic acidosis (low blood pH and low serum HCO_3) in a poisoned patient, the finding of an elevated AG would suggest systemic toxicity from a relatively finite list of chemicals listed in Table 33–4. Additional substances such as benzyl alcohol, carbon monoxide, hydrogen sulfide, metformin, phenformin, formic acid, and other organic or mineral acids, as well as the anti-seizure medication valproic acid, can also result in elevated AG with systemic metabolic acidosis.

TABLE 33–4 Differential Diagnosis of Metabolic Acidosis with Elevated Anion Gap: "AT MUD PILES"

A	Alcohol (ethanol ketoacidosis)
T	Toluene
M	Methanol
U	Uremia
D	Diabetic ketoacidosis
P	Paraldehyde
I	Iron, isoniazid
L	Lactic acid
E	Ethylene glycol
S	Salicylate

The osmol gap is calculated as the numerical difference between the measured serum osmolality and the serum osmolarity calculated from the clinical chemistry measurements of the serum sodium ion, glucose, and blood urea nitrogen (BUN) concentrations. The normal osmol gap is < 10 mOsm. An elevated osmol gap in the setting of a poisoned patient suggests the presence of an osmotically active substance in the plasma that is not accounted for by the Na, glucose, or BUN concentrations. Table 33–5 lists several substances that when ingested can be

associated with an elevated osmol gap in humans.

TABLE 33–5 Differential Diagnosis of Elevated Osmol Gap

Methanol
Ethanol
Ethylene glycol
Isopropanol
Dimethyl sulfoxide (DMSO)
Mannitol
Propylene glycol

Radiographic Examination

The utility of the plain x-ray radiographic examination to diagnose specific poisonings is relatively limited, due to the lack of radiopacity. Generally, plain radiographs can detect a significant amount of ingested oral medication containing ferrous or potassium salts. Certain formulations that have an enteric coating or certain types of sustained release products are radiopaque and can be visualized also.

The most useful radiographs ordered during the initial management of the poisoned patient include chest and abdominal radiographs and computed tomography (CT) of the head. The abdominal radiograph has been used to detect recent lead paint ingestion in children, and the recent ingestion of a halogenated hydrocarbon such as carbon tetrachloride or chloroform that will be visualized as a radiopaque liquid in the gut lumen. Finally, abdominal plain radiographs have been helpful in the setting where foreign bodies are detected in the gastrointestinal tract after accidental (toddler swallows something) or deliberate (body packer smuggling drugs by swallowing storage vesicles filled with illicit drugs) ingestion.

Plain radiography and other types of diagnostic imaging can aid the clinical toxicologist in the ongoing treatment and patient-management phases of the drug overdose. Radiographic detection of drug-induced noncardiac pulmonary edema that is associated with some cases of serious intoxication with salicylates and opioid agonists would increase the severity stratification of the poisoning case and likely alter the therapeutic strategy for the patient. Also, significant exposure to carbon monoxide (CO) has been associated with focal CT lesions of the brain consisting of low-density areas, also referred to as lucencies in the cerebral white matter and in the basal ganglia, especially the globus pallidus. These CT findings in the brain can be useful for estimating the prognosis for a patient who survives the initial phase of CO poisoning.

Prevention of Further Poison Absorption

During the early phases of treatment of a poisoned patient who has had a toxic exposure via the

oral, inhalation, or the topical route, the opportunity to prevent further absorption of the poison to minimize the total amount of chemical that reaches the systemic circulation may be possible. For chemicals presented by the inhalation route, the patient should be moved from the site of exposure to an area with proper ventilation with clean air. Oxygen should be available if needed. For topical exposures, patient clothing containing the chemical must be removed and properly disposed in airtight wrappings or containers to ensure that the rescuers and healthcare providers are adequately protected from secondary exposure. Most topical exposures require gentle washing of the skin with water and mild soap, taking care not to cause cutaneous abrasions of the skin that may enhance dermal absorption.

The optimal time to prevent continued absorption of an oral poison is as soon as possible after the ingestion. Four primary methods for this purpose are induction of emesis with syrup of ipecac, gastric lavage, oral administration of activated charcoal, and whole-bowel irrigation. Ipecac is rarely used to treat poisonings today because of the overall low, and highly variable, effectiveness for gastric decontamination and the potential adverse effects. There is a limited role for the clinical use of syrup of ipecac, mainly in rural areas where the length of time before a poisoned patient can reach medical care is significant. The accepted contraindications for use of syrup of ipecac are (1) children less than 6 months of age, (2) in the ingestion of a caustic agent (acid or alkali), (3) a patient with a depressed level of consciousness or gag reflex or when the toxicant ingested is expected to cause either condition within a short period of time, or (4) when there is a significant risk of aspiration of gastric contents such as for ingestion of a liquid hydrocarbon with high aspiration potential.

The use of gastric lavage, the technique of placing an orogastric tube into the stomach and aspirating fluid and then cyclically instilling fluid and aspirating until the effluent is clear, has also diminished in recent years because of limited effectiveness and the risk of aspiration during the lavage procedure. Like induction of emesis, the efficacy of gastric lavage declines with increasing time interval between the time of ingestion and the use of the procedure.

For many years, orally administered activated charcoal has been routinely incorporated into the initial treatment of a patient poisoned by the oral route. The term “activated” refers to charcoal that has been processed to substantially increase adsorptive capacity. Many organic molecules bind activated charcoal. Proper use of activated charcoal to prevent the absorption of orally administered toxicants requires that the patient’s gag reflex be intact and expected to remain so in order to minimize the risk for aspiration of the administered charcoal.

The procedure of whole bowel irrigation for the treatment of the poisoned patient has gained acceptance in specific poisoning situations. The procedure is accomplished with a poorly absorbed, osmotically neutral polyethylene glycol electrolyte solution that is administered orally to expel the contents of the intestines via the rectal route. This procedure may be used for removal of ingested packets of illegal drugs swallowed by people smuggling the material by concealing the drugs in their intestines. Also, this gastrointestinal decontamination technique can prevent the ongoing absorption of sustained release formulations of drugs and the treatment of ingested iron preparations or other orally ingested metallic poisons.

Enhancement of Poison Elimination

Several methods are available to enhance the elimination of specific poisons or drugs once they have been absorbed into the systemic circulation. The primary methods employed for this use today include alkalinization of the urine, hemodialysis, hemoperfusion, hemofiltration, plasma

exchange or exchange transfusion, and the administration of oral activated charcoal serially during the treatment time course.

Urinary alkalinization enhances the renal clearance of certain weak acids. The basic principle is to increase urinary filtrate pH to a level sufficient to ionize the weak acid and prevent its renal tubular reabsorption. This ion-trapping phenomenon causes the chemical to remain in the urinary filtrate, lessen its reabsorption, and increase its renal elimination. Although similar advantages could be gained from acidification of the urine for weak bases, significant adverse events, including acute renal failure and acidosis and electrolyte disturbances, prevent use of acidification as a therapeutic intervention.

The dialysis techniques, either hemodialysis, continuous venous filtration, or peritoneal dialysis, rely on passage of the toxic chemical through a semipermeable dialysis membrane (or the patient's peritoneal membrane for peritoneal dialysis) so that it can equilibrate with the dialysate and subsequently be removed. Hemodialysis passes blood near a dialysis membrane to allow chemicals permeable to the membrane to pass through and reach equilibrium. For this method to be clinically beneficial, the chemical must have a relatively low volume of distribution, low protein binding, a relatively high degree of water solubility, and low molecular weight. Hemodialysis has been shown to be clinically effective in the treatment of poisoning by the agents shown in Table 33–6.

TABLE 33–6 Chemicals for Which Hemodialysis Has Been Shown Effective as a Treatment Modality for Poisoning

Alcohols	Lithium
Antibiotics	Meprobamate
Boric acid	Metformin
Bromide	Methotrexate
Caffeine	Paraldehyde
Calcium	Phenobarbital
Chloral hydrate	Potassium
Carbamazepine	Salicylates
Diethylene glycol	Strychnine
Fluorides	Theophylline
Iodides	Thiocynates
Isoniazid	Valproic acid
Isopropanol	

Hemoperfusion is similar to hemodialysis except that the patient's blood is pumped through a perfusion cartridge where it is in direct contact with adsorptive material (usually activated

charcoal) that has a coating of material such as cellulose or a heparin-containing gel to prevent the adsorptive material from being carried back to the patient's circulation. The principle characteristics for a drug or other chemical to be successfully removed by this technique are low volume of distribution and adsorption by activated charcoal. This method can be used successfully with lipid-soluble compounds and with higher-molecular-weight compounds. Because of the more direct contact of the patient's blood with the adsorptive material, the medical risks of this procedure include thrombocytopenia, hypocalcemia, and leukopenia. This technique is primarily used for the treatment of serious toxic exposures to theophylline, colchicine, paraquat, meprobamate, and possibly *Amanita phalloides* toxin.

Hemofiltration for the treatment of poisoning has been suggested as an additional modality for enhancement of chemical elimination. During this procedure, the patient's blood is delivered through hollow fiber tubes and an ultrafiltrate of plasma is removed by hydrostatic pressure from the blood side of the membrane. Different membrane pore sizes are available for use so the size of the filtered molecules can be controlled during the procedure. The perfusion pressure for the technique is generated either by the patient's blood pressure (for arteriovenous hemofiltration) or by a blood pump (for venovenous hemofiltration). Fluid and electrolytes removed in the ultrafiltrate are replaced intravenously with sterile solutions.

The use of either plasma exchange or exchange transfusions is limited in clinical toxicology. The techniques afford the potential advantage of being able to remove high-molecular-weight and/or plasma protein–bound toxicants. Plasma exchange or plasma pheresis involves removal of plasma and replacement with frozen donor plasma, albumin, or both with IV fluid. The risks and complications of this technique include allergic-type reactions, infectious complications, and hypotension. Exchange transfusion involves replacement of a patient's blood volume with donor blood. The use of this technique in poison treatment is uncommon and mostly confined to the setting of inadvertent drug overdose in a neonate or premature infant.

Serial oral administration of activated charcoal, also referred to as multiple-dose activated charcoal (MDAC), has been shown to increase the systemic clearance of various drug substances by stimulating translumenal efflux of drug from blood to the charcoal passing through the gastrointestinal tract. MDAC may produce a further enhancement in clearance by interrupting the enteroenteric–enterohepatic circulation of drugs that undergo fecal elimination. The activated charcoal in the gut lumen serves as a "sink" for toxicants. A concentration gradient is maintained as the chemical passes continuously into the gut lumen, where it is adsorbed to charcoal. The characteristics of chemicals that favor enhanced elimination by MDAC include (1) significant enteroenteric–enterohepatic circulation, including active recirculating metabolites, (2) prolonged plasma half-life after an overdose, (3) small (<1.0 L/kg) volume of distribution, (4) limited (<60%) plasma protein binding, (5) a pK_a that maximizes transport of the drug across cell membranes, (6) sustained-release/resin-form tablets and/or capsules, and (7) onset of organ failure (e.g., kidney) that reduces capacity of the major route of elimination of the toxicant (for renally excreted chemicals) so that MDAC may make a considerable contribution to total body clearance. A list of chemicals for which MDAC has been shown as an effective means of enhanced body clearance is provided in Table 33–7.

TABLE 33–7 Drugs for Which Multiple Dose Activated Charcoal Has Been Shown Effective as a Treatment Modality for Poisoning

Carbamazepine	Nadolol
Dapsone	Phenobarbital
Digoxin	Salicylates
Digitoxin	Theophylline

Use of Antidotes in Poisoning

A relatively small number of specific antidotes are available for clinical use in the treatment of poisoning. The two primary reasons for the overall paucity of available specific antidotes are the practical difficulties in performing clinical research in poisoned patients, and the relatively small financial incentives for commercial development of poisoning antidotes.

The mechanism of action of various antidotes may differ. For example, a chelating agent for heavy metal poisoning or Fab fragments specific to digoxin or crotalid venom work by physically binding the toxicant, preventing the chemical from exerting a deleterious effect in vivo, and, in some cases, facilitating body clearance of the chemical. Other antidotes pharmacologically antagonize the effects of the toxicant at the endogenous receptor. Atropine, an antimuscarinic, anticholinergic drug is used to pharmacologically antagonize at the receptor level, the effects of organophosphate insecticides that produce cholinergic, muscarinic effects. Certain chemicals exert their antidote effects by chemically reacting with biological systems to increase the detoxifying capacity for the toxicant. For example, sodium nitrite is given to patients poisoned with cyanide to cause formation of methemoglobin, which serves as an alternative binding site for the cyanide ion. Chemicals, such as l-carnitine, mitigate the biochemical toxicity of high exposures to valproic acid (an antiseizure medication) at the level of the mitochondria.

An example of an efficacious antidote with an uncertain mechanism is intravenous lipid emulsion (ILE) therapy. ILE as a product has been in clinical use for decades as a nutritional supplement for seriously ill patients who were not able to receive nutrition via their gastrointestinal tract. As an antidote, the 20% lipid emulsion solution is most often employed.

Supportive Care of the Poisoned Patient

The supportive care phase of poison treatment is very important. Poisoned patients who are unstable or at risk for significant clinical instability during the later phases of their poisoning are generally admitted to a critical care unit for close monitoring. Not only are there certain poisonings that have delayed toxicity, such as acetaminophen, paraquat, and diphenoxylate, but there are toxicants that exhibit multiple phases of toxicity that include delayed effects (i.e., ethylene glycol, salicylate, and buspirone). Close clinical monitoring can detect later phase poisoning complications and allow for prompt medical intervention to minimize patient morbidity and mortality.

Another important component of the supportive care phase of poison treatment is the psychiatric assessment. For intentional self-poisonings, a formal psychiatric evaluation of the patient should be performed prior to patient discharge. Generally, a patient who has attempted suicide should be constantly observed until they have been evaluated by the psychiatric consultant and judged to be at low risk for being without constant surveillance.

CASE EXAMPLES OF SPECIFIC POISONINGS

Acetaminophen

A 16-year-old female patient arrives in the ED by ambulance after being found by a parent in what appeared to be an intoxicated state with empty pill bottles scattered about her room. The parent reports the patient was despondent recently after breaking up with her boyfriend. The patient is tearful and reports abdominal pain and admits to drinking alcohol and taking over-the-counter (OTC) pills in an apparent suicide attempt. The estimated time of ingestion is 6 hours prior to arrival in the ED. The patient does not use prescriptions, OTC medications, or dietary supplements on a regular basis and is not known to have a history of regular consumption of alcoholic beverages or use illicit drugs. In addition, the patient did not have a history of psychiatric disorders or previous suicide attempt.

On physical examination, the vital signs were blood pressure 118/80 mm Hg, pulse 88/min and regular, respiratory rate 18/min, and temperature 37.0°C. She was awake and oriented to person, place, and time, and responded to questions appropriately with slightly slurred speech. Other pertinent findings included normal bowel sounds with mild epigastric tenderness. The neurological examination was only significant for slightly slurred speech.

Clinical laboratory studies were ordered STAT and included electrolytes, creatinine, BUN, glucose, complete blood count with differential, coagulation studies, urine analysis, urine toxicology screen, an ethanol level, salicylate level, and a plasma acetaminophen level. Chest and abdominal radiography were normal.

The patient was given 1.5 g/kg oral activated charcoal as a slurry in a sorbitol cathartic and placed under the intensive monitoring while the laboratory tests were being performed. Forty minutes later, the laboratory results returned and showed a mildly increased white blood cell count, liver transaminase values were elevated at approximately three times the upper limit of normal, and an acetaminophen concentration was 308 μg/mL. Salicylate was not detected; ethanol level was 89 mg/dL. She denied taking other medications with the acetaminophen and alcohol. Based on the Rumack–Matthew nomogram, a plasma acetaminophen concentration of 308 μg/mL at approximately 6 hours after ingestion is within the “probable hepatic toxicity” range, and therefore treatment with *N*-acetylcysteine (NAC) was required.

The patient received the first dose of IV NAC in the ED and was admitted to the medical ward to complete the treatment course of IV NAC. Hepatic transaminases remained elevated over the ensuing two days of the hospitalization, peaking at five times the upper limit of normal. The patient’s bilirubin remained within the normal range as did indices of synthetic hepatic function (coagulation pathway measurements). The psychiatry consultation service determined she was not actively suicidal; she was discharged from the hospital two days after admission with scheduled psychiatric and medical follow-up appointments.

The clinical presentation of patients poisoned with acetaminophen is sufficiently confusing in some cases; it is difficult to estimate the time of ingestion. Due to the paucity of clinical symptoms with acute overdose, most clinicians will request an acetaminophen concentration be measured for any patient suspected of having a toxic exposure to any substance because the paucity of signs and symptoms associated with an acetaminophen overdose makes inadvertent missing of a potentially fatal overdose until the window for maximum antidote effectiveness has

passed.

Acetaminophen in normal individuals is inactivated by sulfation and glucuronidation. About 2% of the drug is excreted unchanged. About 4% is biotransformed by CYP2E1 to a potentially toxic metabolite that is normally detoxified by conjugation with glutathione and excreted as the mercapturate. Patients who are concurrently using, or have recently used, chemicals that induce CYP2E1 may produce more than 4% of the toxic metabolite. When there is medical history of concurrent chemicals that induce CYP2E1, the treatment nomogram for acetaminophen does not directly apply, and interpretation by the treating physician must be modified to a lower threshold for treatment with NAC.

Follow-up liver biopsy studies of patients who have recovered three months to a year after hepatotoxicity have demonstrated no long-term sequelae or chronic toxicity. A very small percentage (0.25%) of patients may progress to hepatic encephalopathy with subsequent death. The clinical nature of the acetaminophen overdose is one of sharp peaks of serum aspartate transaminase (AST) by day 3, with recovery to less than 100 IU/L by day 7 or 8. Patients with AST levels as high as 20,000 IU/L have shown complete recovery and no sequelae one week after ingestion.

Laboratory evaluation of a potentially poisoned patient is crucial in terms of both hepatic measures of toxicity and plasma levels of acetaminophen. Accurate estimation of acetaminophen in the plasma should be done on samples drawn at least 4 hours after ingestion, when peak plasma concentrations are expected.

Ethylene Glycol

A 37-year-old female was brought to the ED after being found unresponsive in her home. The patient was comatose and unresponsive to pain and without obvious signs of trauma. At the scene, emergency medical personnel administered oxygen and naloxone, and performed a finger stick for glucose (standard procedure for encountering a person with altered mental status and suspected toxic ingestion), which showed a normal value of 95 mg/dL. The patient’s spouse reported that she had been depressed and despondent with the recent loss of her job. No empty pill bottles or liquid containers were found with her at home.

Upon arrival at the hospital, she remained comatose and had the following vital signs: blood pressure 105/65 mm Hg, pulse 78/min, respiratory rate elevated at 32/min, and her body temperature was normal. The remainder of the physical examination was significant as her pupils were 3 mm and sluggishly reactive to light; the lung and heart examinations were normal; the abdominal examination revealed diminished but present bowel sounds, no tenderness, organomegaly, or masses were detected. The rectal examination was normal; the stool was without detectable gross or occult blood. Neuro examination was nonfocal with a diminished gag reflex.

The patient was placed on a cardiac monitor, an IV line was started and clinical laboratory specimens were obtained, was placed on oxygen, and was given naloxone, thiamine, and one dose of dextrose (50%) intravenously. These treatments are standard for patients presenting with an altered mental status to an ED. Chest and abdominal radiography was without abnormality. A 12-lead ECG was also normal. Faced with the uncertainty of oral ingestion versus topical and inhalation exposure, a decision was made to proceed with gastric decontamination. The patient was endotracheally intubated to protect her airway before an orogastric tube was placed. Gastric lavage was performed and no blood or pill fragments were found. The gastric lavage fluid

withdrawn from the stomach was bright yellow in appearance and slightly viscous. When a Wood's lamp illuminated this fluid in a darkened room, fluorescence was observed. This finding suggests the presence of automotive antifreeze that contains ethylene glycol. Activated charcoal (2.0 g/kg) was placed via the orogastric tube into the stomach with a cathartic even though the efficacy for binding ethylene glycol is limited; the use of activated charcoal here was intended for other, potentially unknown coingestants. Clinical laboratory results returned showing the following:

Serum Chemistries—The complete blood count was normal, the urine analysis was normal, measured serum osmolarity was 330 mOsm/kg, and acetaminophen and salicylate levels were below the limits of detection, and the urine toxicology screen was negative.

Na = 140 mEq/L	K = 3.1 mEq/L
Cl = 94 mEq/L	HCO_3 = 8 mEq/L
BUN = 12 mg/dL	Glucose = 100 mg/dL
Arterial blood gas:	
pH = 7.20; pCO_2 = 20 mm Hg; pO_2 = 98 mm Hg	

The laboratory results were interpreted as follows: a metabolic acidosis with elevated AG (AG = 38; normal < 12) and an elevated osmol gap (40 mOsm; normal < 10 mOsm). These findings are consistent with either methanol or ethylene glycol poisoning (Tables 33–4 and 33–5). A blood sample for measurement of methanol and ethylene glycol was sent for analysis but based on the history and finding of fluorescent, yellow fluid in the stomach and the acid–base disorder detected, the working diagnosis of ethylene glycol poisoning was established. The patient was treated with IV fomepizole (4-methylpyrazole) to inhibit alcohol dehydrogenase and IV sodium bicarbonate for the profound metabolic acidosis and the patient was transferred to the regional medical center where she underwent hemodialysis shortly after arrival. After 4 hours of hemodialysis, the acid–base and electrolyte abnormalities were corrected but the patient remained comatose. Approximately 9 hours after the blood specimen was sent from the first hospital, the laboratory reported a "toxic" serum ethylene glycol concentration of 366 mg/dL. The patient underwent a second 4-hour course of hemodialysis 8 hours following the initial hemodialysis treatment to again correct a recurrence of her metabolic acidosis with the appearance of minor renal injury (serum creatinine increased to 1.8 mg/dL). She regained normal consciousness approximately 18 hours after arrival at the second hospital; her renal function recovered completely within 5 days. Subsequently, the patient admitted that she intentionally drank more than half a container of antifreeze with the intent of harming herself. She was evaluated by the psychiatry consultation service and transferred to their service for further care.

This case demonstrates the importance of utilizing the anion and osmol gap calculations in overdose patients as well as all available diagnostic tools (e.g., the Wood lamp) during the initial evaluation of a potentially poisoned patient. Without a readily available drug such as fomepizole (or sterile IV ethanol as a substitute for fomepizole to reduce metabolism by alcohol dehydrogenase) and the ability to rapidly transfer to a facility with definitive care capability (hemodialysis), this patient would have likely succumbed to her poisoning. Initiation of antidote therapy early and providing hemodialysis to increase removal of the poison prior to receipt of the

confirmatory laboratory test (measured serum concentration of the specific poison—ethylene glycol) was also required. Although not considered diagnostic, the presence of both a metabolic acidosis with an AG and an osmol gap is highly suggestive of either methanol or ethylene glycol given the patient's presentation despite a scant history. It is commonplace for hospital clinical laboratories to have to "send out" blood specimens for ethylene glycol and methanol analysis and a turnaround time of 6 to 12 hours is typical in most settings.

Ethylene glycol exerts its primary toxicity after undergoing biotransformation by alcohol dehydrogenase to glycolic acid and then to glyoxylic and oxalic acid by the action of aldehyde dehydrogenase. The latter two acid metabolites are thought to be responsible for both the renal and the acid–base toxicity observed during poisoning by ethylene glycol. If untreated or treated too late, ethylene glycol poisoning can result in fatal cerebral edema with seizures as well as irreversible renal damage. Hemodialysis can remove the ethylene glycol and its toxic metabolites. Administration of an inhibitor or competing substrate for alcohol dehydrogenase (fomepizole or 4-methylperazole) can be instituted before and during transport of the patient to a healthcare facility where hemodialysis is available. Ethanol (sterile, for IV administration) or oral, nonsterile ethanol (when the IV formulation is not readily available) can also be given to effectively inhibit the metabolism of methanol or ethylene glycol and prevent the potentially devastating effects of the poisoning. Many clinical toxicologists will include folic or folinic acid in their therapeutic regimen for methanol-poisoned patients. Poisoning with methanol or ethylene glycol is relatively common.

Valproic Acid

A 33-year-old male is brought to the ED after being found unresponsive by his brother at home with two empty prescription pill bottles of extended release valproic acid at his side. He was last seen 8 hours prior to being found unresponsive and was then in normal health. The patient's pharmacy confirmed that three monthly prescriptions, each containing 30, 250 mg extended release valproic acid tablets had been dispensed within the preceding 3 months.

The patient was unresponsive to verbal or tactile stimulation with vital signs: blood pressure 85/55 mm Hg, pulse 94/min, respiratory rate 20/min, and temperature 33.2°C. IV access was obtained and IV fluids were administered as rapidly as possible. Naloxone was administered without effect. The patient was placed on a cardiac monitor that showed sinus rhythm. The remainder of the physical examination showed the patient to be without obvious signs of trauma; the skin was cool and without track marks. The pupils were 2 mm and poorly reactive to light. Other significant findings included the examination of the abdomen, which showed diminished bowel sounds. The rectal examination was negative for occult blood. The neurological examination revealed coma without focal motor abnormalities and an absent gag reflex.

Initial clinical laboratory results showed mild metabolic acidosis with elevated serum lactate, slightly increased serum ammonia, normal glucose, liver function tests, and renal function tests. The chest and abdominal radiographs were normal. The 12-lead ECG showed a prolonged QT interval without arrhythmia. The patient was endotracheally intubated to protect his airway prior to gastric lavage that yielded some pill fragments only. The patient was placed on a ventilator to support his respiration. Activated charcoal (1.5 g/kg) was administered via the orogastric tube immediately following the lavage procedure. The blood pressure continued to remain low despite IV fluid administration. A STAT valproic acid serum measurement showed the concentration was 572 mcg/mL.

Blood pressure responded to low-dose vasopressors (IV dopamine) with continued IV fluid administration. A repeat serum valproic acid concentration was 890 mcg/mL at 2 hours post admission. Serial oral activated charcoal (every 4 hours) was initiated via the orogastric tube and hemodialysis was started 3 hours after admission. IV l-carnitine was given when a repeat serum ammonia concentration returned further elevated at 94 mg/dL. Subsequent measured plasma concentrations of vaproic acid gradually declined to <100 mcg/mL over the next 48 hours after one additional hemodialysis session was conducted. The patient regained consciousness 24 hours after admission and made a full recovery by hospital day 4. The psychiatry consult service accepted the patient in transfer to their inpatient service after he was medically cleared by the toxicology service.

This case illustrates the significance of increasing plasma concentrations of the toxic substance despite gastric decontamination procedures. The most likely cause for this observation is the ingestion of an extended release formulation, which is designed to slowly dissolve in the gastrointestinal tract and provide for ongoing sustained release of the active drug. Some drugs can demonstrate the “slow release” profile without having been formulated in a special sustained release dosage form. Examples of these drug “bezoars” include salicylates, barbiturates, and some formulations of iron supplements. The presence of a drug bezoar or concretion can be dangerous because, without serial drug concentration measurements, the treating team could erroneously stratify a patient based on the initial measured plasma concentration and be unprepared for severe toxicity or prolonged toxicity time course.

The depletion of l-carnitine is thought to be causative for the hyperammonemia often observed with valproic acid intoxication. The FDA has approved the use of IV l-carnitine for the treatment of valproic acid poisoning in the setting of hepatotoxicity, hyperammonemia, large overdoses of valproate by history, or measured serum concentrations of valproic acid exceeding 450 mcg/mL.

Finally, the benefit of employing multiple methods simultaneously to enhance the elimination of a toxicant is also demonstrated in this case example. Total body clearance of valproic acid is enhanced when serial oral activated charcoal is administered. Hemoperfusion, or the more available method of hemodialysis, has been shown to increase the total body clearance of valproic acid in overdose. Both modalities were likely beneficial in this case.

Salicylate

A 48-year-old female is brought to the ED approximately 2 hours after a witnessed ingestion of approximately 35 aspirin tablets (each 325 mg) at home in an apparent suicide attempt. The patient complained of upper abdominal discomfort, nausea, and tinnitus and reported one episode of spontaneous emesis at home before transport. The patient has a significant past medical history for major depression disorder, mild osteoarthritis, and hypothyroidism. Current medications include sertraline, 50 mg per day, and levothyroxine, 100 mcg per day. The patient has been a heavy cigarette smoker (1.5 packs per day) for at least 20 years. No other information about potential illicit substance or alcohol use is available.

Upon arrival in the ED, the vital signs were blood pressure 130/74 mm Hg, pulse 113/min, respiratory rate 28/min, and body temperature was 38.0°C. She was awake, but oriented only to person. The remainder of the physical examination was significant for slightly dilated pupils (4 mm with normal reactivity to light); an increased heart rate and a normal lung exam; the abdominal examination revealed normal bowel sounds and slight epigastric tenderness; no

organomegaly or masses were detected. The rectal examination was normal; the stool was without detectable gross or occult blood. Neuro examination was altered mental status, non-focal with a diminished gag reflex.

Clinical laboratory analysis upon arrival in the ED found the following abnormalities: white blood cell count mildly elevated, serum potassium was slightly low at 3.1 mEq/L, arterial pH 7.15, and serum bicarbonate 11 mEq/L, and anion gap calculation was 29 (normal < 12). At 2.5 hours after the reported ingestion time, salicylate was 36 mg/dL (therapeutic concentrations 10 to 30 mg/dL), acetaminophen was not detected, serum creatinine 1.9 mg/dL, the urine pH was 5.0, and the urine toxicology screen was negative. Chest and abdominal radiographs were normal.

In the ED the patient received normal saline IV and two bolus dosages of 50 mEq sodium bicarbonate to treat her acidosis. IV anti-emetics were given for nausea. At 6 hours post ingestion, the salicylate level was 51.4 mg/dL and no therapeutic action was taken. Five hours later (approximately 11 hours post ingestion) the patient became restless and displayed a tremor in her upper extremities and cramps in her legs. The vital signs at that time were blood pressure 150/105 mm Hg, pulse 144/min, respiratory rate 30/min, and body temperature 38.5°C. A salicylate level at that time was 81 mg/dL. Thirteen hours after presentation the patient became significantly agitated, the heart rate increased to 224/min. An arterial blood gas demonstrated pH 7.25, pCO_2 12 mm Hg, and calculated serum bicarbonate 6.6 mEq/L, indicating that the significant metabolic acidosis has continued. Endotracheal intubation and mechanical ventilation were initiated; hemodialysis was not performed on this patient. Following a dose of IV labetalol, given to control heart rate, the patient suffered a cardiac arrest and she could not be resuscitated.

This unfortunate case demonstrates several potential pitfalls in the management of serious salicylate ingestion. First, the witnessed dose ingested was 35 tablets, each containing 325 mg, or more than 11 g of salicylate, which by risk stratification and by estimated dose ingested should have alerted the ED staff that this ingestion was very likely to result in severe toxicity. The initial salicylate level was just above the upper limit of normal therapeutic values for salicylate. This lower than expected laboratory result may have steered the ED away from considering this to be a serious and potentially fatal ingestion and toward the obvious under-treatment that occurred. Gastric decontamination did not occur for unclear reasons. Perhaps the ED team assumed that the reported spontaneous emesis was sufficient to prevent significant absorption of the toxicant. Furthermore, the rising salicylate levels over time were apparently overlooked. This phenomenon is commonly seen with extended release formulations (not a factor in this case) or when there is the formation of a drug bezoar. Salicylates, in overdose, are well-known to form drug bezoars in the proximal gastrointestinal tract, which slowly dissolve over time, resulting in rising serum concentrations of salicylate over time. The result of formation of a drug bezoar is that, instead of rapid absorption and a relatively short apparent half-life, the ongoing absorption creates a prolonged and increasing systemic exposure profile for the drug being released from the bezoar. Adequate dosage of activated charcoal upon arrival in the ED, and perhaps use of multi-dose activated charcoal, would likely have reduced the amount of salicylate reaching the systemic circulation. The administration of two bolus doses of sodium bicarbonate was certainly inadequate to overcome the systemic acidosis observed and achieve the needed alkalinizing of the urine to enhance salicylate elimination. The patient should have received a continuous IV sodium bicarbonate infusion with tracking of her urine output and pH with a target pH of 8 for the urine. Involvement of nephrology consultants to initiate hemodialysis to facilitate salicylate elimination should have also taken place once rising plasma concentrations of salicylate, coincident with clinical deterioration, were apparent. In summary, this case reinforces the

importance of timely application of the basic, stepwise, procedures available to treat severe, poison-induced toxicity, and demonstrates the potential consequences of underestimating poisoning severity.

CONCLUSION

Clinical toxicology encompasses the expertise of the specialties of medical toxicology, applied toxicology, and clinical poison information specialists. Basic and clinical science provides new information allowing more effective diagnosis and treatment of the poisoned patient. The incorporation of evidence-based, outcome-driven practice recommendations has improved and standardized the evaluation of treatment modalities for poisonings. Application of a stepwise approach to the poisoned patient remains an essential approach to the effective evaluation and treatment of the poisoned patient. Skillful use of antidotes is an important component of the practice of medical toxicology. Continued research will increase the repertoire of effective treatments for poisoning and ultimately improve patient outcomes.

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QUESTIONS

1. What is the primary goal in taking a history in a poisoned patient?
   - **a.** determining drug allergies.
   - **b.** determining susceptibility to drug overdose.
   - **c.** determining likelihood of an attempted suicide.
   - **d.** determining the ingested substance.
   - **e.** determining the motive behind the poisoning.

2. Who is most likely to give incorrect information while taking a history of a poisoned patient?
   - **a.** patient.
   - **b.** EMT.
   - **c.** employer.
   - **d.** pharmacist.
   - **e.** family members.

3. Which of the following sets of clinical features characterizes an anticholinergic toxic syndrome?

a. increased blood pressure, decreased heart rate, decreased temperature.
b. decreased blood pressure, increased heart rate, decreased temperature.
c. increased blood pressure, increased heart rate, increased temperature.
d. decreased blood pressure, decreased heart rate, decreased temperature.
e. increased blood pressure, decreased heart rate, increased temperature.

4. Which of the following sets of clinical features characterizes a sympathomimetic toxic syndrome?
a. miosis, decreased bowel sounds, decreased alertness.
b. decreased heart rate, increased temperature, mydriasis.
c. hyperalertness, decreased blood pressure, miosis.
d. increased temperature, increased heart rate, miosis.
e. mydriasis, increased blood pressure, hyperalertness.

5. Which of the following drugs CANNOT be tested for in a hospital on a stat basis?
a. ethanol.
b. cocaine.
c. aspirin.
d. phenytoin.
e. digoxin.

6. Which is NOT included in the differential diagnosis of an elevated anion gap?
a. ethanol.
b. methanol.
c. diabetes.
d. ethylene glycol.
e. diarrhea.

7. An elevated osmol gap might suggest which of the following?
a. methanol poisoning.
b. chronic vomiting.
c. lactic acidosis.
d. diabetic ketoacidosis.
e. chronic diarrhea.

8. Which of the following is LEAST likely to prevent further poison absorption?
a. induction of emesis.
b. activated charcoal.
c. gastric lavage.
d. syrup of ipecac.
e. parasympathetic agonist.

9. Which of the following would NOT be used to enhance poison elimination?
a. oral activated charcoal.

b. hemoperfusion.
c. acidification of urine.
d. hemodialysis.
e. plasma exchange.

10. Which of the following might be used as an antidote for patients with cyanide poisoning?
a. syrup of ipecac.
b. atropine.
c. chelating agents.
d. sodium nitrite.
e. quinine.

*Deceased

CHAPTER 34

Occupational Toxicology

Peter S. Thorne

KEY POINTS

- Occupational toxicology is the application of the principles and methodology of toxicology toward chemical and biological hazards encountered at work.
- In occupational environments, exposure is often used as a surrogate for dose.
- Occupational exposure limits do not correspond to the level of exposure below which the probability of impairing the health of the exposed workers is acceptable.
- Diseases arising in occupational environments involve exposure primarily through inhalation, ingestion, or dermal absorption.

INTRODUCTION

Occupational toxicology applies the principles and methodology of toxicology toward understanding and managing chemical and biological hazards encountered at work. As new hazards arise with the emergence of new technologies, occupational toxicologists must be prepared to assess the risks and protect the health of workers. Because the work environment can present exposures to complex mixtures, the occupational toxicologist must also recognize those that are particularly hazardous when occurring in combination.

It is often difficult to establish a causal link between a worker's illness and job. First, the clinical manifestations of occupationally induced diseases are often indistinguishable from those arising from nonoccupational causes. Second, there may be a protracted but biologically predictable latent interval between exposure and the expression of disease. Third, diseases of occupational origin may be multifactorial with other environmental factors or personal risk factors contributing to the disease process. Nevertheless, studies have repeatedly shown that the dose of toxicant is a strong predictor of the likelihood, severity, and type of health effect.

WORKPLACES, EXPOSURES, AND STANDARDS

The Nature of the Workforce

The world's labor force totals 3.45 billion people, with approximately 31.3% engaged in agricultural production. In industrialized nations, workers have transitioned from jobs in heavy industry and agriculture toward jobs in the service sector and high-technology industries. Some manufacturing jobs have moved to less developed countries with less stringent worker health protection. The presence of children in the workforce has important ramifications on body burdens, disease latency, toxicokinetics, and biotransformation of toxicants.

Determinants of Dose

Dose is typically defined as the amount of toxicant that reaches a target tissue over a defined time span. In occupational environments, *exposure* is often used as a proxy for *dose*. The response to a chemical depends on host factors and dose. Figure 34–1 illustrates the pathway from exposure to subclinical disease or to adverse health effect and that shows there are important modifying factors (contemporaneous exposures, genetic and epigenetic susceptibility, age, gender, nutritional status, and behavioral factors) that can influence whether a worker remains healthy, develops subclinical disease that is repaired, or progresses to frank illness. Also, dose is a function of exposure concentration, exposure duration, and exposure frequency. Workplace health protection and surveillance programs (shown in blue) can reduce exposures, disrupt the exposure-dose pathway, or identify internalized dose and early effects before irreparable disease develops, thereby ensuring a safe workplace and a healthier workforce.

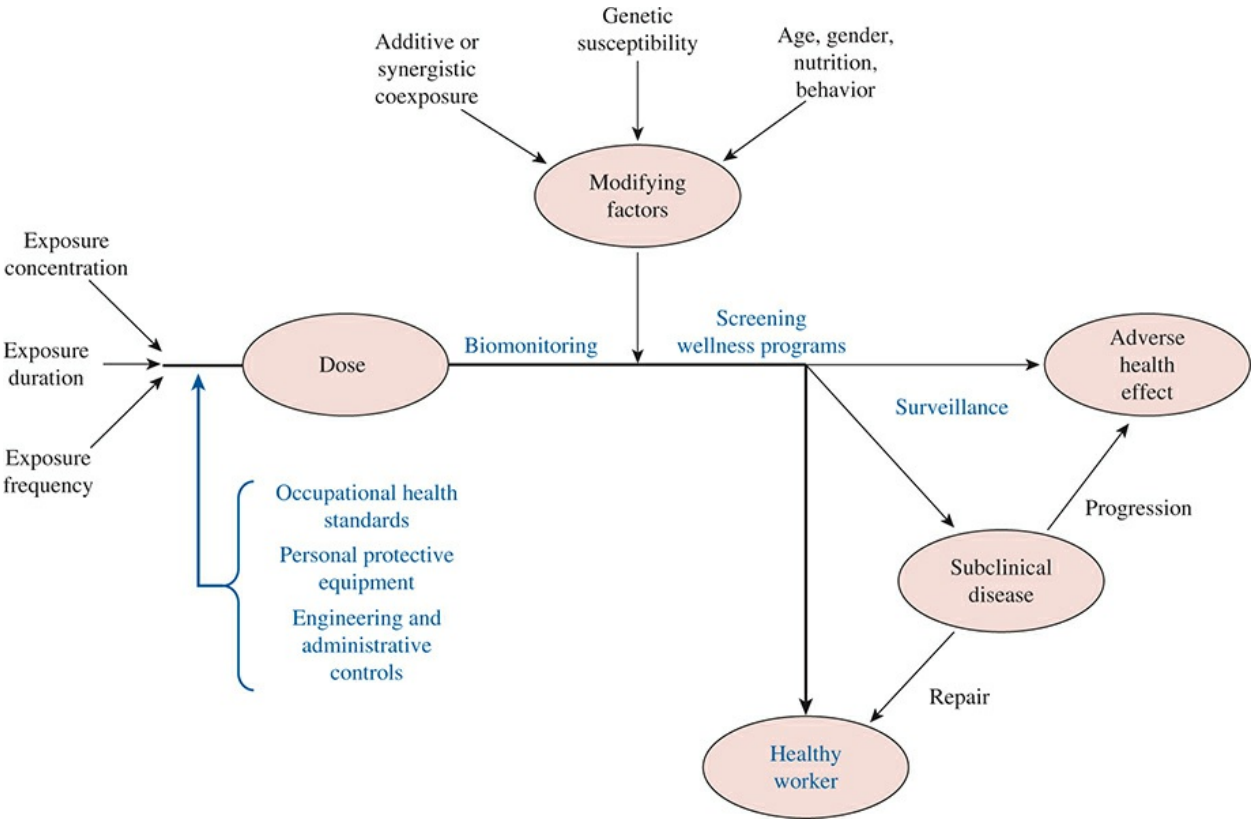

FIGURE 34–1 *Pathway from exposure to disease, showing modifying factors and opportunities for intervention.*

Table 34–1 indicates determinants of dose for exposure via the inhalation and dermal routes. Protection afforded by personal protective equipment (especially respirators) will reduce but not eliminate exposure. For maximal protection, it is important that the glove be constructed of material tailored to the toxicant(s) of concern.

TABLE 34–1 Determinants of Toxicant Dose

Inhalation exposure
Airborne concentration
Particle size distribution
Respiratory rate
Tidal volume
Other host factors
Duration of exposure
Chemical, physical, or biological properties of the hazardous agent
Effectiveness of personal protective devices
Dermal exposure
Concentration in air, droplets, or solutions
Degree and duration of wetness
Integrity of skin
Percutaneous absorption rate
Region of skin exposed
Surface area exposed
Preexisting skin disease
Temperature in the workplace
Vehicle for the toxicant
Presence of other chemicals on skin

Occupational Exposure Limits

Workplace exposure limits for chemical, biological, and physical agents are recommended as guidelines or promulgated as standards in order to promote worker health and safety. For chemical and biological agents, exposure limits are expressed as acceptable ambient concentration levels (occupational exposure limits, OELs) or as concentrations of a toxicant, its metabolites, or a specific marker of its effects in biological systems (biological exposure indices, BEIs).

OELs are established as standards by regulatory agencies or as guidelines by research groups or professional organizations. In the United States, the Occupational Safety and Health Administration (OSHA) under the Department of Labor promulgates legally enforceable standards known as permissible exposure limits (PELs). These standards are supported by the best scientific evidence available and assure "to the extent feasible … that no employee will suffer material impairment of health or functional capacity" with regular exposure during his working life. The National Institute for Occupational Safety and Health (NIOSH), under the Centers for Disease Control and Prevention, publishes recommended exposure limits (RELs) that are more frequently updated and are generally more stringent than PELs. The European Commission has established legally enforceable binding occupational exposure limit values

(BOELV) and biological limit values (BLV) for the protection of health and safety in the workplace.

The American Conference of Governmental Industrial Hygienists (ACGIH) is a non-profit scientific association that publishes OELs for chemicals and for physical agents. These take the form of threshold limit values (TLVs™) and biological exposure indices (BEIs™). They generally reflect current knowledge in occupational toxicology and industrial hygiene. They are developed as guidelines and are not enforceable standards.

Three types of TLVs are suggested, depending on the time scale of adverse effects inducible by the toxicants. The time-weighted average TLV (TLV-TWA) is an OEL for exposures averaged over an 8-hour day and a 5-day work week regimen. These are generally applied to toxicants that exert their effects over long periods. The short-term exposure limit (TLV-STEL) is an OEL for a 15-minute measurement period. The ceiling limit (TLV-C) represents a concentration that should never be exceeded. These are usually applied to toxicants that cause acute effects (such as asphyxia or potent sensory irritation) and for which real-time monitoring devices are available.

The concept of acceptable exposure level must be understood as the level of exposure below which the probability of impairing the health of the exposed workers is acceptable. The process of deciding what is an acceptable risk to occupational or environmental hazards blends the scientific disciplines of exposure assessment and toxicology with often vexing policy issues. To determine that the risks from an occupational hazard are acceptable, it is necessary to characterize the hazard, identify the potential diseases or adverse outcomes, and establish the relationship between exposure intensity or dose and the adverse health effects.

OCCUPATIONAL DISEASES

Routes of Exposure

Diseases arising in occupational environments involve exposure primarily through inhalation, ingestion, or dermal absorption. Inhalation exposures can occur with gases, vapors, liquid aerosols, particulate aerosols, fumes, and mixtures of these. Dermal exposures can arise from airborne materials as well as liquids splashed onto the skin, immersion exposures, or from material handling. Exposure to infectious agents may arise through inhalation or ingestion of microorganisms, needle-sticks in health-care workers or insect bites among those employed out of doors. Poisonings from toxic plants or venomous animals can occur through skin inoculation (e.g., zookeepers, horticulturists, or commercial divers).

Agents Associated with Diseases

Myriad agents are responsible for occupational diseases, a few are listed in Table 34–2.

Table 34–3 lists 39 known human carcinogens for which there has been extensive occupational exposure.

TABLE 34–2 Examples of Occupational Diseases and the Toxicants That Cause Them

Organ System or Disease Group	Disease	Causative Agent
Lung and airways	Acute pulmonary edema, bronchiolitis obliterans	Nitrogen oxides, phosgene, diacetyl
	Allergic rhinitis	Pollens, fungal spores
	Asphyxiation	Carbon monoxide, hydrogen cyanide, inert gas dilution
	Asthma	Toluene diisocyanate, α-amylase, animal urine proteins
	Asthma-like syndrome	Swine barn environments, cotton dust, bioaerosols
	Bronchitis, pneumonitis	Arsenic, chlorine
	Chronic bronchitis	Cotton dust, grain dust, welding fumes
	Emphysema	Coal dust, cigarette smoke
	Fibrotic lung disease	Silica, asbestos
	Hypersensitivity pneumonitis	Thermophilic bacteria, avian proteins, pyrethrum, *Penicillium, Aspergillus*
	Metal fume fever	Zinc, copper, magnesium
	Mucous membrane irritation	Hydrogen chloride, swine barn environments
	Organic dust toxic syndrome	"Moldy" silage, endotoxin
	Reactive airways dysfunction syndrome	Ammonia, chlorine, acetic acid
	Upper respiratory tract inflammation	Endotoxin, peptidoglycan, glucans, viruses
Cancer	Acute myelogenous leukemia	Benzene, ethylene oxide
	Bladder cancer	Benzidine, 2-naphthylamine, 4-biphenylamine
	Gastrointestinal cancers	Asbestos
	Hepatic hemangiosarcoma	Vinyl chloride
	Hepatocellular carcinoma	Aflatoxin, hepatitis B virus
	Mesothelioma, lung carcinoma	Asbestos, arsenic, radon, bis-chloromethyl ether
	Skin cancer	Polycyclic aromatic hydrocarbons, ultraviolet irradiation
Skin	Allergic contact dermatitis	Natural rubber latex, isothiazolins, poison ivy, nickel
	Chemical burns	Sodium hydroxide, hydrogen fluoride
	Chloracne	TCDD†, polychlorinated biphenyls
	Irritant dermatitis	Sodium dodecyl sulfate
Nervous system	Cholinesterase inhibition	Organophosphate insecticides
	Neuronopathy	Methyl mercury
	Parkinsonism	Pesticides, polychlorinated biphenyls, trichloroethylene, carbon disulfide
	Peripheral neuropathy	*N*-hexane, trichloroethylene, acrylamide
Immune system	Autoimmune disease	Vinyl chloride, silica
	Hypersensitivity	See entries for allergic rhinitis, asthma, hypersensitivity pneumonitis, allergic contact dermatitis
	Immunosuppression	TCDD†, lead, mercury, pesticides
Renal disease	Indirect renal failure	Arsine, phosphine, trinitrophenol
	Nephropathy	Paraquat, 1,4-dichlorobenzene, mercuric chloride
Cardiovascular disease	Arrhythmias	Acetone, toluene, methylene chloride, trichloroethylene
	Atherosclerosis	Dinitrotoluene, carbon monoxide
	Coronary artery disease	Carbon disulfide
	Cor pulmonale	Beryllium
	Systemic hypotension	Nitroglycerine, ethylene glycol dinitrate
Liver disease	Fatty liver (steatosis)	Carbon tetrachloride, toluene
	Cirrhosis	Arsenic, trichloroethylene
	Hepatocellular death	Dimethylformamide, TCDD†
Reproductive system	Male	Chlordecone (Kepone), dibromochloropropane, hexane
	Female	Aniline, styrene
	Both sexes	Carbon disulfide, lead, vinyl chloride
Infectious diseases	Arboviral encephalitides	Alphavirus, Bunyavirus, Flavivirus
	Aspergillosis	*Aspergillus niger, A. fumigatus, A. flavus*
	Cryptosporidiosis	*Cryptosporidium parvum*
	Hepatitis B	Hepatitis B virus
	Histoplasmosis	*Histoplasma capsulatum*
	Legionellosis	*Legionella pneumophila*
	Lyme disease	*Borrelia burgdorferi*
	Psittacosis	*Chlamydia psittaci*
	Tuberculosis	*Mycobacterium tuberculosis hominis*

†*TCDD = 2,3,7,8-tetrachlorodibenzo-para-dioxin.*

TABLE 34–3 Occupational Exposure Agents Classified by IARC as Group 1 Definite

Human Carcinogens

Agent	Industries and Occupations Where Some Workers May Be Exposed
Particulate matter	
Asbestos	Miners, abatement workers, construction workers, sheet metal workers, steam fitters, shipyard workers
Crystalline silica (quartz or cristobalite)	Stone and ceramics industry, foundries, construction, abrasives manufacturing
Erionite	Waste treatment workers, building materials manufacturing
Talc containing asbestiform fibers	Ceramics industry
Wood dust	Wood and wood-products industries, pulp and paper industry, wood working trades
Metals	
Arsenic and arsenic compounds	Miners, nonferrous metal smelting, arsenical pesticide manufacturers and applicators
Beryllium	Specialty metallurgy workers, avionics, electronics, nuclear industry
Cadmium and cadmium compounds	Cadmium smelting, battery production, dyes and pigment making, electroplating
Gallium arsenide	Microelectronics manufacturing
Hexavalent chromium compounds	Chromate production plants, dye and pigment making, welders, tanners
Nickel compounds*	Nickel smelting, welding
Organic chemicals	
Aflatoxins	Animal feed industry, grain handling, and processing
4-Aminobiphenyl	Chemical industry, dyestuffs, and pigment manufacturing
Benzene	Refineries, shoe industry, chemical, pharmaceutical and rubber industry, printing industry
Benzidine	Chemical industry, dyestuffs, and pigment manufacturing
Benzo(*a*)pyrene	Coke oven emissions, coal tar pitch volatiles, diesel exhaust, ETS
Bis(chloromethyl) ether and chloromethyl ether (technical grade)	Chemical industry, laboratory reagent, plastic manufacturing
1,3-Butadiene	Chemical industry, petrochemical plants, styrene–butadiene rubber manufacturing
Coal tars and pitches	Coke production, coal gasification, refineries, foundries, road paving, hot tar roofing
Ethylene oxide	Chemical industry, dry vegetable fumigation, hospital sterilizing
Formaldehyde	Textiles, composite wood industry, chemical industry, medical laboratories
4,4′-Methylenebis(2-chloroaniline) [MOCA]	Epoxy resin manufacturing, polyurethane product fabrication
Mineral oils, untreated and mildly treated	Metal machining and honing, roll steel production, printing
2-Naphthylamine	Chemical industry, dyestuffs and pigment manufacturing
2,3,4,7,8-Pentachlorodibenzofuran	Hazardous waste processing, chlorophenoxy herbicide production and use, pulp and paper industry
3,4,5,3′,4′-Pentachlorobiphenyl‡ [PCB126]	Hazardous waste processing, waterway dredging, transformer handling, pulp and paper industry
Shale oils or shale-derived lubricants	Mining and processing, cotton textile industry
Soot	Chimney sweeps, heating and ventilation contractors, firefighters, metallurgical workers
2,3,7,8-Tetrachlorodibenzo-*para*-dioxin (TCDD)	Hazardous waste processing, chlorophenoxy herbicide production and use, pulp and paper industry
Vinyl chloride	Plastics industry, production of polyvinyl chloride products and copolymers
Other agents with occupational exposure	
Environmental tobacco smoke (ETS)	Restaurant, bar, and entertainment industry; other smoke-exposed workers
Occupational exposures as a painter	Commercial painting
Leather dust	Garment industry, auto seat fabrication, saddle, and tack manufacturing
Magenta dye (rosaniline, pararosaniline)	Dye manufacture, textile dying, commercial art, and printing
Mustard gas	Production, soldiers, some research laboratories
Exposures in the rubber industry	Work in rubber manufacturing industries
Strong inorganic acid mists containing sulfuric acid	Steel industry, petrochemical industry, fertilizer industry, pickling industry
Physical agents	
Ionizing radiation†	Radiology and nuclear medicine staff, nuclear workers, miners, hazardous waste workers
Solar radiation	Farmers, gardeners and landscapers, lifeguards, construction workers

*Certain combinations of nickel oxides and sulfides.
†Includes x-rays, γ-rays, neutrons, radon gas, and α and β particle-emitting substances internally deposited.
‡PCBs as a class are listed by IARC as Group 1, carcinogenic to humans as of 2016.
Data from IARC. *Monographs on the Evaluation of Carcinogenic Risks to Humans*. n.d. Available at: http://monographs.iarc.fr/ENG/Monographs/PDFs/index.php; Siemiatycki J, Richardson L, Straif K, et al. Listing occupational carcinogens. *Environ Health Perspect*. 2004;112(15):1447–1459; Cogliano VJ, Baan R, Straif K, et al. Preventable exposures associated with human cancers. *J Natl Cancer Inst*. 2011;103:1827–1839.

Occupational Respiratory Diseases

Diseases affecting the lung and airway are largely responsible for the creation of the occupational regulatory framework. Deaths due to asbestosis, coal workers' pneumoconiosis, silicosis, byssinosis, and occupational asthma continue to have significant associations with morbidity and mortality worldwide. Many of the diseases listed in Table 34–2 are known by other names that refer to a particular occupation or agent. One example is hypersensitivity pneumonitis, an allergic lung disease marked by interstitial lymphocytic pneumonitis and granulomatous lesions. Hypersensitivity pneumonitis is also known as extrinsic allergic alveolitis, farmer's lung disease, bagassosis (sugar cane), humidifier fever, Japanese summer house fever, pigeon breeder's lung, and maple bark stripper's lung, depending upon the occupational setting in which it arises. Although we often think of these as the same disease, it is important to recognize that the exposures and physiological responses they induce are complex and may differ in the manifestation of the disease.

Toxic gas injuries are often characterized by leakage of both fluid and osmotically active proteins from the vascular tissue into the interstitium and airways. The vapors of anhydrous ammonia combine with water in the eyes, sinuses, and upper airways and form ammonium hydroxide, quickly producing liquefaction necrosis. Chemicals with lower solubility, such as nitrogen dioxide, act more on the distal airways and alveoli and take longer to induce tissue damage.

Occupational asthma is characterized by variable airflow limitation and/or airway hyperresponsiveness and/or inflammation due to a particular occupational environment and not to stimuli encountered outside the workplace. Asthma prevalence is much higher for female than male workers and is highest in manufacturing of plastics and rubber, electrical equipment and appliances, chemicals, and transportation equipment. Asthma is prevalent among those engaged in health care and social assistance, education, and personal services. In retail, prevalence is highest among gasoline station workers and employees of furniture and home furnishings stores and businesses selling electronics and appliances.

In chemical-based industries, plastic and rubber polymer precursors, diisocyanates, reactive dyes, and acid anhydrides are recognized low-molecular-weight sensitizing compounds. Biocides and fungicides used in metal fabrication and machining, custodial services, lawn and turf growing, and agriculture are also chemicals associated with occupational asthma. Metals, including chromium, cobalt, nickel, platinum, and zinc, can induce sensitization and asthma. Enzymes include α-amylase among bakery workers and subtilisin used in laundry detergents.

Animal handlers, processors, and laboratory technicians who work with animals can become immunologically sensitized to urine or salivary proteins in many vertebrates; proteins in bat guano and bird droppings; animal dander; proteins in blood products; dust from horns, antlers, and tusks; or crustaceans shells. High rates of sensitization can occur in shellfish processors. Arthropods such as insect larvae, cockroaches, mites, or weevils are recognized inducers of work-related asthma. Plants and plant products (e.g., soy flour, spices, and coffee beans) can also cause asthma among workers. Exposure to fungi, especially of the genera *Aspergillus*, *Penicillium*, *Rhizopus*, *Mucor*, and *Paecilomyces*, is associated with allergic rhinitis and asthma. These are especially present in sawmills, woodchip handling, and composting facilities.

Proteins from the latex of the rubber tree, *Hevea braziliensis*, led to immunologic sensitization. Thirteen of these high-molecular-weight proteins have been characterized as allergens. People with latex allergy may also be sensitive to banana, avocado, chestnut, and kiwi

due to cross-reactivity with the latex allergen fragment hevein.

Agricultural workers exposed to grain dust, cotton dust, or atmospheres in swine or poultry confinement barns are at high risk for development of an asthma-like syndrome. This syndrome is an acute nonallergic airway response characterized by self-limited inflammation with neutrophilic infiltrates and increased pro-inflammatory cytokines and chemokines (e.g., TNFα, IL-6, and IL-8). Asthma-like syndrome includes cough, mild dyspnea, fever, malaise, and cross-shift declines in lung function. Endotoxin in combination with other inflammatory bioaerosols is the likely etiologic agent.

Occupational exposures occur in the manufacture of the nanomaterials and in their use in fabricating materials and consumer products. Exposures can also occur when nanomaterials are cut or shaped and when product waste is discarded. Inhaled nanomaterials may induce pulmonary toxicity or they can cause adverse effects in other tissues through adsorption and transport, generation of toxic substances by their dissolution or degradation, or by crossing key physiological barriers, or cell and nuclear membranes. Mechanisms of nanotoxicity include oxidative stress, inflammation, cytotoxicity, genotoxicity, and developmental toxicity.

Other Occupational Diseases

Occupational diseases of the skin commonly include irritant dermatitis and allergic contact dermatitis. Occupational toxicants may induce diseases in a variety of body sites distant from the lung or skin. These include tumors arising in the liver, bladder, gastrointestinal tract, or hematopoietic system. Nervous system damage can be central or peripheral, or both. It may be acute, as with some organophosphate exposures, or chronic, as with organomercury poisoning or acrylamide-induced neuropathy. Immune system injury may arise from the immunosuppressive effects of chemicals, such as dioxins or toxic metals, or hypersensitivity leading to respiratory or dermal allergy or systemic hypersensitivity reactions. Autoimmune syndromes have been associated with occupational exposures to crystalline silica and vinyl chloride.

Occupational diseases of the heart, liver, and reproductive systems impact workers. Exposures to infectious agents affect veterinarians, health-care workers, biomedical researchers, farmers and foresters. Occupational infectious diseases attributable to the clustering of people affect workers in such facilities as day care centers, schools, health-care settings, correctional facilities, dormitories, military barracks, or shelters for the homeless.

Both industrial and nonindustrial occupational environments may pose occupational hazards due to the presence of chemical or biological agents. Reports of work environments with ineffective ventilation or decreased ventilation rates and increased utilization of synthetic building materials have demonstrated a rise in complaints associated with building occupancy. Volatile and semivolatile chemicals are released from office materials, building materials, floor coverings, furniture, cleaning products, and microorganisms. In some cases, the occupied space of a building may be clean and dry, but local amplification sites for molds may develop in ventilation systems, utility closets, subfloors, or basements that serve as return air plenums, or in local sites of water damage. Airborne viruses, bacteria, and fungi are responsible for several building-related illnesses arising from organisms that are pathogenic to humans.

TOXICOLOGIC EVALUATION OF

OCCUPATIONAL AGENTS

Evaluation of Occupational Risks

To recommend an acceptable exposure level to an industrial chemical, the risk associated with adverse effects in the most sensitive exposed populations must be defined. It then remains to be determined what proportion of exposed subjects may still develop an adverse effect at the proposed acceptable exposure level. This acceptable risk level will vary according to a value judgment of the severity, permanence, and equity of the potential adverse effects and the characteristics of the most susceptible population.

Establishing Causality—In complex occupational environments, it may be difficult to establish a causal relationship between a toxic substance and a disease. Figure 34–2 provides a matrix to evaluate the weight-of-evidence for a causal association between a toxicant and an occupational disease. Evidence from well-conducted in vitro studies, animal toxicology studies, controlled human challenge studies, case reports, and epidemiologic investigations is evaluated regarding data quality and clarity of evidence in support of the establishment of causality. If a chemical were thoroughly studied in animals, humans, and in vitro studies and it produced clear and convincing evidence of an exposure–response relationship in controlled studies that used appropriate models and relevant endpoints, it would constitute compelling evidence of a causal relationship between that chemical and that disease.

	Assessment of exposure to specific agents	Consideration or control of confounders	Evidence of a dose – response relationship	Consistent results from different studies	Objective clinical data	Endpoints related to human pathology	Appropriate subjects or models
In vitro studies							
Animal studies							
Human challenge studies							
Case studies							
Epidemiology studies							

For each type of study listed in the first column weight the quality of data from existing studies based on the criteria listed in the column headings as follows:

0 No evidence or condition is not met
1 Equivocal evidence or condition is partially met
2 Some evidence or condition is mostly met
3 Clear evidence or condition is convincingly met
4 Squares shown in pink should be left blank

FIGURE 34–2 *Matrix for assessing the strength of an association between a toxicant and an occupational disease.*

In Vitro Assays—Useful in vitro assays have been developed to provide screening data and, in some cases, mechanistic insight without the need or expense of exposing animal or human

subjects. There are recommended test method protocols for acute systemic toxicity, endocrine disruptors, eye corrosion and irritation, pyrogenicity, and skin sensitization. In addition, quantitative structure–activity relationships can help suggest potential toxicologic effects for an unstudied compound if structurally similar compounds have been evaluated.

Animal Toxicology Studies—Animal toxicology studies are important in identifying adverse effects, providing mechanistic data, establishing dose–response relationships, and aiding the process of establishing standards. Because animal studies can be conducted before human exposure, these studies play an important role in hazard identification and prevention of human disease. There are numerous animal models for occupational injury and illness; generation of animal toxicology data to predict health effects in workers is a central function of experimental toxicologists. Animal testing can provide only an estimate of the toxicity of a chemical for humans. Animals do not always respond to a chemical exposure in the same way as humans.

Human Challenge Studies—Controlled human challenge studies are used to verify findings from animal toxicology studies in humans and to establish whether biotransformation pathways in the animal models represent those in exposed humans. Human challenge studies with occupational toxicants are usually designed to answer specific questions regarding rates of uptake, biotransformation pathways, the time course of metabolite excretion during and after exposure, evaluation of the threshold concentration for sensory responses (odor, irritation of the nasal mucosa, etc.), and acute effects of toxicant exposure on perception, vigilance, and respiratory, cardiac, or neurobehavioral function. Human challenge studies help to establish biomarkers of exposure. They can be useful for testing therapeutic options. Extreme caution must be exercised to ensure the safety of research subjects, as the scientific benefits must be balanced against the risks to participants.

Case Reports—When new toxicants, new combinations of toxicants, or changes in process conditions occur in the workplace, a case or outbreak of cases can occur. These may be identified through workplace surveillance or workers associating their disease with workplace exposures. In some cases, the problem is identified quickly and resolved, while others take years to resolve. These are often published as case reports and may give rise to animal or epidemiologic studies. Though useful for hazard identification and mitigation, case reports generally do not establish incidence or prevalence of diseases associated with an occupational hazard.

Epidemiology Studies—Epidemiology studies help unravel associations between occupational diseases, exposures, and personal risk factors. Exposure may be characterized using a surrogate measure, such as job classification or via questionnaire, or more directly through exposure monitoring or biomonitoring. Adverse effects may be expressed in terms of mortality, incidence, or prevalence of clinical disease, irreversible or reversible functional changes, or critical biological changes.

Cross-sectional studies compare disease prevalence or health status between groups of workers classified according to job title, work site, or exposure status. Cohort studies compare exposed workers versus unexposed or less exposed workers either prospectively or retrospectively in order to associate the occurrence of disease with exposure. Case–control studies are useful for investigating rare diseases or diseases with long induction periods. Case–control studies compare workers with disease to workers without disease regarding their past exposure intensity, frequency, and duration, plus other postulated risk factors.

Characteristics of observational epidemiology studies are listed in Table 34–4. Occupational epidemiology studies assess relationships between exposures and human health outcomes and, therefore, are particularly useful for risk assessment. Confounding may arise due to exposures of risk factors not associated with the work environment.

TABLE 34–4 Comparison of Epidemiologic Studies and Experimental Exposure Studies

	Observational Epidemiologic Studies	Experimental Animal Exposure Studies
Toxicant Exposure	Reflects true exposure among population at risk	Controlled to represent major toxicant of interest
Character	Complex and variable in space and time May include nonoccupational exposures to toxicant or related compounds	Usually one or two test compounds May not reflect complexity of human exposures
Exposure route	Inhalation, ingestion, percutaneous, or a combination	Injection, inhalation, oral, or dermal; rarely a combination by design
Appropriateness of dose	Reflect the actual range of exposure	Often doses studied are far higher than human exposures
Assessment	Environmental sampling or measurement of biomarkers May be retrospective and based on employer records, group-based approaches, or questionnaires	Measurement of administered dose with or without measurement of biomarkers Sampling of exposure chamber air for inhalation studies
Species Considerations	Humans—cohorts or cases and controls Must protect the safety and confidentiality of subjects	Laboratory animals, usually inbred strains of mice or rats Must ensure proper care and use of animals
Representativeness	May exist a selection bias may exist such that the study population may not represent the occupational workforce	Experimental animal species may not represent humans
Relevance to human health	Directly relevant if appropriate outcomes are studied	Relevant if species differences are known Of limited relevance if species or strain effects on absorption, distribution, metabolism, and disease are unknown
Analytical Challenges	Selection bias, misclassification, and confounding in characterization of outcomes Within- and between-subject variance may be high	Control of genetics, feeding, and housing between exposed and control groups Low variance in outcomes

Because measures of effect may be subtle and may overlay a background level of incidence, results generally require sophisticated statistical comparisons between a group of exposed workers and a similar group of workers without the exposure of interest. Ideally, the group of unexposed workers should be matched on variables such as age, race, gender, socioeconomic status, and smoking habits. They should also undergo the same standardized clinical, biological, or physiological evaluation at the same time as the exposed group. Comparison with the general population is ill-advised because an employed population is a highly selected group and may have a higher degree of physical fitness, called the healthy worker effect.

Animal Toxicology Testing for Establishing Acceptable Levels of Exposure

Animal studies provide valuable data from which to estimate the level of exposure at which the risk of health impairment is acceptable. Table 34–4 presents comparision of the information gained from animal studies to epidemiology studies. To the extent possible, animal studies should employ species for which the metabolic pathways and disease processes reflect those of

humans. Tests include local and systemic acute toxicity tests, tests of toxicity following repeated exposure, investigations of metabolism and mechanism of action, short-term tests for detecting potential mutagens and carcinogens, studies of effect on reproduction and of teratogenic activity, chronic studies to detect carcinogenesis and other long-term effects, interaction studies, tests for immunosuppression, and dermal and pulmonary hypersensitivity tests. The need for performing these testing protocols should be carefully evaluated for the inclusion of any occupational toxicant to which workers will be exposed.

The duration of tests necessary to establish an acceptable level for occupational exposure is primarily a function of the type of toxic action suspected. It is generally recognized that, for systemically acting chemicals, subacute and short-term toxicity studies are usually insufficient for proposing OELs. Subacute and short-term toxicity tests are usually performed to find out whether the compound exhibits immunotoxic properties and cumulative characteristics. They also aid in the selection of the doses for long-term-exposures. Studies designed to evaluate reproductive effects and teratogenicity should also be considered during routine toxicologic testing of occupational toxicants.

Information derived from exposure routes like those experienced by workers is clearly the most relevant. Experimental methodology is much more complicated for inhalation studies than for oral administration experiments and requires more specialized equipment and expertise. Determination of what studies to perform and their routes of administration must be evaluated scientifically for each toxicant. Important considerations include its target sites and mechanism of action, metabolism, the nature of its adverse effects, and how workers are exposed to the toxicant. Investigations that can make use of specific physiological or biochemical tests, based on the knowledge of the principal target organ or function, produce highly valuable information and increase confidence in the derived OEL.

Worker Health Surveillance

The primary objective of worker health surveillance programs is to provide both periodic screening of general health and wellness plus health and exposure monitoring that is tailored to recognize hazards of the workplace. This workplace monitoring of exposures to toxicants may play an important role in detecting excessive exposures before the occurrence of significant biological disturbances and health impairment. When a new chemical is being used on a large scale, careful clinical surveillance of workers and monitoring of workplaces should be instituted.

Evaluation of the validity of the proposed OEL derived from animal experiments through workplace surveillance is the major aim because studies and observations on humans are the final basis for deciding whether an OEL set originally on the basis of animal toxicity testing is truly acceptable as one that will not produce excess health risks. Occupational toxicologists and occupational physicians cannot rely solely on the standard diagnostic tools used in clinical medicine, as they were established primarily to reveal advanced pathological states and not to detect early adverse effects at a stage when they are still reversible.

In cases where a surveillance program was not instituted before the introduction of a new chemical, it is more difficult to establish the efficacy of the OEL. In this situation, evaluation depends on retrospective cohort studies or case–control studies on workers who have already sustained exposure. Evaluation of a “no observed adverse effect level” (NOAEL) is difficult because information on past exposures is often incomplete and frank effects are generally the focus of retrospective or case–control studies. Careful investigation of overexposures resulting

from specific incidents, such as containment breaches, chemical spills, or vessel or pipe ruptures, can provide useful information. Such observations may indicate whether human symptomatology is similar to that found in animals and may suggest functional or biological tests that might prove useful for routine monitoring of exposed workers.

Linkage of Animal Studies and Epidemiologic Studies

In the field of occupational toxicology, cooperation between those conducting animal studies and studies of workers is helpful for examining risks associated with overexposure to chemicals and other toxicants. Several occupational carcinogens have been identified clearly through combined epidemiologic and experimental approaches. For example, the carcinogenicity of vinyl chloride was first demonstrated in rats, and a few years later, epidemiologic studies confirmed the same carcinogenic risk for humans. Several investigations on the metabolism of vinyl chloride in animals and on its mutagenic activity in in vitro systems led to the conclusion that microsomal oxidation forms an epoxide, 2-chloroethylene oxide, which rearranges to 2-chloroacetaldehyde and produces promutagenic etheno-DNA adducts including etheno-guanine, etheno-cytosine, and lesser amounts of etheno-adenine.

Studies of the metabolic handling of occupational toxicants in animals are instrumental in the characterization of reactive intermediates and may suggest unsuspected risks or indicate new methods of biological monitoring. Conversely, clinical observations of workers may stimulate studies of the metabolism or the mechanism of toxicity of a toxicant in animals, thereby revealing the health significance of a biological disturbance.

Arsenic is one of the very few compounds for which there are limited data of predictive value from animal studies to human health effects. Studies among occupationally exposed populations and populations with high arsenic in their drinking water have shown conclusively that arsenic causes human cancers of the skin, lung, bladder, kidney, liver, nasal tissue, and prostate. There is also evidence for arsenic-associated cutaneous effects, cardiovascular and cerebrovascular disease, diabetes mellitus, and adverse reproductive outcomes.

However, many carefully executed cancer bioassays in mice, rats, beagles, and monkeys performed using sodium arsenate, sodium arsenite, lead arsenite, arsenic trioxide, and DMA were uniformly negative for cancer. Subsequent studies that tested for tumor-promotion activity following dosing with recognized tumor initiators also yielded negative results. Some studies using organic arsenicals, susceptible transgenic mice, high dose in utero exposures, or administration of a co-carcinogen have yielded lung adenomas and adenocarcinomas in mice and rats. However, years of negative studies in standard animal carcinogenicity screens were problematic in the face of unquestionable oncogenic activity in humans. Thus, the occupational toxicologist cannot rely solely on animal or epidemiologic studies. A combined approach is necessary in order to identify, elucidate, and prioritize risks and to develop interventions and techniques for worker health surveillance.

EXPOSURE MONITORING

Environmental Monitoring for Exposure Assessment

An important objective of experimental and clinical investigations in occupational toxicology is the proposal of safe levels of occupational exposure. OELs must be reevaluated at regular intervals as new information on the toxicity of industrial chemicals develops. A critical element of establishing OELs is the accurate assessment of exposure. Methodology for exposure assessment must be specifically tailored to the agent under study and the environment in which it appears. To assess airborne exposures for compliance purposes, personal samples taken in the breathing zone are generally used. Repeated random sampling is theoretically the best approach to developing unbiased measures of exposure. Variability in exposure, especially variability over time, is often large. Approaches assessing exposures to groups rather than to individuals are more efficient in terms of measurement effort for obtaining a desired level of accuracy.

Although one cannot assess dose directly through exposure monitoring, it does have several distinct advantages over biomonitoring. Exposure monitoring allows one to quantify workplace exposure by route through selective air monitoring in the breathing zone of the worker and dermal dosimetry using absorptive material affixed to the workers' skin or clothing. Environmental monitoring techniques are generally less expensive and less invasive than techniques involving the collection and analysis of biological samples such as blood or urine. New personal sampling devices incorporate GPS, accelerometers, and smartphone technology to provide enhanced data on location and activity. Spatial, temporal, and work practice associations can be established and can suggest better interventions and engineering controls to reduce exposures. Finally, analytic interferences and variabilities are generally lower with environmental samples than with biological samples.

A fully validated sampling and analysis method requires specification of the sampling methods; sample duration, handling, and storage procedures; the analytic method and measurement technique; the range, precision, accuracy, bias, and limits of detection; quality assurance issues; and known interferences. Documentation of intralaboratory and interlaboratory variability is important. Once a standard method is established, it must be followed in every detail in order to assure consistency of results.

Biological Monitoring for Exposure Assessment

Biomonitoring consists of the measurement of toxicants, their metabolites, or molecular signatures of effect in specimens from humans or animals, including urine, blood, feces, exhaled breath, hair, finger or toenails, bronchial lavage, breast milk, and adipose tissue. These may serve as biomarkers of exposure, biological effect, or susceptibility. New technologies are emerging that will allow measurement and monitoring of chemicals in the body and transmission of the data from indwelling biosensors. Biomonitoring data provide a measurement of exposure based upon internalized dose and, thus, account for all exposures by all routes for the assessed analyte.

The term *internalized dose* may have different meanings. The measured biomarker may reflect the amount of chemical absorbed shortly before sample collection, as with the concentration of a solvent in exhaled air or in a blood sample obtained during the work shift. It may reflect exposure during the preceding day, as with the measurement of a metabolite in blood or urine collected after the end of exposure. For toxicants with a long biological half-life, the measured parameter may reflect exposure accumulated over a period of weeks or months as with arsenic in toenails. *Internal dose* may refer to the amount of chemical stored in one or in several body compartments or in the whole body (*the body burden*).

The greatest advantage of biological measurements is that the biological measure of exposure

is more directly related to the adverse health effects than environmental measurements because it reflects the amount of toxicant absorbed. Biological monitoring accounts for uptake by all exposure routes and may offer a better estimate of risk than that determined from ambient monitoring.

Several factors can influence uptake. Personal hygiene habits vary from one person to another, and there is some degree of individual variation in the absorption rate of a chemical through the lungs, skin, or gastrointestinal tract. Because of its ability to encompass and evaluate the overall exposure (whatever the route of entry), biological monitoring can also be used to test the overall efficacy of personal protective equipment such as respirators, gloves, barrier creams, or aprons. Also, nonoccupational exposures (through hobbies, residential exposures, dietary habits, smoking, and second jobs) may also be expressed in the biological sample. The organism integrates the total external (environmental and occupational) exposure into one internal load. Whereas this is beneficial for worker health and safety, it may be confounding in epidemiologic studies or compliance monitoring.

Relationships between air monitoring and biological monitoring may be modified by genetic or external factors that influence the fate of an occupational toxicant in vivo. Metabolic interactions can occur when workers are exposed simultaneously to chemicals that are biotransformed through identical pathways. Exposure to chemicals that modify the activity of the biotransformation enzymes (e.g., microsomal enzyme inducers or inhibitors) may also influence the fate of another compound. Furthermore, metabolic interferences may occur between occupational toxicants and alcohol, tobacco, food additives, prescription drugs, natural product remedies, or recreational drugs. Changes in any of several biological variables (weight, body mass index, pregnancy, diseases, immune status, etc.) may modify the metabolism of an occupational chemical. These factors must be taken into consideration when the results of biomonitoring are interpreted. Whatever the parameter measured, whether it is the substance itself, its metabolite, or an early biomarker of effect, the test must be sufficiently sensitive and specific to provide meaningful data in the range of workplace exposures.

Table 34–5 lists the approaches most useful for controlling inhalation exposures in the workplace. These include process changes, engineering controls, and use of personal protective equipment. It should be emphasized that process changes and application of engineering controls are preferable to reliance on personal protective equipment. In summary, environmental and biological monitoring should be integrated as much as possible to ensure low levels of contaminants and optimal health for workers.

TABLE 34–5 Control Approaches for Occupational Inhalation Hazards

Change the process to use or produce less hazardous compounds
Automate and enclose the process to isolate the compounds
Incorporate administrative and work practice controls to reduce duration or intensity of exposure
Install or upgrade local exhaust systems and dilution of exhaust
Institute a comprehensive program for personal protective equipment use where necessary

CONCLUSION

The working environment will always have the potential to overexpose workers to toxicants. Recognition of these risks should not wait until epidemiologic studies have uncovered hazardous levels. A combined experimental, clinical, and epidemiologic approach is most effective for evaluating and managing the potential risks. One can then promulgate scientifically based occupational health standards, apply effective workplace controls to ensure adherence to those standards, and institute worker health surveillance programs to identify unexpected effects in susceptible individuals.

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QUESTIONS

1. Which of the following is NOT a modifying factor that can influence the likelihood of disease?
   - **a.** age.
   - **b.** dose.
   - **c.** nutritional status.
   - **d.** gender.
   - **e.** genetic susceptibility.

2. Which of the following is LEAST likely to increase occupational inhalation of a chemical?
   - **a.** increased airborne concentration.
   - **b.** increased respiratory rate.
   - **c.** increased tidal volume.
   - **d.** increased particle size.
   - **e.** increased length of exposure.

3. Which would increase the likelihood of toxic dosage through dermal exposure?
   - **a.** no preexisting skin disease.
   - **b.** toxic exposure to thick skin.
   - **c.** increased percutaneous absorption rate.
   - **d.** low surface area of exposure.
   - **e.** high epidermal intercellular junction integrity.

4. Prolonged arsenic exposure could cause:
   a. infertility.
   b. cirrhosis.
   c. cor pulmonale.
   d. skin cancer.
   e. nephropathy.

5. Which of the following lung diseases has the highest occupational death rate?
   a. asbestosis.
   b. coal workers' pneumoconiosis.
   c. byssinosis.
   d. hypersensitivity pneumonitis.
   e. silicosis.

6. Lyme disease is caused by which of the following?
   a. *B. burgdorferi.*
   b. *H. capsulatum.*
   c. *M. tuberculosis.*
   d. *L. pneumophila.*
   e. *C. psittaci.*

7. Asbestos exposure is unlikely to cause:
   a. lung cancer.
   b. GI cancer.
   c. emphysema.
   d. pulmonary fibrosis.
   e. mesothelioma.

8. Exposure to which of the following can cause autoimmune disease?
   a. mercury.
   b. nitrogen dioxide.
   c. vinyl chloride.
   d. lead.
   e. flavivirus.

9. Which of the following might be linked to parkinsonism?
   a. nitrogen dioxide.
   b. zinc.
   c. copper.
   d. magnesium.
   e. carbon monoxide.

10. Which of the following infectious agents can cause hepatocellular carcinoma?

a. flavivirus.
b. bunyavirus.
c. alphavirus.
d. hepatitis C virus.
e. hepatitis B virus.

Answers to Chapter Questions

Chapter 1

1. b.
2. a.
3. a.
4. d.
5. b.

Chapter 2

1. b.
2. c.
3. b.
4. d.
5. e.
6. e.
7. b.
8. c.
9. d.
10. a.

Chapter 3

1. b.
2. e.
3. a.
4. c.
5. d.
6. e.
7. b.
8. a.
9. e.
10. c.
11. b.
12. b.

Chapter 4

1. d.

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4. c.
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7. c.
8. d.
9. c.
10. b.

Chapter 5

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4. b.
5. d.
6. c.
7. c.
8. d.
9. b.
10. d.

Chapter 6

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3. c.
4. e.
5. b.
6. e.
7. d.
8. a.
9. e.
10. d.

Chapter 7

1. c.
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3. d.
4. d.
5. e.

6. c.
7. b.
8. d.
9. b.
10. c.

Chapter 8

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2. e.
3. e.
4. b.
5. c.
6. a.
7. d.
8. e.
9. c.
10. b.

Chapter 9

1. c.
2. d.
3. b.
4. c.
5. e.
6. b.
7. e.
8. c.
9. c.
10. d.

Chapter 10

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2. c.
3. a.
4. c.
5. d.
6. b.
7. e.
8. c.
9. e.

10. e.

Chapter 11

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3. d.
4. d.
5. a.
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7. d.
8. c.
9. e.
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2. b.
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4. b.
5. c.
6. d.
7. e.
8. b.
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10. a.

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3. b.
4. e.
5. a.
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7. b.
8. d.
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10. e.

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9. d.
10. c.

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8. a.
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10. d.

Chapter 17

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10. d.

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9. d.
10. d.

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10. c.

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6. c.
7. a.
8. e.
9. b.
10. b.

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