

# chapter 1

## History and Scope of Toxicology

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### History of Toxicology

Antiquity  
Middle Ages  
Age of Enlightenment

### 20th Century Toxicology: The Awakening of Understanding

### The Second Half of the 19th Century The 21st Century

Toxicology has been defined as the study of the adverse effects of xenobiotics and thus is a borrowing science that has evolved from ancient poisoners. Modern toxicology goes beyond the study of the adverse effects of exogenous agents to the study of molecular biology, using toxicants as tools. Historically, toxicology formed the basis of therapeutics and experimental medicine. Toxicology in this century (1900 to the present) continues to develop and expand by assimilating knowledge and techniques from most branches of biology, chemistry, mathematics, and physics. A recent addition to the field of toxicology (1975 to the present) is the application of the discipline to safety evaluation and risk assessment.

The contributions and activities of toxicologists are diverse and widespread. In the biomedical area, toxicologists are concerned with mechanisms of action and exposure to chemical agents as a cause of acute and chronic illness. Toxicologists contribute to physiology and pharmacology by using toxic agents to understand physiological phenomena. They are involved in the recognition, identification, and quantification of hazards resulting from occupational exposure to chemicals and the public health aspects of chemicals in air, water, other parts of the environment, foods, and drugs. Traditionally, toxicologists have been intimately involved in the discovery and development of new drugs and pesticides. Toxicologists also participate in the development of standards and regulations designed to protect human health and the environment from the adverse effects of chemicals. Environmental toxicologists (a relatively new subset of the discipline) have expanded toxicology to study the effects of chemicals in flora and fauna. Molecular toxicologists are studying the mechanisms by which toxicants modulate cell growth and differentiation and cells respond to toxicants at the level of the gene. In all branches of toxicology, scientists explore the mechanisms by which chemicals produce adverse effects in biological systems. Clinical toxicologists develop antidotes and treatment regimes to ameliorate poisonings and xenobiotic injury. Toxicologists carry out some or all of these activities as members of academic, industrial, and governmental organizations. In doing so, they share methodologies for obtaining data about the toxicity of materials and the responsibility for using this information to make reasonable predictions regarding the hazards of the material to people

and the environment. These different but complementary activities characterize the discipline of toxicology.

Toxicology, like medicine, is both a science and an art. The science of toxicology is defined as the observational and data-gathering phase, whereas the art of toxicology consists of the utilization of the data to predict outcomes of exposure in human and animal populations. In most cases, these phases are linked because the facts generated by the science of toxicology are used to develop extrapolations and hypotheses to explain the adverse effects of chemical agents in situations where there is little or no information. For example, the observation that the administration of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) to female Sprague–Dawley rats induces hepatocellular carcinoma is a fact. However, the conclusion that it will also do so in humans is a prediction or hypothesis. It is important to distinguish facts from predictions. When we fail to distinguish the science from the art, we confuse facts with predictions and argue that they have equal validity, which they clearly do not. In toxicology, as in all sciences, theories have a higher level of certainty than do hypotheses, which in turn are more certain than speculations, opinions, conjectures, and guesses. An insight into modern toxicology and the roles, points of view, and activities of toxicologists can be obtained by examining the historical evolution of the discipline.

## HISTORY OF TOXICOLOGY

### Antiquity

Toxicology dates back to the earliest humans, who used animal venoms and plant extracts for hunting, warfare, and assassination. Elucidating the mechanisms of the toxicity of venoms continues today in the field of toxinology. The knowledge of these poisons must have predated recorded history. It is safe to assume that prehistoric humans categorized some plants as harmful and others as safe. The same is probably true for the classification of snakes and other animals. The Ebers Papyrus (circa 1500 BC) contains information pertaining to many recognized poisons, including hemlock (the state poison of the Greeks), aconite (a Chinese arrow poison), opium (used as both a poison and an antidote), and metals such

as arsenic lead, copper, and antimony. There is also an indication that plants containing substances similar to digitalis and belladonna alkaloids were known. Hippocrates (circa 400 BC) added a number of poisons and clinical toxicology principles pertaining to bioavailability in therapy and overdosage, while the *Book of Job* (circa 400 BC) speaks of poison arrows (Job 6:4). In the literature of ancient Greece, there are several references to poisons and their use. Some interpretations of Homer have Odysseus obtaining poisons for his arrows (Homer, circa 600 BC). Theophrastus (370–286 BC), a student of Aristotle, included numerous references to poisonous plants in *De Historia Plantarum*. Dioscorides, a Greek physician in the court of the Roman emperor Nero, made the first attempt at a classification of poisons, which was accompanied by descriptions and drawings. His classification into plant, animal, and mineral poisons not only remained a standard for 16 centuries but also is still a convenient classification (Gunther, 1934). Dioscorides also dabbled in therapy, recognizing the use of emetics in poisoning and the use of caustic agents and cupping glasses in snakebite. Poisoning with plant and animal toxins was quite common. Perhaps the best known recipient of poison used as a state method of execution was Socrates (470–399 BC), whose cup of hemlock extract was apparently estimated to be the proper dose. Expeditious suicide on a voluntary basis also made use of toxicological knowledge. Demosthenes (385–322 BC), who took poison hidden in his pen, was one of many examples. The mode of suicide calling for one to fall on his sword, although manly and noble, carried little appeal and less significance for the women of the day. Cleopatra's (69–30 BC) knowledge of natural primitive toxicology permitted her to use the more genteel method of falling on her asp.

The Romans too made considerable use of poisons in politics. One legend tells of King Mithridates VI of Pontus, whose numerous acute toxicity experiments on unfortunate criminals led to his eventual claim that he had discovered an antidote for every venomous reptile and poisonous substance (Guthrie, 1946). Mithridates was so fearful of poisons that he regularly ingested a mixture of 36 ingredients (Galen reports 54) as protection against assassination. On the occasion of his imminent capture by enemies, his attempts to kill himself with poison failed because of his successful antidote concoction, and he was forced to use a sword held by a servant. From this tale comes the term “mithridatic,” referring to an antidotal or protective mixture. The term “theriac” also has become synonymous with “antidote,” although the word comes from the poetic treatise *Theriaca* by Nicander of Colophon (204–135 BC), which dealt with poisonous animals; his poem *Alexipharmaca* was about antidotes.

Poisonings in Rome reached epidemic proportions during the 4th century BC (Livy). It was during this period that a conspiracy of women to remove men from whose death they might profit was uncovered. Similar large-scale poisoning continued until Sulla issued the *Lex Cornelia* (circa 82 BC). This appears to be the first law against poisoning, and it later became a regulatory statute directed at careless dispensers of drugs. Nero (AD 37–68) used poisons to do away with his stepbrother Britannicus and employed his slaves as food tasters to differentiate edible mushrooms from their more poisonous kin.

## Middle Ages

*Come bitter pilot, now at once run on  
The dashing rocks thy seasick weary bark!  
Here's to my love! O true apothecary!  
Thy drugs are quick. Thus with a kiss I die.*

*Romeo and Juliet*, act 5, scene 3

Before the Renaissance, the writings of Maimonides (Moses ben Maimon, AD 1135–1204) included a treatise on the treatment of poisonings from insects, snakes, and mad dogs (*Poisons and their Antidotes*, 1198). Maimonides, like Hippocrates before him, wrote on the subject of bioavailability, noting that milk, butter, and cream could delay intestinal absorption. Maimonides also refuted many of the popular remedies of the day and stated his doubts about others. It is rumored that alchemists of this period (circa AD 1200), in search of the universal antidote, learned to distill fermented products and made a 60% ethanol beverage that had many interesting powers.

In the early Renaissance, the Italians, with characteristic pragmatism, brought the art of poisoning to its zenith. The poisoner became an integral part of the political scene. The records of the city councils of Florence, particularly those of the infamous Council of Ten of Venice, contain ample testimony about the political use of poisons. Victims were named, prices set, and contracts recorded; when the deed was accomplished, payment was made.

An infamous figure of the time was a lady named Toffana who peddled specially prepared arsenic-containing cosmetics (*Agua Toffana*). Accompanying the product were appropriate instructions for its use. Toffana was succeeded by an imitator with organizational genius, Hieronyma Spara, who provided a new fillip by directing her activities toward specific marital and monetary objectives. A local club was formed of young wealthy married women, which soon became a club of eligible young wealthy widows, reminiscent of the matronly conspiracy of Rome centuries earlier. Incidentally, arsenic-containing cosmetics were reported to be responsible for deaths well into the 20th century (Kallet and Schlink, 1933).

Among the prominent families engaged in poisoning, the Borgias were the most notorious. However, many deaths that were attributed to poisoning are now recognized as having resulted from infectious diseases such as malaria. It appears true, however, that Alexander VI, his son Cesare, and Lucrezia Borgia were quite active. The deft application of poisons to men of stature in the Catholic Church swelled the holdings of the papacy, which was their prime heir.

In this period Catherine de Medici exported her skills from Italy to France, where the prime targets of women were their husbands. However, unlike poisoners of an earlier period, the circle represented by Catherine and epitomized by the notorious Marchioness de Brinvilliers depended on developing direct evidence to arrive at the most effective compounds for their purposes. Under the guise of delivering provender to the sick and the poor, Catherine tested toxic concoctions, carefully noting the rapidity of the toxic response (onset of action), the effectiveness of the compound (potency), the degree of response of the parts of the body (specificity, site of action), and the complaints of the victim (clinical signs and symptoms).

The culmination of the practice in France is represented by the commercialization of the service by Catherine Deshayes, a midwife sorceress who earned the title “La Voisin.” Her business was dissolved by her execution in 1680. Her trial was 1 of the most famous of those held by the *Chambre Ardente*, a special judicial commission established by Louis XIV to try such cases without regard to age, sex, or national origin. La Voisin was convicted of many poisonings, with over 2000 infants among her victims.

## Age of Enlightenment

*All substances are poisons; there is none which is not a poison. The right dose differentiates poison from a remedy.*

*Paracelsus*

A significant figure in the history of science and medicine in the late Middle Ages was the renaissance man Philippus Aureolus Theophrastus Bombastus von Hohenheim-Paracelsus (1493–1541). Between the time of Aristotle and the age of Paracelsus, there was little substantial change in the biomedical sciences. In the 16th century, the revolt against the authority of the Catholic Church was accompanied by a parallel attack on the godlike authority exercised by the followers of Hippocrates and Galen. Paracelsus personally and professionally embodied the qualities that forced numerous changes in this period. He and his age were pivotal, standing between the philosophy and magic of classical antiquity and the philosophy and science willed to us by figures of the 17th and 18th centuries. Clearly, one can identify in Paracelsus's approach, point of view, and breadth of interest numerous similarities to the discipline that is now called toxicology.

Paracelsus, a physician–alchemist and the son of a physician, formulated many revolutionary views that remain an integral part of the structure of toxicology, pharmacology, and therapeutics today (Pagel, 1958). He promoted a focus on the “toxicon,” the primary toxic agent, as a chemical entity, as opposed to the Grecian concept of the mixture or blend. A view initiated by Paracelsus that became a lasting contribution held as corollaries that (1) experimentation is essential in the examination of responses to chemicals, (2) one should make a distinction between the therapeutic and toxic properties of chemicals, (3) these properties are sometimes but not always indistinguishable except by dose, and (4) one can ascertain a degree of specificity of chemicals and their therapeutic or toxic effects. These principles led Paracelsus to introduce mercury as the drug of choice for the treatment of syphilis, a practice that survived 300 years but led to his famous trial. This viewpoint presaged the “magic bullet” (arsphenamine) of Paul Ehrlich and the introduction of the therapeutic index. Further, in a very real sense, this was the first sound articulation of the dose–response relation, a bulwark of toxicology (Pachter, 1961).

The tradition of the poisoners spread throughout Europe, and their deeds played a major role in the distribution of political power throughout the Middle Ages. Pharmacology as it is known today had its beginnings during the Middle Ages and early Renaissance. Concurrently, the study of the toxicity and the dose–response relationship of therapeutic agents were commencing.

The occupational hazards associated with metalworking were recognized during the 15th century. Early publications by Ellenbog (circa 1480) warned of the toxicity of the mercury and lead exposures involved in goldsmithing. Agricola published a short treatise on mining diseases in 1556. However, the major work on the subject, *On the Miners' Sickness and Other Diseases of Miners* (1567), was published by Paracelsus. This treatise addressed the etiology of miners' disease, along with treatment and prevention strategies. Occupational toxicology was further advanced by the work of Bernardino Ramazzini. His classic, published in 1700 and entitled *Discourse on the Diseases of Workers*, set the standard for occupational medicine well into the 19th century. Ramazzini's work broadened the field by discussing occupations ranging from miners to midwives and including printers, weavers, and potters.

The developments of the industrial revolution stimulated a rise in many occupational diseases. The recognition, in 1775, of the renowned 18th-century English surgeon Percival Pott (1714–1788) of the role of soot in scrotal cancer among chimney sweeps was the first reported example of polyaromatic hydrocarbon (PAH) carcinogenicity, a problem the mechanism of which still intrigues

toxicologists today. These findings led to improved medical practices, particularly in prevention of occupationally related diseases. It should be noted that Paracelsus and Ramazzini also pointed out the toxicity of smoke and soot.

The 19th century dawned in a climate of industrial and political revolution. Organic chemistry was in its infancy in 1800, but by 1825 phosgene ( $\text{COCl}_2$ ) and mustard gas (bis[ $\beta$ -chloroethyl]sulfide) had been synthesized. These 2 agents, along with chlorine gas, were used by the German forces in World War I as chemical warfare agents. They were stockpiled throughout World War II, and used by Iraq in the Iran–Iraq War in the 1980s (*Marine Corps History*). By 1880 over 10,000 organic compounds had been synthesized including chloroform, carbon tetrachloride, diethyl ether, and carbonic acid, and petroleum and coal gasification by-products were used in trade. Determination of the toxicological potential of these newly created chemicals became the underpinning of the science of toxicology as it is practiced today. However, there was little interest during the mid-19th century in hampering industrial development. Hence, the impact of industrial toxicology discoveries was not felt until the passage of worker's insurance laws, first in Germany (1883), then in England (1897), and later in the United States (1910). Experimental toxicology accompanied the growth of organic chemistry and developed rapidly during the 19th century. Magendie (1783–1885), Orfila (1787–1853), and Bernard (1813–1878) carried out truly seminal research in experimental toxicology and medicine, and laid the groundwork for pharmacology, drug safety toxicology, and experimental therapeutics as well as occupational toxicology.

Orfila, a Spanish physician in the French court, was the first toxicologist to use autopsy material and chemical analysis systematically as legal proof of poisoning. His introduction of this detailed type of analysis survives as the underpinning of forensic toxicology (Orfila, 1818). Orfila published the first major work devoted expressly to the toxicity of natural agents (1814–1815). Magendie, a physician and experimental physiologist, studied the mechanisms of action of emetine, strychnine, and “arrow poisons” (Olmsted, 1944). His research into the absorption and distribution of these compounds in the body (the precursor to ADME studies today) remains a classic in toxicology and pharmacology. One of Magendie's more famous students, Claude Bernard, continued the study of arrow poisons (Bernard, 1850) but also added works on the mechanism of action of carbon monoxide. Bernard's treatise, *Introduction to the Study of Experimental Medicine* (translated by Greene and Schuman in 1949), is a classic in the development of toxicology.

Many German scientists contributed greatly to the growth of toxicology in the late 19th and early 20th centuries. Among the giants of the field are Oswald Schmiedeberg (1838–1921) and Louis Lewin (1850–1929). Schmiedeberg made many contributions to the science of toxicology, not the least of which was the training of approximately 120 students who later populated the most important laboratories of pharmacology and toxicology throughout the world. Schmiedeberg's research focused on the synthesis of hippuric acid in the liver and the detoxification mechanisms of the liver in several animal species (Schmiedeberg and Koppe, 1869). Lewin, who was educated originally in medicine and the natural sciences, trained in toxicology under Liebreich at the Pharmacological Institute of Berlin (1881). His contributions on the chronic toxicity of narcotics and other alkaloids remain a classic. Lewin also published much of the early work on the toxicity of methanol, glycerol, acrolein, and chloroform (Lewin, 1920, 1929).

## 20TH CENTURY TOXICOLOGY: THE AWAKENING OF UNDERSTANDING

*The doubter is a true man of science; he doubts only himself and his interpretations, but he believes in science.*

*Claude Bernard*

The latter part of the 19th century saw the introduction of “patent medicines” in many parts of the world.

Toxicology evolved rapidly during the 20th century. The early controversies focusing on patent medicines and sale of consumer products of questionable safety (Kallett and Schlink) were followed by the rapid advances in analytical chemistry methods that fostered the advancement of forensic toxicology specifically at the New York City Medical Examiner’s Office. However, the exponential growth of the discipline can be traced to the World War II era with its marked increase in the production of drugs, pesticides, munitions, synthetic fibers, and industrial chemicals. The history of many sciences represents an orderly transition based on theory, hypothesis testing, and synthesis of new ideas. Toxicology, as a gathering and an applied science, has, by contrast, developed in fits and starts. It calls on almost all the basic sciences to test its hypotheses. This fact, coupled with the health and occupational regulations that have driven toxicology research since 1900, has made toxicology exceptional in the history of science. The differentiation of toxicology as an art and a science, though arbitrary, permits the presentation of historical highlights along 2 major lines.

Modern toxicology can be viewed as a continuation of the development of the biological and physical sciences in the late 19th and 20th centuries (Table 1-1). During the second half of the 19th century, the world witnessed an explosion in science that produced the beginning of the modern era of medicine, synthetic chemistry, physics, and biology. Toxicology has drawn its strength and diversity from its proclivity to borrowing. With the advent of anesthetics and disinfectants and the advancement of experimental pharmacology in the late 1850s, toxicology as it is currently understood got its start. The introduction of ether, chloroform, and carbonic acid led to several iatrogenic deaths. These unfortunate outcomes spurred research into the causes of the deaths and early experiments on the physiological mechanisms by which these compounds caused both beneficial and adverse effects. By the late 19th century the use of organic chemicals was becoming more widespread, and benzene, toluene, and the xylenes, as well as the chlorinated solvents related to chloroform, went into large-scale commercial production. During this period, the use of patent medicines, consisting primarily of “medicinal herbs,” nonsugar sweeteners, and alcohol, was prevalent, and there were several incidents of poisonings from these medicaments. In 1902 Congress approved \$5000 to fund the “Poison Squad,” professional tasters under the direction of Harvey Washington Wiley that harkened back to the food tasters used by royalty to avoid intentional poisoning from their foods. The case of “Doctor” Munyan versus Harvey Wiley, MD (1844–1930), a classic battle between the federal government and the most infamous purveyor of patent medicines, over mislabeling, false advertisement, lack of efficacy, and serious toxicity led to further Congressional action. The adverse reactions to patent medicines and mislabeled foods coupled with the response to Upton Sinclair’s exposé of the Chicago meat-packing industry in *The Jungle* (1905) culminated in the passage of the Wiley Bill (*The Pure Foods Act of 1906*).

**Table 1-1**

### Selection of Developments in Toxicology

#### *Development of early advances in analytical methods*

- Marsh: development of method for arsenic analysis
- Reinh: combined method for separation and analysis of As and Hg
- Fresenius and von Babo: development of screening method for general poisons
- Stas-Otto: detection and identification of phosphorus

#### *Early mechanistic studies*

- F. Magendie: study of “arrow poisons,” mechanism of action of emetine and strychnine
- C. Bernard (1850): carbon monoxide combination with hemoglobin, study of mechanism of action of strychnine, site of action of curare
- R. Bohm (ca. 1890): active anthelmintics from fern, action of croton oil catharsis, poisonous mushrooms

#### *Introduction of new toxicants and antidotes*

- R. A. Peters, L. A. Stocken, and R. H. S. Thompson: development of British anti-Lewisite (BAL) as a relatively specific antidote for arsenic, toxicity of monofluorocarbon compounds
- K. K. Chen: introduction of modern antidotes (nitrite and thiosulfate) for cyanide toxicity
- C. Voegtlin *et al.* (1923): mechanism of action of As and other metals on the SH groups
- P. Müller: introduction and study of dichlorodiphenyltrichloroethane (DDT) and related insecticide compounds
- G. Schrader: introduction and study of organophosphorus compounds
- R. N. Chopra: indigenous drugs of India

#### *Miscellaneous toxicological studies*

- R. T. Williams: study of detoxication mechanisms and species variation
- A. Rothstein: effects of uranium ion on cell membrane transport
- R. A. Kehoe: investigation of acute and chronic effects of lead
- A. Vorwald: studies of chronic respiratory disease (beryllium)
- H. Hardy: community and industrial poisoning (beryllium)
- A. Hamilton: introduction of modern industrial toxicology
- H. C. Hodge: toxicology of uranium, fluorides; standards of toxicity
- A. Hoffman: introduction of lysergic acid and derivatives; psychotomimetics
- R. A. Peters: biochemical lesions, lethal synthesis
- A. E. Garrod: inborn errors of metabolism
- T. T. Litchfield and F. Wilcoxon: simplified dose–response evaluation
- C. J. Bliss: method of probits, calculation of dosage–mortality curves



This was the first of many US pure food and drug laws (see Hutt and Hutt, 1984, for regulatory history of foods). The Wiley Bill as it was known was widely supported in Congress at its passage. However, the support did not last. The Bill required prior toxicity testing, the establishment of a government analytical laboratory, and the removal of toxic compounds, particularly ethanol, herbal mixtures, and coloring agents. It also prohibited false advertising. After enactment of the Bill, individual federal leaders including Congressmen and judges, as well as “Dr” Munyan (who claimed his remedies were effective and had cured thousands), campaigned against its enforcement. Parts of the Bill were overturned by Justice Oliver Wendell Holmes and the US Supreme Court in 1911 stating that “hype is not false advertising.” In part because of the opposition to the Bill, Wiley left the government to direct the fledgling Consumer Union in 1912. Today, a century later, similar battles are being fought over dietary supplements and food additives.

A working hypothesis about the development of toxicology is that the discipline expands in response to legislation, which itself is a response to a real or perceived tragedy. The Wiley Bill was the first such reaction in the area of food and drugs, and the workers’ compensation laws cited above were a response to occupational toxicities. In addition, the National Safety Council was established in 1911, and the Division of Industrial Hygiene was established by the US Public Health Service in 1914. A corollary to this hypothesis might be that the founding of scientific journals and/or societies is sparked by the development of a new field. The *Journal of Industrial Hygiene* began in 1918. The major chemical manufacturers in the United States (Dow, Union Carbide, and Du Pont) established internal toxicology research laboratories to help guide decisions on worker health and product safety.

During the 1890s and early 1900s, European scientists Becquerel, Roentgen, and the Curies reported the discovery of radioactivity and x-rays. This opened up for exploration a very large area in physics, biology, and medicine. Interestingly, many of these early researchers died of radiation poisoning. Radiation exposure became widespread in consumer usage. Radium-containing rocks were touted as health cures, and the Radiothor and radium dial watches were widespread. The adverse effects were virtually unknown and radiation would not actively affect the science of toxicology until the World War II era.

However, another discovery, that of vitamins, or “vital amines,” led to the use of the first large-scale bioassays (multiple animal studies) to determine whether these new synthetic chemicals were beneficial or harmful to laboratory animals, and by extension to humans. The initial work in this area took place at around the time of World War I in several laboratories, including the laboratory of Philip B. Hawk in Philadelphia. Hawk and a young associate, Bernard L. Oser, were responsible for the development and verification of many early toxicological assays that are still used in a slightly amended form. The results from these animal studies formed the underpinnings of risk assessment. Oser’s contributions to food and regulatory toxicology were extraordinary. These early bioassays were made possible by a major advance in toxicology: the availability of developed and refined strains of inbred laboratory rodents (Donaldson, 1912) and the rapid development of analytical chemistry.

The 1920s saw many events that began to mold the fledgling field of toxicology. The discovery by Paul Ehrlich (1854–1915) of arsenicals for the treatment of syphilis (arsenicals had been used in agriculture since the mid-19th century) resulted in acute

and chronic toxicity. Arsenic remains a major toxicant in many developing nations. Prohibition of alcoholic beverages in the United States opened the door for early studies of neurotoxicology, with the discovery that triorthocresyl phosphate (TOCP), methanol, and lead (all found in bootleg liquor) are neurotoxins. TOCP, which was a recent gasoline additive, caused a syndrome that became known as “ginger-jake” walk, a spastic gait resulting from drinking ginger beer adulterated with TOCP. Methanol, used as a cheap ethanol substitute, blinded and killed many unsuspecting people (see *Poisoners Handbook*, 2011). Mueller’s discovery of dichlorodiphenyltrichloroethane (DDT) and several other organohalides, such as hexachlorobenzene and hexachlorocyclohexane, during the late 1920s resulted in widespread use of these insecticidal agents. Toxicity testing of the new organohalide compounds was in its infancy. Understanding of the modes of action and persistence of these compounds would have to wait 40 years.

Other scientists were hard at work attempting to elucidate the structures and activity of the estrogens and androgens. Work on the steroid hormones led to the use of several assays for the determination of the biological activity of organ extracts and synthetic compounds. Allen and Doisy published the first uterotrophic assay (1928) that accelerated the study of estrogenic chemicals. Modifications of this assay are used today in studying endocrine disruption by xenobiotics. Efforts to synthesize estrogen-active chemicals were spearheaded by E. C. Dodds and his co-workers, one of whom was Leon Golberg, a young organic chemist and a future leader in toxicology. Dodds’s work on the bioactivity of the estrogenic compounds resulted in the synthesis of diethylstilbestrol (DES), hexestrol, other stilbenes, and bisphenol A (BPA) and the discovery of the strong estrogenic activity of substituted stilbenes. Golberg’s intimate involvement in this work stimulated his interest in biology, leading to degrees in biochemistry and medicine and a career in toxicology in which he oversaw the creation of the laboratories of the British Industrial Biological Research Association (BIBRA) and the Chemical Industry Institute of Toxicology (CIIT). Interestingly, the initial observations that led to the discovery of DES were the findings of feminization of animals treated with the experimental carcinogen 7,12-dimethylbenz[*a*]anthracene (DMBA).

*Occupational illnesses became more pronounced after the 1920s, and occupational toxicology developed into a field of its own. The seminal works of Alice Hamilton (1869–1970) (Exploring the Dangerous Trades) and Ethel Browning (Toxicity of Industrial Solvents, 1937) are critical readings.*

The 1930s saw the world preparing for World War II and a major effort by the pharmaceutical and chemical industry in Europe and the United States to manufacture the first mass-produced antibiotics. One of the first journals expressly dedicated to experimental toxicology, *Archiv für Toxikologie*, began publication in Europe in 1930, the same year that Herbert Hoover signed the act that established the National Institutes of Health (NIH) in the United States.

The discovery of sulfanilamide was heralded as a major event in combating bacterial diseases. However, for a drug to be effective, there must be a reasonable delivery system, and sulfanilamide is highly insoluble in an aqueous medium. Therefore, it was originally prepared in ethanol (elixir). However, it was soon discovered that the drug was more soluble in diethylene glycol. The drug was sold in glycol solutions but was labeled as an elixir, and several patients (mostly children) died of acute kidney failure resulting from the metabolism of the glycol to oxalic acid

and glycolic acid, with the acids and the active drug crystallizing in the kidney tubules. This tragic event led to the passage of the Copeland Bill in 1938, the second major bill involving the formation of the US Food and Drug Administration (FDA). The sulfanilamide disaster played a critical role in the further development of toxicology, resulting in work by Eugene Maximilian Geiling in the Pharmacology Department of the University of Chicago that elucidated the mechanism of toxicity of both sulfanilamide and diethylene glycol. Studies of the glycols were simultaneously carried out at the US FDA by a group led by Arnold Lehman. The scientists associated with Lehman and Geiling were to become the leaders of toxicology (especially the Society of Toxicology) over the next 40 years. With few exceptions, toxicology in the United States owes its heritage to Geiling's innovativeness and ability to stimulate and direct young scientists, and Lehman's vision of the use of experimental toxicology in public health decision making. Because of Geiling's reputation, the US government turned to this group for help in the war effort. There were 3 main areas in which the Chicago group took part during World War II: the toxicology and pharmacology of organophosphate (OP) chemicals, antimalarial drugs, and radionuclides. Each of these areas produced teams of toxicologists who became academic, governmental, and industrial leaders in the field.

It was also during this time that DDT and the phenoxy herbicides were developed for increased food production and, in the case of DDT, control of insect-borne diseases. These efforts between 1940 and 1946 led to an explosion in toxicology. Thus, in line with the hypothesis advanced above, the crisis of World War II caused the next major leap in the development of toxicology.

If one traces the history of the toxicology of metals over the past 45 years, the role of the Chicago and Rochester groups is quite visible. This story commences with the use of uranium for the "bomb" and continues today with research on the role of metals in their interactions with DNA, RNA, and growth factors. Indeed, the Manhattan Project created a fertile environment that resulted in the initiation of quantitative biology, radiotracer technology, and inhalation toxicology. These innovations have revolutionized modern biology, chemistry, therapeutics, and toxicology.

Inhalation toxicology began at the University of Rochester under the direction of Stafford Warren, who headed the Department of Radiology. He developed a program with colleagues such as Harold Hodge (pharmacologist), Herb Stokinger (chemist), Sid Laskin (inhalation toxicologist), and Lou and George Casarett (toxicologists). These young scientists were to go on to become giants in the field. The other sites for the study of radionuclides were Chicago for the "internal" effects of radioactivity and Oak Ridge, Tennessee, for the effects of "external" radiation. The work of the scientists on these teams gave the scientific community data that contributed to the early understanding of macromolecular binding of xenobiotics, cellular mutational events, methods for inhalation toxicology and therapy, and toxicological properties of trace metals, along with a better appreciation of the complexities of the dose-response curve.

Another seminal event in toxicology that occurred during the World War II era was the discovery by Lange and Schrader in 1938 of OP cholinesterase inhibitors including sarin, tabun (chemical warfare agents), and less potent OP insecticides. This class of chemicals was destined to become a driving force in the study of neurophysiology and toxicology for several decades. Again, the scientists in Chicago played major roles in elucidating the mechanisms of action of this new class of compounds. Geiling's group, Kenneth DuBois in particular, was the leader in this area of toxicology and pharmacology. DuBois's colleagues, particularly

Sheldon Murphy, continued to be in the forefront of this special area. The importance of the early research on the OPs has taken on special meaning in the years since 1960, when these nonbioaccumulating insecticides were destined to replace DDT and other organochlorine insecticides. Today, a third generation of insecticides has replaced much of the OP use.

Early in the 20th century, it was demonstrated experimentally that quinine has a marked effect on the malaria parasite (it had been known for centuries that cinchona bark extract is efficacious for "Jesuit fever" [malaria]). This discovery led to the development of quinine derivatives for the treatment of the disease and the formulation of the early principles of chemotherapy. The Pharmacology Department of the University of Chicago was charged with the development of antimalarials for the war effort. The original protocols called for testing of efficacy and toxicity in rodents and perhaps dogs and then the testing of efficacy in human volunteers. One of the investigators charged with generating the data needed to move a candidate drug from animals to humans was Fredrick Coulston. This young parasitologist and his colleagues, working in Chicago, were to evaluate potential drugs in animal models and then establish human clinical trials. It was during these experiments that the use of nonhuman primates came into vogue for toxicology testing. It had been noted by Russian scientists that some antimalarial compounds caused retinopathies in humans but did not apparently have the same adverse effect in rodents and dogs. This finding led the Chicago team to add 1 more step in the development process: toxicity testing in rhesus monkeys just before efficacy studies in people. This resulted in the prevention of blindness in untold numbers of volunteers and perhaps some of the troops in the field. It also led to the school of thought that nonhuman primates may be one of the better models for humans and the establishment of primate colonies for the study of toxicity. Coulston pioneered this area of toxicology and remained committed to it.

Another area not traditionally thought of as toxicology but one that evolved during the 1940s as an exciting and innovative field is experimental pathology. This branch of experimental biology developed from bioassays of estrogens and early experiments in chemical- and radiation-induced carcinogenesis. It is from these early studies that hypotheses on tumor promotion and cancer progression have evolved.

Toxicologists today owe a great deal to the researchers of chemical carcinogenesis of the 1940s. Much of today's work can be traced to Elizabeth and James Miller at Wisconsin. This husband and wife team started under the mentorship of Professor Rusch, the director of the newly formed McArdle Laboratory for Cancer Research, and Professor Baumann. The seminal research of the Millers led to the discovery of the role of reactive intermediates in carcinogenicity and that of mixed-function oxidases in the endoplasmic reticulum. These findings, which initiated the great works on the cytochrome P450 family of proteins, were aided by 2 other major discoveries for which toxicologists (and all other biological scientists) are deeply indebted: paper chromatography in 1944 and the use of radiolabeled dibenzanthracene in 1948. Other major events of note in drug metabolism included the work of Bernard Brodie on the metabolism of methyl orange in 1947. This piece of seminal research led to the examination of blood and urine for chemical and drug metabolites. It became the tool with which one could study the relationship between blood levels and biological action. The classic treatise of R. T. Williams, *Detoxication Mechanisms*, was published in 1947. This text described the many pathways and possible mechanisms of detoxication and opened the field to several new areas of study.

The decade after World War II was not as boisterous as the period from 1935 to 1945. The first major US pesticide act was signed into law in 1947. The significance of the initial Federal Insecticide, Fungicide, and Rodenticide Act was that for the first time in US history a substance that was neither a drug nor a food had to be shown to be safe and efficacious. This decade, which coincided with the Eisenhower years, saw the dispersion of the groups from Chicago, Rochester, and Oak Ridge and the establishment of new centers of research. Adrian Albert's classic *Selective Toxicity* was published in 1951. This treatise, which has appeared in several editions, presented a concise documentation of the principles of the site-specific action of chemicals.

## THE SECOND HALF OF THE 19TH CENTURY

*You too can be a toxicologist in two easy lessons, each of ten years.*

*Arnold Lehman (circa 1955)*

The mid-1950s witnessed the strengthening of the US FDA's commitment to toxicology under the guidance of Arnold Lehman. Lehman's tutelage and influence are still felt today. The adage "You too can be a toxicologist" is as important a summation of toxicology as the often-quoted statement of Paracelsus: "The dose makes the poison." The period from 1955 to 1958 produced 2 major events that would have a long-lasting impact on toxicology as a science and a professional discipline. Lehman, Fitzhugh, and their co-workers formalized the experimental program for the appraisal of food, drug, and cosmetic safety in 1955, updated by the US FDA in 1982, and the Gordon Research Conferences established a conference on toxicology and safety evaluation, with Bernard L. Oser as its initial chairman. These 2 events led to close relationships among toxicologists from several groups and brought toxicology into a new phase. At about the same time, the US Congress passed and the president of the United States signed the additives amendments to the Food, Drug, and Cosmetic Act. The Delaney Clause (1958) of these amendments stated broadly that any chemical found to be carcinogenic in laboratory animals or humans could not be added to the US food supply. The impact of this legislation cannot be overstated. Delaney became a battle cry for many groups and resulted in the inclusion at a new level of biostatisticians and mathematical modelers in the field of toxicology. It fostered the expansion of quantitative methods in toxicology and led to innumerable arguments about the "1-hit" theory of carcinogenesis. Regardless of one's view of Delaney, it has served as an excellent starting point for understanding the complexity of the biological phenomenon of carcinogenicity and the development of risk assessment models. One must remember that at the time of Delaney, the analytical detection level for most chemicals was 20 to 100 ppm (today, parts per quadrillion). Interestingly, the Delaney Clause has been invoked only on a few occasions, and it has been stated that Congress added little to the food and drug law with this clause (Hutt and Hutt, 1984).

Shortly after the Delaney amendment and after 3 successful Gordon Conferences, the first American journal dedicated to toxicology was launched by Coulston, Lehman, and Hayes. *Toxicology and Applied Pharmacology* has been a flagship journal of toxicology ever since. The founding of the Society of Toxicology (1961) followed shortly afterward, and this journal became its official publication of the SOT through the 20th century. The society's founding members were Fredrick Coulston, William Deichmann, Kenneth DuBois, Victor Drill, Harry Hayes, Harold Hodge, Paul Larson, Arnold Lehman, and C. Boyd Shaffer. These researchers

deserve a great deal of credit for the growth of toxicology. DuBois and Geiling published their *Textbook of Toxicology* in 1959.

In 1975, Louis Casarett and John Doull followed this short text with what has become the most widely accepted general toxicology text—*Toxicology: The Basic Science of Poisons*. This volume is the eighth addition of the classic text.

The 1960s were a tumultuous time for society, and toxicology was swept up in the tide, starting with the tragic thalidomide incident, in which several thousand children were born with serious birth defects. Dr Frances O. Kelsey, a protégé of E. M. K. Geiling, was instrumental in keeping this notorious teratogen off the US market. Interestingly, as the mechanism of thalidomide became known later in the 20th century, the drug and its derivatives were developed for several life-threatening diseases. The publication of Carson's *Silent Spring* (1962) energized the field of environmental toxicology that developed at a feverish pitch. Attempts to understand the effects of chemicals on the embryo and fetus and on the environment as a whole gained momentum. New legislation was passed, and new journals were founded. The education of toxicologists spread from the deep traditions of Chicago and Rochester to Harvard, Miami, Albany, Iowa, Jefferson, and beyond. Geiling's fledglings spread as Schmiedeberg's had a half century before. Many new fields were influencing and being assimilated into the broad scope of toxicology, including environmental sciences, aquatic and avian biology, cell biology, analytical chemistry, and genetics.

During the 1960s the analytical tools used in toxicology were developed to a level of sophistication that allowed the detection of chemicals in tissues and other substrates at part per billion concentrations (see *The Vanishing Zero*, 1972). Today parts per quadrillion and less are detected. Pioneering work in the development of point mutation assays that were replicable, quick, and inexpensive led to a better understanding of the genetic mechanisms of carcinogenicity (Ames, 1983). The combined work of Ames and the Millers (Elizabeth C. and James A.) at McArdle Laboratory allowed the toxicology community to make major contributions to the understanding of the carcinogenic process.

The low levels of detection of chemicals and the ability to detect point mutations rapidly created several problems and opportunities for toxicologists and risk assessors that stemmed from interpretation of the Delaney amendment. Cellular and molecular toxicology developed as a subdiscipline, and risk assessment became a major product of toxicological investigations.

The establishment of the National Center for Toxicologic Research (NCTR), the expansion of the role of the US FDA, and the establishment of the US Environmental Protection Agency (EPA) and the National Institute of Environmental Health Sciences (NIEHS) were considered clear messages that the government had taken a strong interest in toxicology. Several new journals appeared during the 1960s, and new legislation was written quickly after *Silent Spring* and the thalidomide disaster.

Elwood Jensen and his colleagues discovered the high-affinity estradiol-binding protein (the estrogen receptor) in the mid-1960s. The end of the 1960s witnessed the "discovery" of TCDD as a contaminant in the herbicide Agent Orange (the original discovery of TCDD toxicity was reported in 1957). The research on the toxicity of this compound has produced some very good and some very poor research in the field of toxicology. The discovery of a high-affinity cellular binding protein, using techniques established by Jensen, was designated the "Ah" receptor (see Poland and Knutsen, 1982, for a review) at the McArdle Laboratory. Works on the genetics of the receptor at NIH (Nebert and Gonzalez, 1987) revolutionized the field of toxicology. The importance of TCDD



to toxicology lies in the fact that it forced researchers, regulators, and the legal community to look at the role of mechanisms of toxic action in a different fashion.

At least one other event precipitated a great deal of legislation during the 1970s: Love Canal. The “discovery” of Love Canal led to major concerns regarding hazardous wastes, chemical dump sites, and disclosure of information about those sites. Soon after Love Canal, the EPA listed several equally contaminated sites in the United States. The agency was given the responsibility to develop risk assessment methodology to determine health risks from exposure to effluents and to attempt to remediate these sites. These combined efforts led to broad-based support for research into the mechanisms of action of individual chemicals and complex mixtures. Love Canal and similar issues created the legislative environment that led to the Toxic Substances Control Act and eventually to the Superfund Bill. These omnibus bills were created to cover the toxicology of chemicals from initial synthesis to disposal (cradle to grave).

The expansion of legislation, journals, and new societies involved with toxicology was exponential during the 1970s and 1980s and shows no signs of slowing down. Currently, in the United States there are dozens of professional, governmental, and other scientific organizations with thousands of members and over 120 journals dedicated to toxicology and related disciplines.

In addition, toxicology continues to expand in stature and in the number of programs worldwide. The International Congress of Toxicology is made up of toxicology societies from Europe, South America, Asia, Africa, and Australia and brings together the broadest representation of toxicologists.

The original Gordon Conference series that celebrated its 50th anniversary in 2007 has evolved to *Cellular & Molecular Mechanisms of Toxicity*, and several other conferences related to special areas of toxicology are now in existence. The Society of Toxicology formed specialty sections and regional chapters to accommodate the over 6000 scientists involved in toxicology today. The SOT celebrated its 50th anniversary in March 2011. Texts and reference books for toxicology students and scientists abound. Toxicology has evolved from a borrowing science to a seminal discipline seeding the growth and development of several related fields of science and science policy.

The history of toxicology has been interesting and varied but never dull. Perhaps as a science that has grown and prospered by borrowing from many disciplines, it has suffered from the absence of a single goal, but its diversification has allowed for the interpersions of ideas and concepts from higher education, industry, and government. As an example of this diversification, one now finds toxicology graduate programs in medical schools, schools of public health, and schools of pharmacy as well as programs in environmental science and engineering and undergraduate programs in toxicology at several institutions. Surprisingly, courses in toxicology are now being offered in several liberal arts undergraduate schools as part of their biology and chemistry curricula. This has resulted in an exciting, innovative, and diversified field that is serving science and the community at large.

## THE 21ST CENTURY

*Genetics loads the gun but the environment pulls the trigger.*

Judith Stein

The sequencing of the human genome and several other genomes has markedly affected all biological sciences. Toxicology is

no exception. Today new animal models, especially zebrafish, *C. elegans*, and *D. melanogaster* (all of which have orthologs of human genes), are widely used in toxicology. The understanding of epigenetics is opening novel approaches to the fetal origin of adult diseases including cancers, diabetes, and neurodegenerative diseases and disorders. These are discussed in subsequent chapters.

Few disciplines can point to both basic sciences and direct applications at the same time. Toxicology—the study of the adverse effects of xenobiotics—may be unique in this regard. The mechanisms of action of the xenobiotics studied by toxicologists, in the tradition of Claude Bernard, continue to be the tools of modern biology.

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# chapter 2

## Principles of Toxicology

David L. Eaton and Steven G. Gilbert

### Introduction to Toxicology

Different Areas of Toxicology  
Toxicology and Society  
General Characteristics  
of the Toxic Response

### Classification of Toxic Agents

### Spectrum of Undesired Effects

Allergic Reactions  
Idiosyncratic Reactions  
Immediate versus Delayed Toxicity  
Reversible versus Irreversible  
Toxic Effects  
Local versus Systemic Toxicity  
Interaction of Chemicals  
Tolerance

### Characteristics of Exposure

Route and Site of Exposure  
Duration and Frequency of Exposure

### Dose–Response Relationship

Individual, or Graded,  
Dose–Response Relationships

Quantal Dose–Response  
Relationships

Shape of the Dose–Response Curve

*Essential Nutrients*

*Hormesis*

*Threshold*

*Nonmonotonic Dose–Response Curves*

Assumptions in Deriving the  
Dose–Response Relationship

Evaluating the Dose–Response  
Relationship

*Comparison of Dose Responses*

*Therapeutic Index*

*Margins of Safety and Exposure*

*Potency versus Efficacy*

### Variation in Toxic Responses

Selective Toxicity  
Species Differences  
Individual Differences in Response

### Descriptive Animal

### Toxicity Tests

Acute Toxicity Testing  
Skin and Eye Irritations

Sensitization

Subacute (Repeated-Dose Study)

Subchronic

Chronic

Developmental and Reproductive  
Toxicity

Mutagenicity

Oncogenicity Bioassays

Neurotoxicity Assessment

Immunotoxicity Assessment

Other Descriptive Toxicity Tests

### Toxicogenomics

Genomics

Epigenetics/Epigenomics

Transcriptomics

Proteomics

Metabonomics/Metabolomics

Bioinformatics

Challenges in Using “Omics”

Technologies for Predictive  
Toxicology and Risk  
Assessment

## INTRODUCTION TO TOXICOLOGY

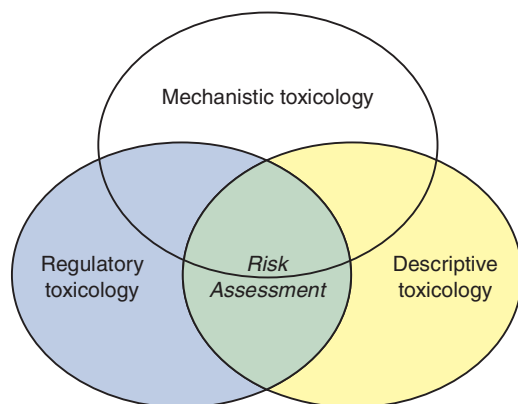
*Toxicology* is the study of the adverse effects of chemical or physical agents on living organisms. A *toxicologist* is trained to examine and communicate the nature of those effects on human, animal, and environmental health. Toxicological research examines the cellular, biochemical, and molecular mechanisms of action as well as functional effects such as neurobehavioral and immunological, and assesses the probability of their occurrence. Fundamental to this process is characterizing the relation of exposure (or dose) to the response. *Risk assessment* is the quantitative estimate of the potential effects on human health and environmental significance of various types of chemical exposures (eg, pesticide residues in food, contaminants in drinking water). The variety of potential adverse effects and the diversity of chemicals in the environment make toxicology a broad science, which often demands specialization in one area of toxicology. Our society's dependence on chemicals and the need to assess potential hazards have made toxicologists an increasingly important part of the decision-making processes.

## Different Areas of Toxicology

The professional activities of toxicologists fall into 3 main categories: descriptive, mechanistic, and regulatory (Fig. 2-1). Although each has distinctive characteristics, each contributes to the other, and all are vitally important to chemical risk assessment (see Chap. 4).

A *mechanistic toxicologist* is concerned with identifying and understanding the cellular, biochemical, and molecular mechanisms by which chemicals exert toxic effects on living organisms (see Chap. 3 for a detailed discussion of mechanisms of toxicity). The results of mechanistic studies are very important in many areas of applied toxicology. In risk assessment, mechanistic data may be very useful in demonstrating that an adverse outcome (eg, cancer, birth defects) observed in laboratory animals is directly relevant to humans. For example, the relative toxic potential of organophosphorus (OP) insecticides in humans, rodents, and insects can be accurately predicted on the basis of an understanding of common mechanisms (inhibition of acetylcholinesterase) and differences in biotransformation for these insecticides among the different species. Similarly, mechanistic data may be very useful in identifying





**Figure 2-1.** Graphical representation of the interconnections between different areas of toxicology.

adverse responses in experimental animals that may not be relevant to humans. For example, the propensity of the widely used artificial sweetener saccharin to cause bladder cancer in rats may not be relevant to humans at normal dietary intake rates. This is because mechanistic studies have demonstrated that bladder cancer is induced only under conditions where saccharin is at such a high concentration in the urine that it forms a crystalline precipitate (Cohen, 1998). Dose–response studies suggest that such high concentrations would not be achieved in the human bladder even after extensive dietary consumption.

Mechanistic data are also useful in the design and production of safer alternative chemicals and in rational therapy for chemical poisoning and treatment of disease. For example, the drug thalidomide was originally marketed in Europe and Australia as a sedative agent for pregnant women. However, it was banned for clinical use in 1962 because of devastating birth defects that occurred if the drug was ingested during a critical period in pregnancy. But mechanistic studies over the past several decades have demonstrated that this drug may have a unique molecular mechanism of action that interferes with the expression of certain genes responsible for blood vessel formation (angiogenesis). With an understanding of this mechanism, thalidomide has been “rediscovered” as a valuable therapeutic agent that may be highly effective in the treatment of certain infectious diseases (eg, leprosy) and multiple myeloma. This provides an interesting example of how a highly toxic drug with selectivity toward a specific population (pregnant women) can be used relatively safely with proper precautions. Following its approval for therapeutic use in 1998, a program was established that required all clinicians, pharmacists, and patients who receive thalidomide to enroll in a specific program (System for Thalidomide Education and Prescribing Safety [STEPS]). The population at risk for the potential teratogenic effects of thalidomide (all women of childbearing age) was required to use 2 forms of birth control, and also have a negative pregnancy test within 24 hours of beginning therapy, and periodically thereafter. Among the patients registered with the STEPS program, 6000 were females of childbearing age. Remarkably, after 6 years of use, only 1 patient actually received thalidomide during her pregnancy. She initially tested negative at the beginning of therapy; on a subsequent test she was identified as positive, and the drug was stopped. The pregnancy ended up as a miscarriage (Uhl *et al.*, 2006). Thus, a clear understanding of mechanism of action led to the development of strict prescribing guidelines and patient monitoring, thereby allowing a potentially dangerous drug to be used safely and effectively to treat disease in tens of thousands of patients who would otherwise not have benefited from the therapeutic actions of the drug (Lary *et al.*, 1999).

In addition to aiding directly in the identification, treatment, and prevention of chemical toxicity, an understanding of the mechanisms of toxic action contributes to the knowledge of basic physiology, pharmacology, cell biology, and biochemistry. The advent of new technologies in molecular biology and genomics now provides mechanistic toxicologists with the tools to explore exactly how humans may differ from laboratory animals in their response to toxic substances. These same tools are also being utilized to identify individuals who are genetically susceptible to factors in the environment or respond differently to a chemical exposure. For example, a small percentage of the population genetically lacks the ability to detoxify the chemotherapeutic drug, 6-mercaptopurine, used in the treatment of some forms of leukemia. Young children with leukemia who are homozygous for this genetic trait (about 1 in 300) may experience serious toxic effects from a standard therapeutic dose of this drug (Weinshilboum *et al.*, 1999). Numerous genetic tests for polymorphisms in drug-metabolizing enzymes and transporters are now available that can identify genetically susceptible individuals in advance of pharmacological treatment (Eichelbaum *et al.*, 2006).

The development of new approaches to identifying associations between diseases or adverse outcomes and common genetic variants (polymorphisms) has changed from a focus on individual candidate genes to “genome-wide association studies” (GWAS). GWAS are based on a rapid 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 (eg, disease state or adverse drug response). These tools have resulted in the discovery of many “gene–environment interactions,” including associations between adverse drug responses and particular genetic polymorphisms (Wang *et al.*, 2011). Moving from the single, “candidate gene” approach to genome-wide studies has led to the development of the relatively new fields of pharmacogenomics and toxicogenomics. These areas provide an exciting opportunity for mechanistic toxicologists to identify and protect genetically susceptible individuals from harmful environmental exposures, and to customize drug therapies that enhance efficacy and minimize toxicity, based on an individual’s genetic makeup.

A *descriptive toxicologist* is concerned directly with toxicity testing, which provides information for safety evaluation and regulatory requirements. The appropriate toxicity tests (as described later in this chapter and other chapters) in cell culture systems or experimental animals are designed to yield information to evaluate risks posed to humans and the environment from exposure to specific chemicals. The concern may be limited to effects on humans, as in the case of drugs and food additives. Toxicologists in the chemical industry, however, must be concerned not only with the risk posed by a company’s chemicals (insecticides, herbicides, solvents, etc) to humans but also with potential effects on fish, birds, and plants, as well as other factors that might disturb the balance of the ecosystem. Descriptive toxicology is of course not divorced from mechanistic studies, as such studies provide important clues to a chemical’s mechanism of action, and thus contribute to the development of mechanistic toxicology through hypothesis generation. Such studies are also a key component of risk assessments that are used by regulatory toxicologists. The development of so-called omics technologies (genomics, transcriptomics, proteomics, metabonomics/metabolomics, etc) forms the basis of the subdiscipline of toxicogenomics. The application of these technologies to toxicity testing is in many ways “descriptive” in nature, yet affords great mechanistic insights into how chemicals produce their toxic effects. This exciting area of toxicology is discussed in more detail later in the chapter.

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 or subsequent human or environmental exposure resulting from its use. The Food and Drug Administration (FDA) is responsible for allowing drugs, cosmetics, and food additives to be sold in the market according to the Federal Food, Drug and Cosmetic Act (FFDCA). The US Environmental Protection Agency (EPA) is responsible for regulating most other chemicals according to a variety of different legislative acts, including the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), the Toxic Substances Control Act (TSCA), the Resource Conservation and Recovery Act (RCRA), the Safe Drinking Water Act, and the Clean Air Act. In 1996, the US Congress passed the Food Quality Protection Act (FQPA) that fundamentally changed the pesticide and food safety laws to consider stricter safety standards particularly for infants and children, who were recognized as more susceptible to health effects of pesticides. The EPA is also responsible for enforcing the Comprehensive Environmental Response, Compensation and Liability Act (CERCLA, later revised as the Superfund Amendments Reauthorization Act [SARA]), more commonly called the Superfund Act. This regulation provides direction and financial support for the cleanup of waste sites that contain toxic chemicals that may present a risk to human health or the environment. The Occupational Safety and Health Administration (OSHA) of the Department of Labor was established to ensure that safe and healthful conditions exist in the workplace. The National Institute for Occupational Safety and Health (NIOSH) as part of the Centers for Disease Control and Prevention (CDC) in the Department of Health and Human Services (DHHS) is responsible for conducting research and making recommendations for the prevention of work-related injury and illness. The Consumer Product Safety Commission (CPSC) is responsible for protecting consumers from hazardous household substances, whereas the Department of Transportation (DOT) ensures that materials shipped in interstate commerce are labeled and packaged in a manner consistent with the degree of hazard they present. The Nuclear Regulatory Commission (NRC), established in 1974, regulates the civilian use of nuclear material to protect public health and safety, and the environment. 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 (see Chap. 4).

In addition to the above categories, there are other specialized areas of toxicology such as forensic, clinical, and environmental toxicology. *Forensic toxicology* is a hybrid of analytic chemistry and fundamental toxicological principles. It is concerned primarily with the medicolegal aspects of the harmful effects of chemicals on humans and animals. The expertise of forensic toxicologists is invoked primarily to aid in establishing the cause of death and determining its circumstances in a post-mortem investigation (see Chap. 31). *Clinical toxicology* designates an area of professional emphasis in the realm of medical science that is concerned with disease caused by or uniquely associated with toxic substances (see Chap. 32). 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 those intoxications. Public information about treatment and prevention is often provided through the national network of poison

control centers. *Environmental toxicology* focuses on the impacts of chemical pollutants in the environment on biological organisms. Although toxicologists concerned with the effects of environmental pollutants on human health fit into this definition, it is most commonly associated with studies on the impacts of chemicals on nonhuman organisms such as fish, birds, terrestrial animals, and plants. *Ecotoxicology* is a specialized area within environmental toxicology that focuses more specifically 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.

## Toxicology and Society

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. Knowledge about the toxicological effects of a compound affects consumer products, drugs, manufacturing processes, waste cleanup, regulatory action, civil disputes, and broad policy decisions. The expanding influence of toxicology on societal issues is accompanied by the responsibility to be increasingly sensitive to the ethical, legal, and social implications of toxicological research and testing.

The convergence of multiple elements has highlighted the evolving ethical dynamics of toxicology. First, experience and new discoveries in the biological sciences have emphasized our interconnectedness with nature and the need for well-articulated visions of human, animal, and environmental health. One vision is that we have “condition(s) that ensure that all living things have the best opportunity to reach and maintain their full genetic potential” (Gilbert, 2005a). Second, we have experience with the health consequences of exposure to such things as lead, asbestos, and tobacco, along with the detailed mechanistic research to understand the long-term risks to individuals and society. This has precipitated many regulatory and legal actions and public policy decisions, not to mention costly and time-consuming lawsuits. Third, we have an increasingly well-defined framework for discussing our social and ethical responsibilities. There is growing recognition that ethics play a crucial role in public health decision making that involves conflicts between individual, corporate, and social justice goals (Callahan and Jennings, 2002; Kass, 2001; Lee, 2002). Fourth is the appreciation that all research involving humans or animals must be conducted in a responsible and ethical manner. Fifth is managing both the uncertainty and biological variability inherent in the biological sciences. Decision making often includes making judgments with limited or uncertain information, which often includes an overlay of individual values and ethics. Finally, individuals involved in toxicological research must be aware of and accountable to their own individual biases and possible conflicts of interest and adhere to the highest ethical standards of the profession (Maurissen *et al.*, 2005; Coble *et al.*, 2009; Gilbert and Eaton, 2009).

Ethical reasoning and philosophy has a long and deep history, but more pragmatic bioethical reasoning can be traced to Leopold, who is arguably America’s first bioethicist: “A thing is right when it tends to preserve the integrity, stability, and beauty of the biotic community. It is wrong when it tends otherwise” (Leopold, 1949). The essence of toxicology is to understand the effects of chemicals on the biotic community. This broader definition of an ethic became more focused with examples such as the mercury poisoning in Minamata Bay, Japan, thalidomide, and the effects of pesticides as brought to public awareness by Carson’s *Silent Spring* (Carson, 1962). In the United States, these events supported the public and

political will to establish the EPA and strengthen the FDA and other regulations designed to protect human and environmental health. The knowledge that some segments of our society were deferentially at risk from chemical exposures evolved into an appreciation of environmental justice (Corburn, 2002; EPA, 2005; Lee, 2002; Morello-Frosch *et al.*, 2002). The EPA defines environmental justice as “the fair treatment and meaningful involvement of all people regardless of race, color, national origin, or income with respect to the development, implementation, and enforcement of environmental laws, regulations, and policies...” (EPA, 2005). Environmental justice is now an important component of numerous community-based programs of interest, and is relevant to the field of toxicology (Nweke, 2011). There is growing recognition of the direct financial and indirect costs to individuals and society from environmental exposures that are not equally distributed across society (Landrigan *et al.*, 2002).

On a parallel track, biomedical ethics developed out of the lessons of World War II and related abuses of human subjects. The 4 principle of biomedical ethics—respect for autonomy, beneficence (do good), nonmaleficence (do no harm), and justice (be fair)—became well established as a basis for decision making in health care settings (Beauchamp and Childress, 1994). These principles formed the basis of rules and regulations regarding the conduct of human research. The demands of ethics and science made it clear that the highest standards of care produced the best results in both human and animal research. Rules and regulations regarding the housing and conduct of animal studies evolved similarly. Professional toxicology societies now require their members to adhere to the highest ethical standards when conducting research with humans or animals. A further refinement and expansion of biomedical ethical principles is the development of community-based participatory research that takes into consideration community needs to ensure the best results and benefit to the community (Arcury *et al.*, 2001; Gilbert, 2006; O’Fallon and Dearry, 2002).

A glance at the daily newspaper confirms the number of current, sometimes controversial issues that are relevant to the field of toxicology. Decisions and action are often demanded or required even when there is a certain level of uncertainty in the toxicological data. The classic example of this challenge is establishing causation of the health effects of tobacco products. In part to address issues related to the health effects of tobacco products, Hill, a distinguished epidemiologist, defined a set of guidelines for evaluating “causation”—for example, whether a causal connection between a particular “exposure” and a particular outcome, condition, or disease can be scientifically established (Hill, 1965). These criteria are briefly summarized as follows:

1. Strength of association (relationship between independent and dependent variables)
2. Consistency of findings (replication of results by different studies)
3. Biological gradient (strength of the dose–response relationship)
4. Temporal sequence (“cause” before effect)
5. Biological or theoretical plausibility (mechanism of action)
6. Coherence with established knowledge (no competing hypotheses)
7. Specificity of association (cause is tightly linked to an outcome)

Although the guidelines provided by Hill were originally designed for interpretation of epidemiological data, they are equally applicable to establishing causation in toxicology, which often relies on a mix of both epidemiological and toxicological data.

Quantitative risk assessment was developed in part to address issues of uncertainty related to potential harm. The risk assessment process summarizes data for risk managers and other decision makers, who must take into consideration to some degree the qualitative elements of ethical, social, and political issues. Whereas risk management clearly has an ethical and values-based aspect, risk assessment is not immune from the influence of one’s values, bias, or perspective. Ultimately action is required and as Hill (1965) noted: “All scientific work is incomplete—whether it be observational or experimental. All scientific work is liable to be upset or modified by advancing knowledge. That does not confer upon us a freedom to ignore the knowledge we already have or postpone the action that it appears to demand at a given time.” These so-called Bradford Hill criteria were developed largely as a “weight-of-evidence” approach for interpreting a body of epidemiology data, yet are relevant as well to toxicology. Guzelian *et al.* (2005) provided a more detailed, evidence-based approach for determining causation in toxicology, primarily for application in the legal arena.

Although the scientific data may be the same, there are substantial differences in how toxicological data are used in a regulatory framework to protect public health versus establishing individual causation in the courtroom (Eaton, 2003). The approach to regulatory decision making is in part directed by policy. For example, the experience with thalidomide and other drugs motivated the US Congress to give the FDA broad power to ensure the efficacy and safety of new medicines or medical procedures. In this situation the pharmaceutical company or proponents of an activity must invest in the appropriate animal and human studies to demonstrate safety of the product. In general, a relatively precautionary approach has historically been taken with regard to drugs and medical devices. The approach to industrial chemicals is defined by the Toxic Substance Control Act and does not stipulate such a rigorous approach when introducing a new chemical into commerce.

Building on the work of Hill and others particularly from Europe, the Precautionary Principle was defined at the Wingspread Conference, in 1998: “When an activity raises threats of harm to human health or the environment, precautionary measures should be taken even if some cause and effect relationships are not fully established scientifically” (Gilbert, 2005b; Myers and Raffensperger, 2006; Raffensperger and Tickner, 1999). The precautionary principle incorporates elements of science and ethical philosophy into a single statement, acknowledging that ethics and values are part of the decision-making process. Although the conceptual value of the precautionary principle to public health protection is obvious, its actual implementation in toxicological risk assessment is not straightforward, and remains a point of considerable debate (Marchant, 2003; Goldstein, 2006; Peterson, 2006). The challenge remains to develop a regulatory environment that is responsive to issues of public health and the stewardship of societal resources (Simon, 2011).

With the increased relevance of toxicological data and evaluation in issues fundamental to society, there has been increased awareness of the possibility of conflicts of interest influencing the decision-making process (Maurissen *et al.*, 2005). The disclosure of conflicts of interest as well as the development of appropriate guidelines continues to be a challenge (NAS, 2003; Goozner, 2004; Krinsky and Rothenberg, 2001). These issues go to the core of one’s individual values and integrity in the interpretation and communication of research results. Many professional societies, including the Society of Toxicology (<http://www.toxicology.org/ai/asot/ethics.asp>), have developed codes of ethics for their members. Conflict of interest has also been addressed by most publishers of toxicology journals (Krinsky and Sweet, 2009).



Table 2-1

**Approximate Acute LD<sub>50</sub>s of Some Representative Chemical Agents**

AGENT	LD <sub>50</sub> (mg/kg)*
Ethyl alcohol	10,000
Sodium chloride	4000
Ferrous sulfate	1500
Morphine sulfate	900
Phenobarbital sodium	150
Picrotoxin	5
Strychnine sulfate	2
Nicotine	1
D-Tubocurarine	0.5
Hemicholinium-3	0.2
Tetrodotoxin	0.10
Dioxin (TCDD)	0.001
Botulinum toxin	0.00001

\*LD<sub>50</sub> is the dosage (mg/kg body weight) causing death in 50% of exposed animals.

As the field of toxicology has matured and its influence on societal issues has increased, so has the need for the profession to make a commitment to examine the ethical, legal, and social implications of research and practice of toxicology.

## General Characteristics of the Toxic Response

One could define a poison as any agent capable of producing a deleterious response in a biological system, seriously injuring function or producing death. This is not, however, a useful working definition for the very simple reason that virtually every known chemical has the potential to produce injury or death if it is present in a sufficient amount. Paracelsus (1493–1541), a Swiss/German/Austrian physician, scientist, and philosopher, phrased this well when he noted, “What is there that is not poison? All things are poison and nothing [is] without poison. Solely the dose determines that a thing is not a poison.”

Among chemicals there is a wide spectrum of doses needed to produce deleterious effects, serious injury, or death. This is demonstrated in Table 2-1, which shows the dosage of chemicals needed to produce death in 50% of treated animals (lethal dose 50 [LD<sub>50</sub>]). Some chemicals produce death in microgram doses and are commonly thought of as being extremely poisonous. Other chemicals may be relatively harmless after doses in excess of several grams. It should be noted, however, that measures of acute lethality such as LD<sub>50</sub> do not accurately reflect the full spectrum of toxicity, or hazard, associated with exposure to a chemical. For example, some chemicals with low acute toxicity may have carcinogenic, teratogenic, or neurobehavioral effects at doses that produce no evidence of acute toxicity. In addition, there is growing recognition that genetic factors can account for individual susceptibility to a range of responses. Finally, it should be recognized that, for a given chemical, multiple different effects can occur in a given

organism, each with its own “dose–response relationship.” In some circumstances, effects that occur at low doses may not be evident at higher doses because other adverse responses overwhelm or mask more subtle effects that may occur at low doses. Although some have argued that such low-dose effects, not seen at higher doses, make the classical interpretation of the “dose–response” relationship no longer relevant, such low-dose effects also follow their own “dose–response” relationship, but with a “saturation” of the effect occurring at higher doses that induces other molecular, biochemical, and cellular effects that tend to obscure the effects seen at lower doses. The effects of exogenous chemicals that bind to and activate or inhibit endogenous hormone receptors (so-called endocrine disruptors—see Chap. 21) may often have “low-dose” effects that are quite different from those seen at much higher doses.

## CLASSIFICATION OF TOXIC AGENTS

Toxic agents are classified in a variety of ways, depending on the interests and needs of the classifier. In this textbook, for example, toxic agents are discussed in terms of their target organs (liver, kidney, hematopoietic system, etc), use (pesticide, solvent, food additive, etc), source (animal and plant toxins), and effects (cancer, mutation, liver injury, etc). The term *toxin* generally refers to toxic substances that are produced by biological systems such as plants, animals, fungi, or bacteria. The term *toxicant* is used in speaking of toxic substances that are produced by or are a by-product of anthropogenic (human-made) activities. Thus, zearalenone, produced by a mold, is a toxin, whereas “dioxin” (2,3,7,8-tetrachlorodibenzo-*p*-dioxin [TCDD]), produced during the production and/or combustion of certain chlorinated organic chemicals, is a toxicant. Some toxicants can be produced by both natural and anthropogenic activities. For example, polyaromatic hydrocarbons are produced by the combustion of organic matter, which may occur both through natural processes (eg, forest fires) and through anthropogenic activities (eg, combustion of coal for energy production; cigarette smoking). Arsenic, a toxic metalloid, may occur as a natural contaminant of groundwater or may contaminate groundwater secondary to industrial activities. Generally, such toxic substances are referred to as toxicants, rather than toxins, because, although they are naturally produced, they are not produced by biological systems. Distinguishing a “toxin” from a “toxicant” is not always easy. For example, many pesticides, such as the pyrethroids, are synthetic analogs of natural products, such that one would call the pyrethrum found in the chrysanthemum flower a “toxin,” but the synthetic (and slightly altered in structure) form produced for use in pesticide formulations would be a “toxicant.” Thus, although technically incorrect, many physicians and others involved in the diagnosis and treatment of poisonings often use the term “toxin” to refer to any toxic substance, regardless of origin.

Toxic agents may also be classified in terms of their physical state (gas, dust, liquid, size, eg, nanotoxicology), their chemical stability or reactivity (explosive, flammable, oxidizer), general chemical structure (aromatic amine, halogenated hydrocarbon, etc), or poisoning potential (extremely toxic, very toxic, slightly toxic, etc). Classification of toxic agents on the basis of their biochemical mechanisms of action (eg, alkylating agent, cholinesterase inhibitor, endocrine disruptor) is usually more informative than classification by general terms such as irritants and corrosives. But more general classifications such as air pollutants, occupation-related agents, and acute and chronic poisons can provide a useful focus on a specific problem. It is evident from this discussion that no single classification is applicable to the entire spectrum of toxic agents and that a combination of classification systems or a classification based on

other factors is generally needed to provide the best characterization of a toxic substance. Nevertheless, classification systems that take into consideration both the chemical and the biological properties of an agent and the exposure characteristics are most likely to be useful for regulatory or control purposes and for toxicology in general.

## SPECTRUM OF UNDESIRABLE EFFECTS

The spectrum of undesired effects of chemicals is often broad. Some effects are deleterious and others are not. In therapeutics, for example, each drug produces a number of effects, but usually only one effect is associated with the primary objective of the therapy; all the other effects are referred to as *undesirable* or *side effects* of that drug for that therapeutic indication. However, some of these side effects may be desired for another therapeutic indication. For example, the “first-generation” antihistamine diphenhydramine (Benadryl) is effective in reducing histamine responses associated with allergies, but it readily enters the brain and causes mild central nervous system (CNS) depression (drowsiness, delayed reaction time). With the advent of selective histamine receptor antagonists that do not cross the blood–brain barrier and thus do not have this CNS-depressant side effect, diphenhydramine is used less commonly today as an antihistamine. However, it is widely used as an “over-the-counter” sleep remedy, often in combination with analgesics (eg, Tylenol PM, Excedrin PM), taking advantage of the CNS-depressant effects. Some side effects of drugs are never desirable and are always deleterious to the well-being of humans. These are referred to as the *adverse*, *deleterious*, or *toxic* effects of the drug.

## Allergic Reactions

*Chemical allergy* is an immunologically mediated adverse reaction to a chemical resulting from previous sensitization to that chemical or to a structurally similar one. The term *hypersensitivity* is most often used to describe this allergic state, but *allergic reaction* and *sensitization reaction* are also used to describe this situation when preexposure of the chemical is required to produce the toxic effect (see Chap. 12). Once sensitization has occurred, allergic reactions may result from exposure to relatively very low doses of chemicals; therefore, population-based dose–response curves for allergic reactions have seldom been obtained. Because of this omission, some people assumed that allergic reactions are not dose-related. Thus, they do not consider the allergic reaction to be a true toxic response. However, for a given allergic individual, allergic reactions are dose-related. For example, it is well known that the allergic response to pollen in sensitized individuals is related to the concentration of pollen in the air. In addition, because the allergic response is an undesirable, adverse, deleterious effect, it obviously is also a toxic response. Sensitization reactions are sometimes very severe and may be fatal.

Most chemicals and their metabolic products are not sufficiently large to be recognized by the immune system as a foreign substance and thus must first combine with an endogenous protein to form an antigen (or immunogen). A molecule that must combine with an endogenous protein to elicit an allergic reaction is called a *hapten*. The hapten–protein complex (antigen) is then capable of eliciting the formation of antibodies, and usually at least one or two weeks is required for the synthesis of significant amounts of antibodies. Subsequent exposure to the chemical results in an antigen–antibody interaction, which provokes the typical manifestations of allergy. The manifestations of allergy are numerous.

They may involve various organ systems and range in severity from minor skin disturbance to fatal anaphylactic shock. The pattern of allergic response differs in various species. In humans, involvement of the skin (eg, dermatitis, urticaria, and itching) and involvement of the eyes (eg, conjunctivitis) are most common, whereas in guinea pigs, bronchiolar constriction leading to asphyxia is the most common. However, chemically induced asthma (characterized by bronchiolar constriction) certainly does occur in some humans, and the incidence of allergic asthma has increased substantially in recent years. Hypersensitivity reactions are discussed in more detail in Chap. 12.

## Idiosyncratic Reactions

*Chemical idiosyncrasy* refers to a genetically determined abnormal reactivity to a chemical (Goldstein *et al.*, 1974; Levine, 1978; Uetrecht, 2007). The response observed is usually qualitatively similar to that observed in all individuals but may take the form of extreme sensitivity to low doses or extreme insensitivity to high doses of the chemical. However, while some people use the term *idiosyncratic* as a catchall to refer to all reactions that occur with low frequency, it should not be used in that manner (Goldstein *et al.*, 1974). A classic example of an idiosyncratic reaction is provided by patients who exhibit prolonged muscular relaxation and apnea (inability to breathe) lasting several hours after a standard dose of succinylcholine. Succinylcholine usually produces skeletal muscle relaxation of only short duration because of its very rapid metabolic degradation by an enzyme that is present normally in the bloodstream called plasma butyrylcholinesterase (also referred to as pseudocholinesterase). Patients exhibiting this idiosyncratic reaction have a genetic polymorphism in the gene for the enzyme butyrylcholinesterase, which results in a protein that is less active in breaking down succinylcholine. Family pedigree and molecular genetic analyses have demonstrated that the presence of low plasma butyrylcholinesterase activity is due to the presence of one or more single-nucleotide polymorphisms (SNPs) in this gene (Bartels *et al.*, 1992). Similarly, there is a group of people who are abnormally sensitive to nitrites and certain other chemicals that have in common the ability to oxidize the iron in hemoglobin to produce *methemoglobin*, which is incapable of carrying oxygen to the tissues. The unusual phenotype is inherited as an autosomal recessive trait and is characterized by a deficiency in NADH-cytochrome  $b_5$  reductase activity. The genetic basis for this idiosyncratic response has been identified as a single nucleotide change in codon 127, which results in replacement of serine with proline (Kobayashi *et al.*, 1990). The consequence of this genetic deficiency is that these individuals may suffer from a serious lack of oxygen delivery to tissues after exposure to doses of methemoglobin-producing chemicals that would be harmless to individuals with normal NADH-cytochrome  $b_5$  reductase activity.

It is now recognized that many of the so-called idiosyncratic adverse drug reactions and many drug–drug interactions are due to specific genetic polymorphisms in drug-metabolizing enzymes, transporters, or receptors. As discussed previously, the growing field of pharmacogenomics and toxicogenomics has helped to identify the molecular basis for many previously described idiosyncratic responses to drugs and other toxic substances (Wang *et al.*, 2011). However, not all “idiosyncratic” responses to toxic substances are easily described by a single genetic polymorphism in a drug-metabolizing enzyme. It is generally thought that most, but not all, idiosyncratic drug responses are due to a combination of individual differences in the ability to: (1) form a reactive intermediate (usually through oxidation to an electrophilic intermediate), (2) detoxify

that reactive intermediate (usually through hydrolysis or conjugation), and/or (3) exhibit differences in immune response to adducted proteins (Uetrecht, 2007). The role of the immune system in mediating rare drug-induced toxic reactions in the liver, skin, and other organ systems is widely recognized, and specific genetic variants in certain parts of the genome that code for the major histocompatibility complexes (MHCs) give rise to specific immune responses to proteins that have been damaged by reactive intermediates of certain drugs. Thus, it is only the individuals who genetically form sufficient amounts of a reactive drug metabolite, and who then have an immune response to the modified protein, who have an adverse response to the drug (Uetrecht, 2007).

For example, troglitazone, introduced into the marketplace in 1997 as an effective treatment for type II diabetes, was subsequently withdrawn from the market because of a relatively rare (1 adverse response per 30,000 patients) but often fatal hepatotoxic response. Subsequent studies of tissues from patients who had developed hepatotoxic responses at the normal therapeutic doses revealed that individuals who lacked functional genes for 2 forms of glutathione *S*-transferase (GSTM1 and GSTT1) were more than 3 times as likely to develop troglitazone-induced hepatotoxicity than individuals with 1 or more functional GSTM1 or T1 genes (Ikeda, 2011). However, this does not explain the rarity of the adverse response, since there were many individuals who lacked GSTM1 and T1 genes who took troglitazone with no evident hepatotoxicity. Further studies have suggested that the idiosyncratic hepatotoxicity from troglitazone also has an immune system component, and genetic differences in specific human lymphocyte antigen (HLA) loci might contribute to idiosyncratic drug-induced hepatotoxicity (Ikeda, 2011).

## Immediate versus Delayed Toxicity

Immediate toxic effects can be defined as those that occur or develop rapidly after a single administration of a substance, whereas delayed toxic effects are those that occur after the lapse of some time. Carcinogenic effects of chemicals usually have a long latency period, often 20 to 30 years after the initial exposure, before tumors are observed in humans. For example, daughters of mothers who took diethylstilbestrol (DES) during pregnancy have a greatly increased risk of developing vaginal cancer, in young adulthood, approximately 20 to 30 years after their in utero exposure to DES (Hatch *et al.*, 1998). Also, delayed neurotoxicity is observed after exposure to some OP insecticides that act by covalent modification of an enzyme referred to as *neuropathy target esterase* (NTE), a neuronal protein with serine esterase activity (Glynn *et al.*, 1999). Binding of certain OP chemicals to this protein initiates degeneration of long axons in the peripheral and CNS. The most notorious of the compounds that produce this type of neurotoxic effect is triorthocresylphosphate (TOCP). The effect is not observed until at least several days after exposure to the toxic compound. In contrast, most substances produce immediate toxic effects but do not produce delayed effects.

## Reversible versus Irreversible Toxic Effects

Some toxic effects of chemicals are reversible, and others are irreversible. If a chemical produces pathological injury to a tissue, the ability of that tissue to regenerate largely determines whether the effect is reversible or irreversible. Thus, for a tissue such as liver, which has a high ability to regenerate, most injuries are reversible, whereas injury to the CNS is largely irreversible because differentiated cells of the CNS cannot divide and be replaced (although recovery from chemically induced damage to the CNS can occur,

primarily through the “plasticity” of the brain that allows developed neurons to learn new functions; see Chap. 16). Carcinogenic and teratogenic effects of chemicals, once they occur, are usually considered irreversible toxic effects.

## Local versus Systemic Toxicity

Another distinction between types of effects is made on the basis of the general site of action. Local effects are those that occur at the site of first contact between the biological system and the toxicant. Such effects are produced by the ingestion of caustic substances or the inhalation of irritant materials. For example, chlorine gas reacts with lung tissue at the site of contact, causing damage and swelling of the tissue, with possibly fatal consequences, even though very little of the chemical is absorbed into the bloodstream. The alternative to local effects is systemic effects. Systemic effects require absorption and distribution of a toxicant from its entry point to a distant site at which deleterious effects are produced. Most substances except highly reactive materials produce systemic effects. For some materials, both effects can be demonstrated. For example, tetraethyl lead produces effects on skin at the site of absorption and then is transported systemically to produce its typical effects on the CNS and other organs. If the local effect is marked, there may also be indirect systemic effects. For example, kidney damage after a severe acid burn is an indirect systemic effect because the toxicant does not reach the kidney.

Most chemicals that produce systemic toxicity do not cause a similar degree of toxicity in all organs; instead, they usually elicit their major toxicity in only 1 or 2 organs. These sites are referred to as the *target organs* of toxicity of a particular chemical. The target organ of toxicity is often not the site of the highest concentration of the chemical. For example, lead is concentrated in bone, but its toxicity is due to its effects in soft tissues, particularly the brain. DDT is concentrated in adipose tissue but produces no known toxic effects in that tissue.

The target organ of toxicity most frequently involved in systemic toxicity is the CNS (brain and spinal cord). Even with many compounds having a prominent effect elsewhere, damage to the CNS can be demonstrated by the use of appropriate and sensitive methods. Next in order of frequency of involvement in systemic toxicity are the circulatory system; the blood and hematopoietic system; visceral organs such as the liver, kidney, and lung; and the skin. Muscle and bone are least often the target tissues for systemic effects. With substances that have a predominantly local effect, the frequency with which tissues react depends largely on the portal of entry (skin, gastrointestinal tract, or respiratory tract).

## Interaction of Chemicals

Because of the large number of different chemicals an individual may come in contact with at any given time (workplace, drugs, diet, hobbies, etc), it is necessary, in assessing the spectrum of responses, to consider how different chemicals may interact with each other. Interactions can occur in a variety of ways. Chemical interactions are known to occur by a number of mechanisms, such as alterations in absorption, protein binding, and the biotransformation and excretion of 1 or both of the interacting toxicants. In addition to these modes of interaction, the response of the organism to combinations of toxicants may be increased or decreased because of toxicological responses at the site of action.

The effects of 2 chemicals given simultaneously produce a response that may simply be additive of their individual responses or may be greater or less than that expected by addition of their individual responses. The study of these interactions often leads to a



better understanding of the mechanism of toxicity of the chemicals involved. A number of terms have been used to describe pharmacological and toxicological interactions. An *additive* effect occurs when the combined effect of 2 chemicals is equal to the sum of the effects of each agent given alone (eg,  $2 + 3 = 5$ ). The effect most commonly observed when 2 chemicals are given together is an additive effect. For example, when 2 OP insecticides are given together, the cholinesterase inhibition is usually additive. A *synergistic* effect occurs when the combined effects of 2 chemicals are much greater than the sum of the effects of each agent given alone (eg,  $2 + 2 = 20$ ). For example, both carbon tetrachloride and ethanol are hepatotoxic compounds, but together they produce much more liver injury than the mathematical sum of their individual effects on liver at a given dose would suggest. *Potentiation* occurs when 1 substance does not have a toxic effect on a certain organ or system but when added to another chemical makes that chemical much more toxic (eg,  $0 + 2 = 10$ ). Isopropanol, for example, is not hepatotoxic, but when it is administered in addition to carbon tetrachloride, the hepatotoxicity of carbon tetrachloride is much greater than when it is given alone. *Antagonism* occurs when 2 chemicals administered together interfere with each other's actions or 1 interferes with the action of the other (eg,  $4 + 6 = 8$ ;  $4 + (-4) = 0$ ;  $4 + 0 = 1$ ). Antagonistic effects of chemicals are often very desirable in toxicology and are the basis of many antidotes. There are 4 major types of antagonism: functional, chemical, dispositional, and receptor. *Functional antagonism* occurs when 2 chemicals counterbalance each other by producing opposite effects on the same physiological function. For example, advantage is taken of this principle in that the blood pressure can markedly fall during severe barbiturate intoxication, which can be effectively antagonized by the intravenous administration of a vasopressor agent such as norepinephrine or metaraminol. Similarly, many chemicals, when given at toxic dose (TD) levels, produce convulsions, and the convulsions often can be controlled by giving anticonvulsants such as the benzodiazepines (eg, diazepam). *Chemical antagonism or inactivation* is simply a chemical reaction between 2 compounds that produces a less toxic product. For example, 2,3-dimercaptosuccinic acid (DMSA; Succimer) chelates with metal ions such as arsenic, mercury, and lead and decreases their toxicity. The use of antitoxins in the treatment of various animal toxins is also an example of chemical antagonism. The use of the strongly basic low-molecular-weight protein protamine sulfate to form a stable complex with heparin, which abolishes its anticoagulant activity, is another example. *Dispositional antagonism* occurs when the disposition—that is, 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 are diminished. Thus, the prevention of absorption of a toxicant by ipecac or charcoal and the increased excretion of a chemical by administration of an osmotic diuretic or alteration of the pH of the urine are examples of dispositional antagonism. If the parent compound is responsible for the toxicity of the chemical (such as the anticoagulant warfarin) and its metabolic breakdown products are less toxic than the parent compound, increasing the compound's biotransformation (metabolism) by administering a drug that increases the activity of the metabolizing enzymes (eg, a "microsomal enzyme inducer" such as phenobarbital) will decrease its toxicity. However, if the chemical's toxicity is largely due to a metabolic product (as in the case of the organophosphate insecticide parathion), inhibiting its biotransformation by an inhibitor of microsomal enzyme activity (SKF-525A or piperonyl butoxide) will decrease its toxicity. *Receptor antagonism* occurs when 2 chemicals that bind to the same receptor produce less of an effect when given together than the addition of their separate effects

(eg,  $4 + 6 = 8$ ) or when 1 chemical antagonizes the effect of the second chemical (eg,  $0 + 4 = 1$ ). Receptor antagonists are often termed *blockers*. This concept is used to advantage in the clinical treatment of poisoning. For example, the receptor antagonist naloxone is used to treat the respiratory depressive effects of morphine and other morphine-like narcotics by competitive binding to the same receptor. Another example of receptor antagonism is the use of the antiestrogen drug tamoxifen to lower breast cancer risk among women at high risk for this estrogen-related cancer. Tamoxifen competitively blocks estradiol from binding to its receptor. Treatment of organophosphate insecticide poisoning with atropine is an example not of the antidote competing with the poison for the receptor (cholinesterase) but of blocking the receptor (cholinergic receptor) for the excess acetylcholine that accumulates by poisoning of the cholinesterase by the organophosphate (see Chap. 22).

## Tolerance

Tolerance is a state of decreased responsiveness to a toxic effect of a chemical resulting from prior exposure to that chemical or to a structurally related chemical. Two major mechanisms are responsible for tolerance: 1 is due to a decreased amount of toxicant reaching the site where the toxic effect is produced (*dispositional tolerance*) and the other is due to a reduced responsiveness of a tissue to the chemical. Comparatively less is known about the cellular mechanisms responsible for altering the responsiveness of a tissue to a toxic chemical than is known about dispositional tolerance. Two chemicals known to produce dispositional tolerance are carbon tetrachloride and cadmium. The barbiturate, phenobarbital, produces tolerance to itself by increasing the expression of enzymes in the liver that are responsible for its biotransformation to pharmacologically inactive products, a process known as "biotransformation enzyme induction." The mechanism of cadmium tolerance is explained by induction of metallothionein, a metal-binding protein. Subsequent binding of cadmium to metallothionein rather than to critical cellular macromolecules decreases its toxicity.

## CHARACTERISTICS OF EXPOSURE

Toxic effects in a biological system are not produced by a chemical agent unless that agent or its metabolic breakdown (biotransformation) products reach appropriate sites in the body at a concentration and for a length of time sufficient to produce a toxic manifestation. Many chemicals are of relatively low toxicity in the "native" form but, when acted on by enzymes in the body, are converted to intermediate forms that interfere with normal cellular biochemistry and physiology. Thus, whether a toxic response occurs is dependent on the chemical and physical properties of the agent, the exposure situation, how the agent 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 or subject. Thus, to characterize fully the potential hazard of a specific chemical agent, we need to know not only what type of effect it produces and the dose required to produce that effect but also information about the agent, the exposure, and its disposition by the subject. Two major factors that influence toxicity as it relates to the exposure situation for a specific chemical are the route of exposure and the duration and frequency of exposure.

## Route and Site of Exposure

The major routes (pathways) by which toxic agents gain access to the body are through the gastrointestinal tract (ingestion), the lungs (inhalation), or the skin (topical, percutaneous, or dermal). Toxic

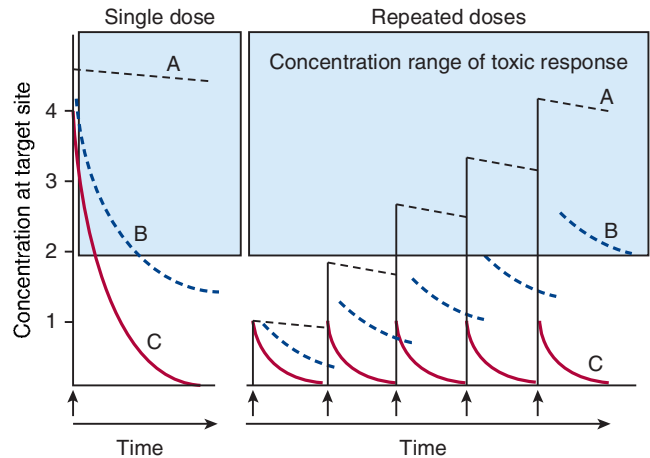
agents generally produce the greatest effect and the most rapid response when given directly into the bloodstream (the intravenous route). An approximate descending order of effectiveness for the other routes would be inhalation, intraperitoneal, subcutaneous, intramuscular, intradermal, oral, and dermal. The “vehicle” (the material in which the chemical is dissolved) and other formulation factors can markedly alter absorption after ingestion, inhalation, or topical exposure. In addition, the route of administration can influence the toxicity of agents. For example, an agent 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 of the dose pass through the liver before reaching the systemic circulation and then the CNS.

Occupational exposure to toxic agents 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 poisoning occurs most frequently by oral ingestion. Comparison of the toxic dose (TD) of a toxic substance by different routes of exposure often provides useful information about its extent of absorption. In instances when the TD after oral or dermal administration is similar to the TD after intravenous administration, the assumption is that the toxic agent is absorbed readily and rapidly. Conversely, in cases where the TD by the dermal route is several orders of magnitude higher than the oral TD, it is likely that the skin provides an effective barrier to absorption of the agent. Toxic effects by any route of exposure can also be influenced by the concentration of the agent in its vehicle, the total volume of the vehicle and the properties of the vehicle to which the biological system is exposed, and the rate at which exposure occurs. Studies in which the concentration of a chemical in the blood is determined at various times after exposure are often needed to clarify the role of these and other factors in the toxicity of a compound. For more details on the absorption of toxicants, see Chap. 5.

### Duration and Frequency of Exposure

Toxicologists usually divide the exposure of experimental animals to chemicals into 4 categories: acute, subacute, subchronic, and chronic. Acute exposure is defined as exposure to a chemical for less than 24 hours, and examples of exposure routes are intraperitoneal, intravenous, and subcutaneous injection; oral intubation; and dermal application. Whereas 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 3 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 3 categories of repeated exposure can be by any route, but most often they occur by the oral route, with the chemical added directly to the diet.

In human exposure situations, the frequency and duration of exposure are usually not as clearly defined as in controlled animal studies, but 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).



**Figure 2-2.** 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 (eg, half-life of 1 year). (Line B) A chemical with a rate of elimination equal to frequency of dosing (eg, 1 day). (Line C) Rate of elimination faster than the dosing frequency (eg, 5 hours). Blue shaded area is representative of the concentration of chemical at the target site necessary to elicit a toxic response.

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. Acute exposure to chemicals that are rapidly absorbed is likely to produce immediate toxic effects but also can produce delayed toxicity that may or may not be similar to the toxic effects of chronic exposure. Conversely, 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. In characterizing the toxicity of a specific chemical, it is evident that information is needed not only for the single-dose (acute) and long-term (chronic) effects but also for exposures of intermediate duration. The other time-related factor that is important in the temporal characterization of repeated exposures is the frequency of exposure. The relationship between elimination rate and frequency of exposure is shown in Fig. 2-2. 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. 2-2, 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 2 concentration units in Fig. 2-2) is not reached until the fourth dose, whereas that concentration is reached with only 2 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 may also, in fact, be totally 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 Chaps. 5 and 7.

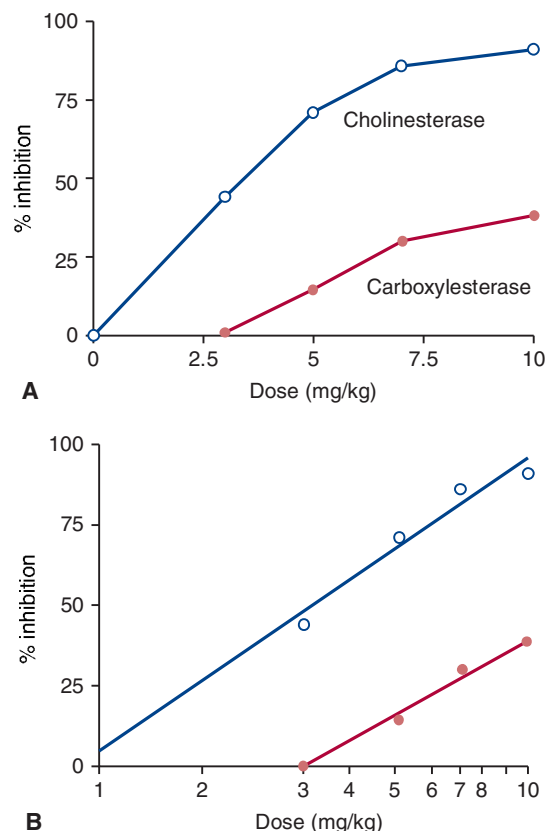
## DOSE-RESPONSE RELATIONSHIP

The characteristics of exposure and the spectrum of toxic effects come together in a correlative relationship customarily referred to as the *dose-response relationship*. Whatever response is selected for measurement, the relationship between the degree of response of the biological system and the amount of toxicant administered assumes a form that occurs so consistently as to be considered the most fundamental and pervasive concept in toxicology.

From a practical perspective, there are 2 types of dose-response relationships: (1) the individual dose-response relationship, which describes the response of an *individual* organism to varying 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 individual organisms. It is also important to recognize that a given chemical may have multiple different molecular, biochemical, and cellular effects, each with its own “dose-response” relationship. Thus, the nature of a toxic response might very well be different at low doses than at higher doses. In the case of population-level “dose-response” characterization, the observed response is an integration of multiple individual “dose-response relationships” occurring in different cell types, and at different molecular sites within those cells. Subtle effects that occur at low doses may be masked or overwhelmed by more evident responses occurring at higher doses.

### Individual, or Graded, Dose-Response Relationships

Individual dose-response relationships are characterized by a dose-related increase in the severity of the response. The dose-relatedness of the response often results from an alteration of a specific biochemical process. For example, Fig. 2-3 shows the dose-response relationship between different dietary doses of the organophosphate insecticide chlorpyrifos and the extent of inhibition of 2 different enzymes in the brain and liver: acetylcholinesterase and carboxylesterase. In the brain, the degree of inhibition of both enzymes is clearly dose-related and spans a wide range, although the amount of inhibition per unit dose is different for the 2 enzymes. From the shapes of these 2 dose-response curves it is evident that, in the brain, cholinesterase is more easily inhibited than carboxylesterase. The toxicological response that results 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 similar to that for brain cholinesterase. However, as noted above, for many chemicals, more than 1 effect may result because of multiple different target sites in different tissues. Thus, the observed response to varying doses of a chemical in the whole organism is often complicated by the fact that most toxic substances have multiple sites or mechanisms of toxicity, each with its own “dose-response” relationship and subsequent adverse effect. Note that when these dose-response data are plotted using the base 10 log of the dose on the abscissa (Fig. 2.3B), a better “fit” of the data to a straight line usually occurs. This is typical of many graded as well as quantal dose-response relationships.



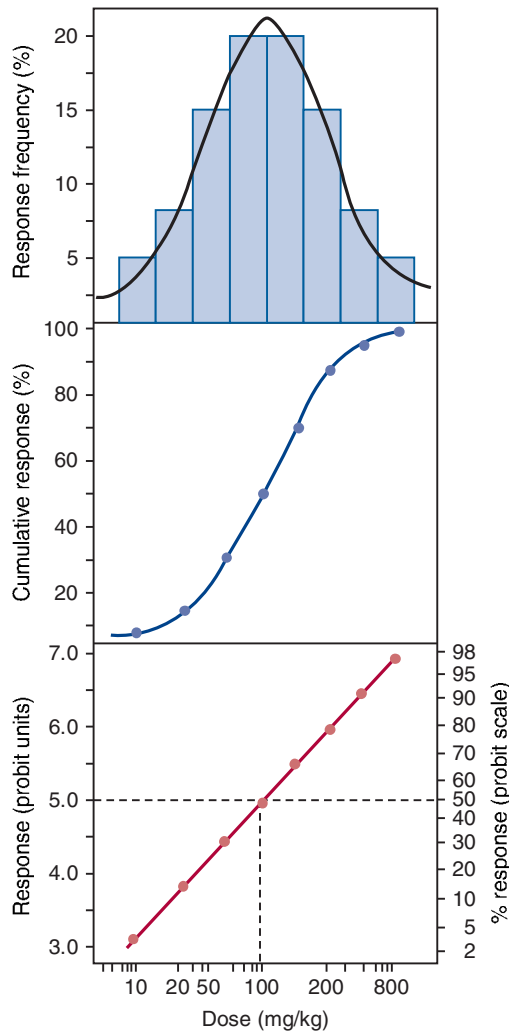
**Figure 2-3.** Dose-response relationship between different doses of the organophosphate insecticide chlorpyrifos and esterase enzyme inhibition in the brain. Open circles and blue lines represent acetylcholinesterase activity and closed circles represent carboxylesterase activity in the brains of pregnant female Long-Evans rats given 5 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 *et al.*, 1999, with permission.)

### 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 by definition 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 2 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 in administered doses.

A widely used statistical approach for estimating the response of a population to a toxic exposure is the “effective dose” (ED). Generally, the midpoint, or 50%, response level is used, giving rise to the “ED<sub>50</sub>” value. However, any response level, such as an ED<sub>01</sub>, ED<sub>10</sub>, or ED<sub>30</sub>, could be chosen. A graphical representation of an approximate ED<sub>50</sub> is shown in Fig. 2-4. Note that these data are “quantal.” Where death is the measured end point, the ED<sub>50</sub> would be referred to as the LD<sub>50</sub>. Historically, determination of the LD<sub>50</sub> was often the first experiment performed with a new chemical. Today, it is widely recognized that the LD<sub>50</sub> is of marginal value as a measure of hazard, although it does provide a useful “ball park” indication of the relative hazard of a compound to cause serious, life-threatening poisoning from a single exposure. Although death is an obvious quantal end point to measure, it should be noted that any





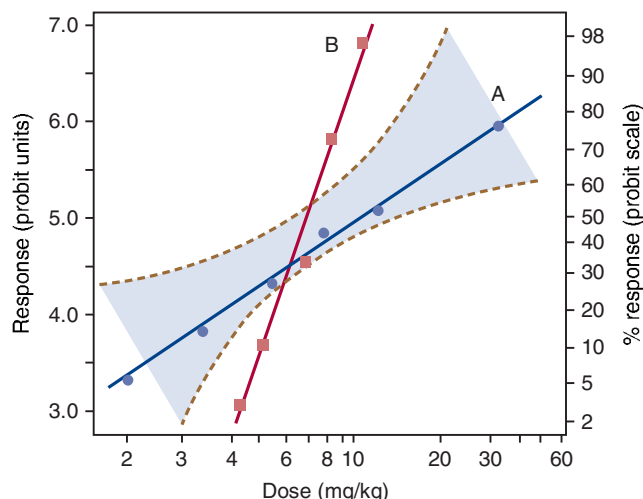
**Figure 2-4.** Diagram of quantal dose-response relationship. The abscissa is a log dosage of the chemical. In the top panel the ordinate is response frequency, in the middle panel the ordinate is percent response, and in the bottom panel the response is in probit units (see text).

quantal response could be used. For example, the  $LD_{50}$  of lead or DDT is not a relevant end point when characterizing hazards of the agents to children or wildlife, respectively. Even continuous variables can be converted to quantal responses if desired. For example, an antihypertensive drug that lowers blood pressure might be evaluated in a population by assigning a “responder” as an individual whose blood pressure was lowered by 10 mm Hg or more. Note that, in this example, an individual who responded to a change in blood pressure of 50 mm Hg would be classified the same as an individual with a change in only 10 mm Hg, yet an individual with a change in 8 mm Hg would be classified as a “nonresponder.” The top panel of Fig. 2-4 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 2 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 susceptibility to chemicals among individuals; 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 called *resistant*. If the numbers 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 is used with a large number of animals per dose, a sigmoid dose-response curve is observed, as depicted in the middle panel of Fig. 2-4. With the lowest dose (6 mg/kg), 1% of the animals respond. A normally distributed sigmoid curve such as this one 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 minimally ED of any chemical that evokes a stated all-or-none response is called the *threshold dose* even though it cannot be determined experimentally.

For a normally distributed population response, the sigmoid curve has a relatively linear portion between 16% and 84%. These values represent the limits of 1 standard deviation (SD) of the mean (and the median) in a population with truly normal or Gaussian distribution. However, it is usually not practical to describe the dose-response curve from this type of plot because one does not usually have large enough sample sizes to define the sigmoid curve adequately. In a normally distributed population, the mean  $\pm 1$  SD represents 68.3% of the population, the mean  $\pm 2$  SD represents 95.5% of the population, and the mean  $\pm 3$  SD equals 99.7% of the population. Because quantal dose-response phenomena are usually normally distributed, 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; an NED of +1 is equated with an 84.1% response. Traditionally, units of NED are converted by the addition of 5 to the value to avoid negative numbers; these converted units are called *probit units* (Bliss, 1957). The probit (from the contraction of *probability unit*), then, is an 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 2 panels of Fig. 2-4 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. In essence, what is accomplished in a probit transformation is an adjustment of quantal data to an assumed normal population distribution, resulting in a straight line. The  $ED_{50}$  is obtained by drawing a horizontal line from the 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. It is evident from the line that 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. 2-5). In addition to the  $ED_{50}$ , the slope of the dose-response curve can also be obtained. Fig. 2-5 demonstrates the dose-response curves for the response of 2 compounds. Compound A exhibits a “flat” dose-response curve, showing that a large change in dosage 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 dosage 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 2-5.** Comparison of dose-response relationship for 2 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. 2-4 and 2-5 the dosage has been given on a log basis. Although the use of the log of the dosage is empiric, log dosage plots for normally distributed quantal data provide a more nearly linear representation of the data. It must be remembered, however, that this is not universally the case. Some radiation effects, for example, give a better probit fit when the dose is expressed arithmetically rather than logarithmically. There are other situations in which other functions (eg, exponentials) of dosage provide a better fit to the data than does the log function. It is also conventional to express the dosage in milligrams per kilogram. It might be argued that expression of dosage on a mole-per-kilogram basis would be better, particularly for making comparisons among a series of compounds. Although such an argument has considerable merit, dosage is usually expressed in milligrams per kilogram.

One might also view dosage on the basis of body weight as being less appropriate than other bases, such as surface area. The term *allometry* refers to the field of study that examines the relationships between body weight and other biological and physical parameters such as rate of basal metabolism (caloric consumption),

heart rate, blood flow, etc. Allometric studies revealed that the relationship between body weight and various other physiological parameters can be closely estimated by the following formula:  $Y = aW^b$ , where  $Y$  is the biological parameter of interest and  $a$  and  $b$  are constants that relate  $Y$  to body weight (Rodricks *et al.*, 2008). 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. The relationship between body surface area and body weight across most mammalian species is closely described by the formula  $SA = 10.5 \times (\text{body weight [grams]})^{0.67}$  (Harkness and Wagner, 1995). Empirical comparisons of toxicity data across species confirm that this relationship is appropriate for toxicological scaling. For example, Travis and White (1988) analyzed a number of toxicity testing data sets for 27 different chemotherapeutic drugs for which toxicity data were available in mouse, rat, hamster, dog, monkey, and human. They found that the exponent of body weight that gave the best correlation with toxicity was 0.73, with 95% confidence bounds of 0.69 to 0.77 (Rodricks *et al.*, 2008). Table 2-2 illustrates the differences in comparative doses when scaling is done by body weight (mg/kg) versus an allometric approach that uses an exponent of either 0.67 or 0.75. Thus, if a scaling factor of  $(BW)^{2/3}$  is used, a mouse would need to receive a dose 13 times greater than that required for humans for an equivalent toxic response, whereas the dose would be 7 times greater if a scaling factor of  $(BW)^{3/4}$  was used. However, not all toxic responses will necessarily scale across species in the same way. For example, acute lethality seemed to correlate better across species when body weight, rather than body surface area, was used (Rhomberg and Wolff, 1998). The selection of the most appropriate scaling factor should also take into account pharmacokinetic differences, including physiologically based pharmacokinetic modeling (PBPK). When toxicity is attributable to the formation of a toxic metabolite, or when xenobiotic biotransformation is saturated at high doses, a scaling factor of 1 may be more appropriate than 0.75 (Kirman *et al.*, 2003).

## Shape of the Dose-Response Curve

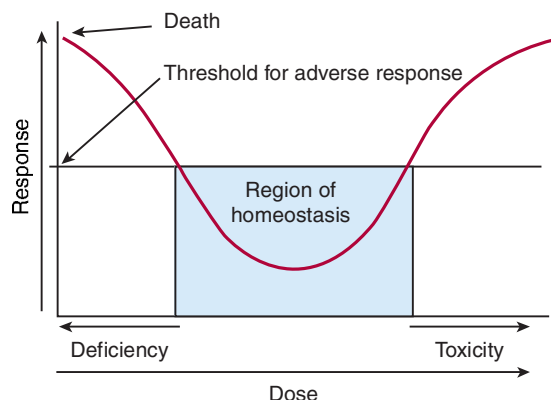
**Essential Nutrients** The shape of the dose-response relationship has many important implications in toxicity assessment. For example, for substances that are required for normal physiological

**Table 2-2**

### Allometric Scaling of Dose Across Different Species

SPECIES	WEIGHT (kg)	SURFACE AREA (cm <sup>2</sup> )*	FOLD DIFFERENCE, RELATIVE TO HUMANS, NORMALIZED BY BODY WEIGHT		
			mg/kg	(BW) <sup>2/3</sup>	(BW) <sup>3/4</sup>
Mouse	0.30	103	1	13.0	7.0
Rat	0.2	365	1	6.9	4.3
Guinea pig	0.4	582	1	5.5	3.6
Rabbit	1.5	1410	1	3.5	2.6
Cat	2	1710	1	3.2	2.4
Monkey	4	2720	1	2.6	2.0
Dog	12	5680	1	1.8	1.5
Human	70	18,500	1	1.0	1.0

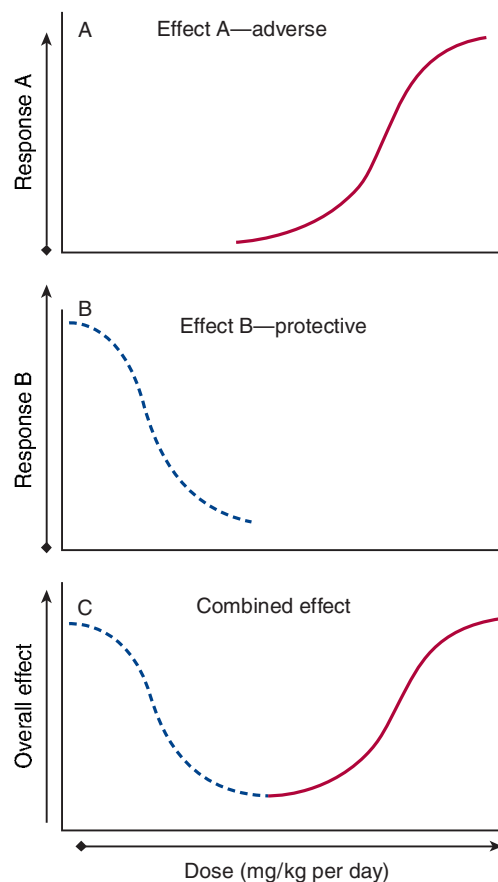
\*Surface area of animals is closely approximated by the following formula:  $SA = 10.5 \times (\text{body weight [grams]})^{2/3}$ .



**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.

function and survival (eg, vitamins and essential trace elements such as chromium, cobalt, and selenium), the “graded” dose-response relationship in an individual over the entire dose range is actually U-shaped (Fig. 2-6). That is, at very low doses, there is a high level of adverse effect, which decreases with an increasing dose. This region of the dose-response relationship for essential nutrients is commonly referred to as a *deficiency*. As the dose is increased to a point where the deficiency no longer exists, no adverse response is detected and the organism is in a state of homeostasis. However, as the dose is increased to abnormally high levels, an adverse response (usually qualitatively different from that observed at deficient doses) appears and increases in magnitude with increasing dose, just as with other toxic substances. Thus, it is recognized that high doses of vitamin A can cause liver toxicity and birth defects, high doses of selenium can affect the brain, and high doses of estrogens may increase the risk of breast cancer, even though low doses of all these substances are essential for life.

**Hormesis** There is considerable evidence to suggest that some nonnutritional toxic substances may also impart beneficial or stimulatory effects at low doses but that, at higher doses, they produce adverse effects. This concept of “hormesis” was first described for radiation effects but may also pertain to most chemical responses (Calabrese and Blaine, 2005). Thus, in plotting dose versus response over a wide range of doses, the effects of hormesis may also result in a “U-shaped” dose-response curve. In its original development, the concept of hormesis pertained to the ability of substances to stimulate biological systems at low doses but to inhibit them at high doses. The application of the concept of hormesis to whole-animal toxicological dose-response relationships may also be relevant but requires that the “response” on the ordinate be variant with dose. 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 (curve A, Fig. 2-7). 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 (curve B, Fig. 2-7) (Hanna *et al.*, 1997). Thus, when all responses are plotted on the ordinate, a “U-shaped” dose-response curve is obtained (curve C, Fig. 2-7). U-shaped dose-response relationships



**Figure 2-7.** Hypothetical dose-response relationship depicting characteristics of hormesis. Hormetic effects of a substance are hypothesized to occur when relatively low doses result in the stimulation of a beneficial or protective response (B), such as induction of enzymatic pathways that protect against oxidative stress. Although low doses provide a potential beneficial effect, a threshold is exceeded as the dose increases and the net effects will be detrimental (A), resulting in a typical dose-related increase in toxicity. The complete dose-response curve (C) is conceptually similar to the individual dose-response relationship for essential nutrients shown in Fig. 2-6.

have obvious implications for the process of low-dose extrapolation in risk assessment.

**Threshold** Another important aspect of the dose-response relationship at low doses is the concept of the threshold. It has long been recognized that acute toxicological responses are associated with thresholds, that is, there is some dose below which the probability of an individual responding is zero. Obviously, the identification of a threshold depends on the particular response that is measured, the sensitivity of the measurement, and the number of subjects studied. For the individual dose-response relationship, thresholds for most toxic effects certainly exist, although interindividual variability in response and qualitative changes in response pattern with dose make it difficult to establish a true “no effects” threshold for any chemical. The biological basis of thresholds for acute responses is well established and frequently can be demonstrated on the basis of mechanistic information (Aldridge, 1986). The traditional approaches to establishing acceptable levels of exposure to chemicals are inherently different for threshold versus nonthreshold responses. The existence of thresholds for chronic responses is less well defined, especially in the area of chemical carcinogenesis. It is, of course, impossible to scientifically prove the absence of a threshold, as one can never prove a negative.

Nevertheless, for the identification of “safe” levels of exposure to a substance, the absence or presence of a threshold is important for practical reasons (see Chap. 4). A classic example of the difficulty of establishing thresholds experimentally is provided by the “ED<sub>01</sub>” study, where over 24,000 mice and 81 different treatment groups were used to determine the shape of the dose–response relationship for the prototypical carcinogen 2-acetylaminofluorene (2-AAF). The study was designed to identify a statistically significant response of 1% (0.01 probability). The mice were exposed to 2-AAF at 1 of 7 different doses in the dose range of 30 to 150 ppm (plus 0 dose control) (Littlefield *et al.*, 1979). Eight “sacrifice intervals” were used to determine how quickly tumors developed. Both types of tumors demonstrated increasing incidence with increasing dose, but the shapes of the 2 curves are dramatically different. For liver tumors, no clear threshold was evident, whereas for bladder tumors, an apparent threshold was evident. However, the apparent threshold, or “no observable adverse effect level” (NOAEL), for bladder cancer was lower at 33 months (45 ppm) than at 24 months (75 ppm). Of course, the ability to detect a low incidence of tumors depends on the number of animals used in the study. Thus, although a threshold (a dose below which no response occurs) appears evident for bladder tumors, one cannot say for certain that tumors would not occur if more animals had been included in the lower-dose groups. A different animal model that relies on relatively brief exposure of rainbow trout embryos to carcinogens has allowed an even more statistically stringent analysis of the shape of the dose–response curve at low doses for mutagenic carcinogens. Using this model with 2 different genotoxic carcinogens, dibenzo[*d,e,f*,*p*] chrysene (DBC, also referred to as dibenzo[*a,l*]pyrene) and aflatoxin B<sub>1</sub> (AFB<sub>1</sub>), estimates of the shape of the dose–response curve down to a response level of 1 additional tumor in 5000 animals could be obtained, because very large numbers of animals could be exposed. In both studies, over 40,000 trout were exposed to different doses ranging over a factor of 200 (AFB<sub>1</sub>, lowest dose 0.5 ppb, highest dose 110 ppb) to 500 (DBC, lowest dose 0.45 ppm, highest dose 225 ppm), with over 8000 animals in the control and low-dose groups (Williams, 2012). Both of these chemicals are potent mutagens, so it was assumed that both the rate of DNA adduct formation and the tumor incidence would be linear throughout the dose range. However, for DBC, there was a clear deviation from linearity at the lower doses, such that the extrapolated dose–response curve crossed the y-axis at 1 cancer in a million exposed animals at a dose 500- to 1500-fold (depending on the statistical model) higher than would have been predicted from the linear extrapolation below the 10% response range (ED<sub>10</sub>) (Bailey *et al.*, 2009) (Fig. 2-8). Remarkably, although the tumor response exhibited a clear “threshold,” the formation of DBC–DNA adducts was quite linear through the lowest dose used. In contrast, in a similarly designed study using the potent carcinogen, AFB<sub>1</sub>, both tumor response and AFB–DNA adduct formation appear approximately linear down through the lowest dose; the liver tumor response to AFB<sub>1</sub> remained linear to the lowest dose, although the slope was about 1.5 and the predicted dose resulting in 1 cancer in a million exposed animals was about 10-fold higher than that predicted from the extrapolated LED<sub>10</sub> line, although the lowest doses tested yielded tumor incidence that was close (within a factor of 2) to the background tumor rate (Fig. 2-9) (Williams *et al.*, 2009a; Williams, 2012).

(See Chap. 4 for more discussion on statistical issues related to extrapolation of dose–response curves and the determination of NOAELs.)

In evaluating the shape of the dose–response relationship in populations, it is realistic to consider inflections in the shape of the dose–response curve rather than absolute thresholds. That is, the

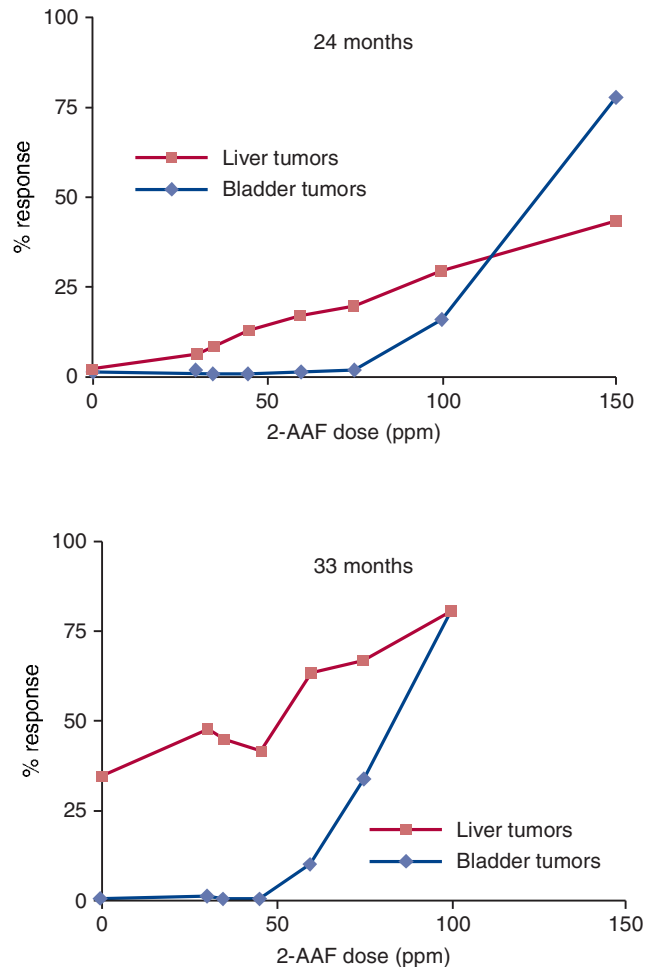


Figure 2-8. Dose–response relationship for carcinogens—rodents and 2-AAF.

slope of the dose–response relationship at high doses may be substantially different from the slope at low doses, usually because of dispositional differences in the chemical. Saturation of biotransformation pathways, protein-binding sites or receptors, and depletion of intracellular cofactors represent some reasons why sharp inflections in the dose–response relationship may occur. For example, the widely used analgesic acetaminophen has a very low rate of liver toxicity at normal therapeutic doses. Even though a toxic metabolite (*N*-acetyl-*p*-benzoquinone imine [NAPQI]) is produced in the liver

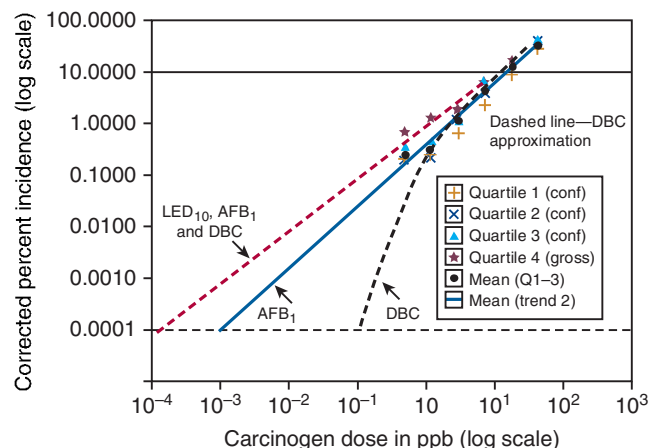


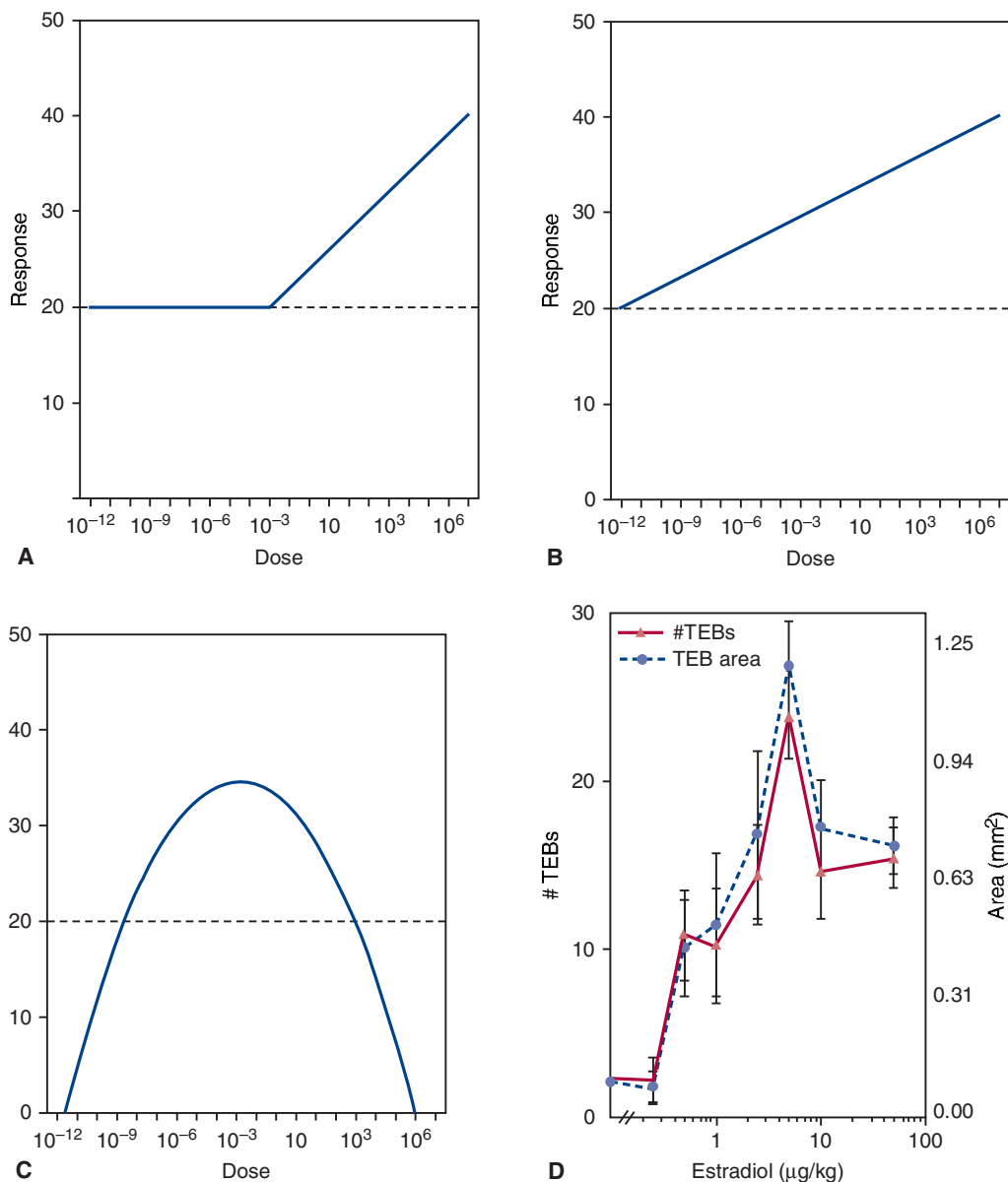
Figure 2-9. Dose–response relationship for carcinogens—fish and aflatoxin B<sub>1</sub>. (Reproduced with permission from Williams, 2012.)



at therapeutic doses, it is rapidly detoxified through conjugation with the intracellular antioxidant glutathione. However, at very high doses, the level of intracellular glutathione in the liver is depleted and NAPQI accumulates, causing serious and potentially fatal liver toxicity. This effect is analogous to the rapid change in pH of a buffered solution that occurs when the buffer capacity is exceeded. Some toxic responses, most notably the development of cancer after the administration of genotoxic carcinogens, are often considered to be linear at low doses and thus do not exhibit a threshold. In such circumstances, there is no dose with “zero” risk, although the risk decreases proportionately with a decrease in the dose. The existence or lack of existence of a threshold dose for carcinogens has many regulatory implications and is a point of considerable controversy and research in the field of quantitative risk assessment for chemical carcinogens (see Chap. 4).

**Nonmonotonic Dose–Response Curves** For chemicals that exert their primary toxic effects via modification of hormonal responses (endocrine disruptors), it is possible that effects occur at relatively low doses that are not seen at higher doses, thereby seemingly defying the traditional concept of “dose–response”.

The characterization of so-called nonmonotonic dose–response (NMDR) curves is an important refinement in our understanding of dose–response relationships in toxicology (Fig. 2-10). Indeed, some chemicals, such as the plastics monomer bisphenol A (BPA), exhibit relatively little evident toxicity at high doses in traditional acute toxicity testing procedures, yet may have important biological effects when exposure occurs during sensitive periods of development, even at doses well below those shown to cause evident toxicity. For example, human pituitary cells cultured in the presence of BPA elicited significant responses at concentrations of 0.001 and



**Figure 2-10.** Hypothetical dose–response curves for the (A) threshold responses, (B) nonthreshold linear response, and (C and D) nonmonotonic dose–response (NMDR). Curves A and B reflect traditional dose–response relationships. However, in the NMDR curve (C), an increase in dose does not necessarily correspond to an increase in response, such that, in this example, doses from  $10^{-12}$  to  $10^{-3}$  dose units result in an increase in response, and doses from  $10^{-3}$  to  $10^6$  dose units result in a decrease in response. Curve D represents the NMDR curves observed in mammary gland morphological parameters after administration of estradiol to ovariectomized females. The left y-axis is the number of terminal end buds (TEBs), and the right y-axis is total area of all TEBs; the TEB is an estrogen-dependent structure. (Based on Vandenberg *et al.*, 2009.)

0.01 nM, but not at 1 and 10 nM, yet the response was seen at 100 nM (Vandenberg *et al.*, 2009). Several other studies have found that BPA and other endocrine-active xenobiotics can elicit NMDR relationships for a variety of other specific receptors and/or cell signaling pathways (reviewed in Vandenberg *et al.*, 2009).

Specific cellular/molecular mechanisms that might explain NMDR curves include: (1) upregulation of some receptors at low concentrations, with downregulation of the same receptors at higher levels, and/or (2) integration of 2 or more monotonic dose–response curves that occur through different molecular/cellular pathways with common end points but opposite effects (Vandenberg *et al.*, 2009). Since endocrine-active xenobiotics may act as weak agonists for specific hormone receptors, it is reasonable that low doses could have different effects than high doses if, as partial agonists, they competitively inhibit endogenous ligands at higher concentrations, but have either no or positive agonist effects at low concentrations. Another explanation for NMDR curves is that we simply do not understand all the varied and interconnected molecular pathways that work in concert to produce an observable response at the organismal level. Indeed, BPA has been shown to have multiple different effects on a myriad of putative molecular pathways involved in hormone function, so it perhaps is not surprising to see NMDR functions over dose ranges of many orders of magnitude (Vandenberg *et al.*, 2009).

## Assumptions in Deriving the Dose–Response Relationship

A number of assumptions must be considered before dose–response relationships can be used appropriately. The first is that the response is due to the chemical administered. To describe the relationship between a toxic material and an observed effect or response, one must know with reasonable certainty that the relationship is indeed a causal one. For some data, it is not always apparent that the response is a result of chemical exposure. For example, an epidemiological study might result in the discovery of an “association” between a response (eg, disease) and 1 or more variables. Frequently, the data are presented similarly to the presentation of “dose response” in pharmacology and toxicology. Use of the dose response in this context is suspect unless other convincing evidence supports a causal connection between the estimated dose and the measured end point (response). Unfortunately, in nearly all retrospective and case–control studies and even in many prospective studies, the dose, duration, frequency, and routes of exposure are seldom quantified, and other potential etiologic factors are frequently present. In its most strict usage, then, 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. Perhaps because of its apparent simplicity, this assumption is often a source of misunderstanding. It is really a composite of 3 other assumptions that recur frequently:

1. There is a molecular target site (or sites) with which the chemical interacts to initiate the response.
2. The production of a response and the degree of response are related to the concentration of the chemical at the target site.
3. The concentration at the site is, in turn, related to the dose administered.

The third assumption in using the dose–response relationship is that there exist both a quantifiable method of measuring and a precise means of expressing the toxicity. For any given dose–response

relationship, a great variety of criteria or end points of toxicity could be used. The ideal criterion would be one closely associated with the molecular events resulting from exposure to the toxicant. It follows from this that a given chemical may have a family of dose–response relationships, 1 for each toxic end point. For example, a chemical that produces cancer through genotoxic effects, liver damage through inhibition of a specific enzyme, and CNS effects via a different mechanism, may have 3 distinct dose–response relationships, 1 for each end point. Early in the assessment of toxicity, little mechanistic information is usually available; thus, establishing a dose–response relationship based on the molecular mechanism of action is usually impossible. Indeed, it might not be approachable even for well-known toxicants. In the absence of a mechanistic, molecular ideal criterion of toxicity, one looks to a measure of toxicity that is unequivocal and clearly relevant to the toxic effect. Such measures are often referred to as “effects-related biomarkers.” For example, with a new compound chemically related to the class of organophosphate insecticides, one might approach the measurement of toxicity by measuring the inhibition of cholinesterase in blood. In this way, one would be measuring, in a readily accessible system and using a technique that is convenient and reasonably precise, a prominent effect of the chemical and one that is usually pertinent to the mechanism by which toxicity is produced.

The selection of a toxic end point for measurement is not always so straightforward. Even the example cited above may be misleading, as an organophosphate may produce a decrease in blood cholinesterase, but this change may not be directly related to its toxicity. As additional data are gathered to suggest a mechanism of toxicity for any substance, other measures of toxicity may be selected. Although many end points are quantitative and precise, they are often indirect measures of toxicity. Changes in enzyme levels in blood can be indicative of tissue damage. For example, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are used to detect liver damage. Use of these enzymes in serum is yet another example of an effects-related biomarker because the change in enzyme activity in the blood is directly related to damage to liver cells. Much of clinical diagnostic medicine relies on effects-related biomarkers, but to be useful the relationship between the biomarker and the disease must be carefully established. Patterns of isozymes and their alteration may provide insight into the organ or system that is the site of toxic effects. As discussed later in this chapter, the new tools of toxicogenomics provide an unprecedented opportunity to discover new “effects-related biomarkers” in toxicology.

Many direct measures of effects are also not necessarily related to the mechanism by which a substance produces harm to an organism but have the advantage of permitting a causal relation to be drawn between the chemical and its action. For example, measurement of the alteration of the tone of smooth or skeletal muscle for substances acting on muscles represents a fundamental approach to toxicological assessment. Similarly, measures of heart rate, blood pressure, and electrical activity of heart muscle, nerve, and brain are examples of the use of physiological functions as indices of toxicity. Measurement can also take the form of a still higher level of integration, such as the degree of motor activity or behavioral change.

The measurements used as examples in the preceding discussion all assume prior information about the toxicant, such as its target organ or site of action or a fundamental effect. However, such information is usually available only after toxicological screening and testing based on other measures of toxicity. With a new substance, the customary starting point is a single-dose acute toxicity test designed to provide preliminary identification of target organ

toxicity. Studies specifically designed with lethality as an end point are no longer recommended by the United States or international agencies. Data from acute studies provide essential information for choosing doses for repeated dosing studies as well as choosing specific toxicological end points for further study. Key elements of the study design must be a careful, disciplined, detailed observation of the intact animal extending from the time of administration of the toxicant to any clinical signs of distress, which may include detailed behavioral observations or physiological measures. It is recommended that these observations be taken over a 14-day period. From properly conducted observations, immensely informative data can be gathered by a trained toxicologist. Second, an acute toxicity study ordinarily is supported by histological examination of major tissues and organs for abnormalities. From these observations, one can usually obtain more specific information about the events leading to the various end points, the target organs involved, and often a suggestion about the possible mechanism of toxicity at a relatively fundamental level.

## Evaluating the Dose–Response Relationship

**Comparison of Dose Responses** Fig. 2-11 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, ED) such as liver injury, and the lethal dose (LD). As depicted in Fig. 2-11, a parallelism is apparent between the ED curve and the curve depicting mortality (LD). It is tempting to view the parallel dose–response curves as indicative of identity of mechanism—that is, to conclude that the lethality is a simple extension of the therapeutic effect. Whereas this conclusion may ultimately prove to be correct in any particular case, it is not warranted solely on the basis of the 2 parallel lines. The same admonition applies to any pair of parallel “effect” curves or any other pair of toxicity or lethality curves.

**Therapeutic Index** The hypothetical curves in Fig. 2-11 illustrate 2 other interrelated points: the importance of the selection of the toxic criterion and the interpretation of comparative effect. The concept of the “therapeutic index” (TI), which was introduced by Paul Ehrlich in 1913, can be used to illustrate this relationship. Although the TI is directed toward a comparison of the therapeutically ED to the TD of a chemical, it is equally applicable to considerations of comparative toxicity. The TI in its broadest sense is defined as the ratio of the dose required to produce a toxic effect to

the dose needed to elicit the desired therapeutic response. Similarly, an index of comparative toxicity is obtained by the ratio of doses of 2 different materials to produce an identical response or the ratio of doses of the same material necessary to yield different toxic effects.

The most commonly used index of effect, whether beneficial or toxic, is the median effect dose ( $ED_{50}$ ). The TI of a drug is an approximate statement about the relative safety of a drug expressed as the ratio of the adverse end point or TD (historically the LD) to the therapeutic dose:

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

From Fig. 2-11 one can approximate a TI by using these median doses. The larger the ratio, the greater is the relative safety. 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 tell nothing about the slopes of the dose–response curves for therapeutic and toxic effects.

**Margins of Safety and Exposure** One way to overcome this deficiency is to use the  $ED_{99}$  for the desired effect and the  $TD_1$  for the undesired effect. These parameters are used in the calculation of the margin of safety (MOS):

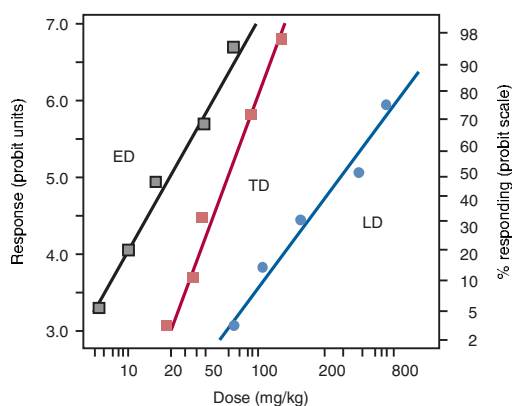
$$MOS = \frac{TD_1}{ED_{99}}$$

The quantitative comparisons described above have been used mainly after a single administration of chemicals. However, for chemicals for which there is no beneficial or effective dose and exposures are likely to occur repeatedly, the ratio of  $TD_1$  to  $ED_{99}$  has little relevance. Thus, for nondrug chemicals, the term *MOS* has found use in risk assessment procedures as an indicator of the magnitude of the difference between an estimated “exposed dose” to a human population and the NOAEL or other benchmark dose determined in experimental animals.

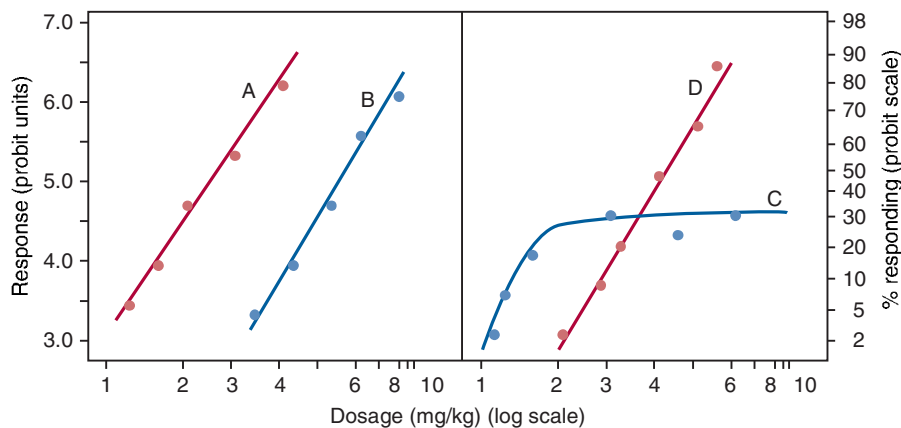
A measure of the degree of accumulation of a chemical and/or its toxic effects can also be estimated from quantal toxicity data. The *chronicity index* of a chemical is a unitless value obtained by dividing its 1-dose  $TD_{50}$  by its 90-dose (90-day)  $TD_{50}$ , with both expressed in milligrams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

Historically, statistical procedures similar to those used to calculate the  $LD_{50}$  can also be used to determine the lethal time 50 ( $LT_{50}$ ), or the time required for half the animals to die (Litchfield, 1949). The  $LT_{50}$  value for a chemical indicates the time course of the toxic effects but does not indicate whether 1 chemical is more toxic than another.

Frequently, dose–response curves from repeated-dose experimental animal studies (subacute, subchronic, or chronic) are used to estimate the NOAEL, or some other “benchmark” measure of minimal toxic response, such as the dose estimated to produce toxic effects in 10% of the population ( $TD_{10}$ ) (see also Chap. 4). These estimates of minimal TD, derived from quantal dose–response curves, can be used in risk assessment to derive a “margin of exposure” (MOE) index. This index compares the estimated daily exposure, in milligrams per kilogram per day, that might occur under a given set of circumstances with some estimated value from the quantal dose–response relationship (eg, NOAEL or  $TD_{10}$ ). Like the



**Figure 2-11.** Comparison of effective dose (ED), toxic dose (TD), and lethal dose (LD). The plot is of log dosage versus percentage of population responding in probit units.



**Figure 2-12.** Schematic representation of the difference in the dose-response curves for 4 chemicals (A-D), illustrating the difference between potency and efficacy (see text).

MOS, the MOE is often expressed as a ratio of these 2 values. Thus, for example, if an estimate of human exposure to a pesticide residue yielded a value of 0.001 mg/kg per day, and a  $TD_{10}$  of 1 mg/kg per day was determined for that same pesticide, the MOE would be 1000. This value indicates that the estimate of daily exposure under the described set of conditions is 1/1000 the estimated daily dose that would cause evident toxicity in 10% of exposed animals. (See Chap. 4 for a more complete discussion of benchmark doses, NOAELs, and MOE.)

**Potency versus Efficacy** To compare the toxic effects of 2 or more chemicals, the dose response to the toxic effects of each chemical must be established. One can then compare the potency and maximal efficacy of the 2 chemicals to produce a toxic effect. These 2 important terms can be explained by reference to Fig. 2-12, which depicts dose-response curves to 4 different chemicals for the frequency of a particular toxic effect, such as the production of tumors. Chemical A is said to be more potent than chemical B because of their relative positions along the dosage axis. Potency thus refers to the range of doses over which a chemical produces increasing responses. Thus, A is more potent than B and C is more potent than D. Maximal efficacy reflects the limit of the dose-response relationship on the response axis to a certain chemical. Chemicals A and B have equal maximal efficacy, whereas the maximal efficacy of C is less than that of D.

## VARIATION IN TOXIC RESPONSES

### Selective Toxicity

*Selective toxicity* means that a chemical produces injury to 1 kind of living matter without harming another form of life even though the 2 may exist in intimate contact (Albert, 1973). The living matter that is injured is termed the *uneconomic form* (or undesirable), and the matter protected is called the *economic form* (or desirable). They may be related to each other as parasite and host or may be 2 tissues in 1 organism. This biological diversity interferes with the ability of ecotoxicologists to predict the toxic effects of a chemical in 1 species (humans) from experiments performed in another species (laboratory animals). However, by taking advantage of the biological diversity, it is possible to develop chemicals that are lethal for an undesired species and harmless for other species. In agriculture, for example, there are fungi, insects, and even competitive plants that injure the crop, and thus selective pesticides are needed. Similarly, animal husbandry and human medicine require

chemicals, such as antibiotics, that are selectively toxic to the undesirable form but do not produce damage to the desirable form.

Drugs and other chemicals used for selective toxic purposes are selective for 1 of 2 reasons. Either (1) the chemical is equally toxic to both economic and uneconomic cells but is accumulated mainly by uneconomic cells or (2) it reacts fairly specifically with a cytological or a biochemical feature that is absent from or does not play an important role in the economic form (Albert, 1973). Selectivity resulting from differences in distribution usually is caused by differences in the absorption, biotransformation, or excretion of the toxicant. The selective toxicity of an insecticide spray may be partly due to a larger surface area per unit weight that causes the insect to absorb a proportionally larger dose than does the mammal being sprayed. The effectiveness of radioactive iodine in the treatment of hyperthyroidism (as well as its thyroid carcinogenicity) is due to the selective ability of the thyroid gland to accumulate iodine. A major reason why chemicals are toxic to one, but not to another, type of tissue is that there are differences in accumulation of the ultimate toxic compound in various tissues. This, in turn, may be due to differences in the ability of various tissues to transport or biotransform the chemical into the ultimate toxic product.

Selective toxicity caused by differences in comparative cytology is exemplified by a comparison of plant and animal cells. Plants differ from animals in many ways—for example, absence of a nervous system, an efficient circulatory system, and muscles as well as the presence of a photosynthetic mechanism and cell walls. The fact that bacteria contain cell walls and humans do not has been utilized in developing selective toxic chemotherapeutic agents, such as penicillin and cephalosporins, that kill bacteria but are relatively nontoxic to mammalian cells.

Selective toxicity can also be a result of a difference in biochemistry in the 2 types of cells. For example, bacteria do not absorb folic acid but synthesize it from *p*-aminobenzoic acid, glutamic acid, and pteridine, whereas mammals cannot synthesize folic acid but have to absorb it from the diet. Thus, sulfonamide drugs are selectively toxic to bacteria because the sulfonamides, which resemble *p*-aminobenzoic acid in both charge and dimensions, antagonize the incorporation of *p*-aminobenzoic acid into the folic acid molecule—a reaction that humans do not carry out.

### Species Differences

Although a basic tenet of toxicology is that “experimental results in animals, when properly qualified, are applicable to humans,” it is important to recognize that both quantitative and qualitative



differences in response to toxic substances may occur among different species. As discussed above, there are many reasons for selective toxicity among different species. Even among phylogenetically similar species (eg, rats, mice, guinea pigs, and hamsters), large differences in response may occur. For example, the LD<sub>50</sub> for the highly toxic dioxin, TCDD, differs by more than 1000-fold between guinea pigs and hamsters. Not only the lethal dose for TCDD but also the particular target organs affected vary widely among species. Species differences in response to carcinogenic chemicals represent an important issue in regulatory risk assessment. As discussed in Chap. 4, extrapolation of laboratory animal data to infer human cancer risk is currently a key component of regulatory decision making. The validity of this approach of course depends on the relevance of the experimental animal model to humans. Large differences in carcinogenic response between experimental animal species are not unusual. For example, mice are highly resistant to the hepatocarcinogenic effects of the fungal toxin AFB<sub>1</sub>. Dietary doses as high as 10,000 ppb failed to produce liver cancer in mice, whereas in rats dietary doses as low as 15 ppb produced a significant increase in liver tumors (Wogan *et al.*, 1974). The mechanistic basis for this dramatic difference in response appears to be entirely related to species differences in the expression of a particular form of glutathione *S*-transferase (mGSTA3-3) that has unusually high catalytic activity toward the carcinogenic epoxide of aflatoxin (Eaton and Gallagher, 1994). Mice express this enzyme constitutively, whereas rats normally express a closely related form with much less detoxifying activity toward aflatoxin epoxide. Interestingly, rats do possess the gene for a form of glutathione *S*-transferase with high catalytic activity toward aflatoxin epoxide (rGSTA5-5) that is inducible by certain dietary antioxidants and drugs. Thus, dietary treatment can dramatically change the sensitivity of a species to a carcinogen.

Other examples in which large species differences in response to carcinogens have been observed include the development of renal tumors from 2,3,5-trimethylpentane and D-limonene in male rats (Lehman-McKeeman and Caudill, 1992), the production of liver tumors from “peroxisomal proliferators” such as the antilipidemic drug clofibrate and the common solvent trichloroethylene (Roberts, 1999), and the induction of nasal carcinomas in rats after inhalation exposure to formaldehyde (Monticello and Morgan, 1997).

Identifying the mechanistic basis for species differences in response to chemicals is an important part of toxicology because only through a thorough understanding of these differences can the relevance of animal data to human response be verified.

## Individual Differences in Response

Even within a species, large interindividual differences in response to a chemical can occur because of subtle genetic differences. Hereditary differences in a single gene that occur in more than 1% of the population are referred to as *genetic polymorphism* and may be responsible for idiosyncratic reactions to chemicals, as discussed earlier in this chapter. However, genetic polymorphism may have other important but less dramatic effects than those described for acute idiosyncratic responses (such as that occurring in pseudocholinesterase-deficient individuals after succinylcholine exposure). For example, it is recognized that approximately 50% of the Caucasian population has a gene deletion for the enzyme glutathione *S*-transferase M1. This enzyme has no apparent significant physiological function, and thus homozygotes for the gene deletion (eg, those who lack both copies of the normal gene) are functionally and physiologically normal. However, epidemiological studies have indicated that smokers who are homozygous for the null

allele may be at slightly increased risk of developing lung cancer compared with smokers who have 1 or both copies of the normal gene (Mohr *et al.*, 2003). Chap. 6 provides additional examples of genetic differences in biotransformation enzymes that may be important determinants of variability in individual susceptibility to chemical exposures.

Genetic polymorphism in physiologically important genes may also be responsible for interindividual differences in toxic responses. For example, studies in transgenic mice have shown that mice possessing 1 copy of a mutated *p53* gene (a so-called tumor suppressor gene; see Chap. 8) are much more susceptible to some chemical carcinogens than are mice with 2 normal copies of the gene (Tennant *et al.*, 1999). In humans, there is evidence that possessing 1 mutated copy of a tumor suppressor gene greatly increases the risk of developing certain cancers. For example, retinoblastoma is a largely inherited form of cancer that arises because of the presence of 2 copies of a defective tumor suppressor gene (the Rb gene) (Wiman, 1993). Individuals with 1 mutated copy of the Rb gene and 1 normal copy are not destined to acquire the disease (as are those with 2 copies of the mutated gene), although their chance of acquiring it is much greater than that of persons with 2 normal Rb genes. This is the case because both copies of the gene must be nonfunctional for the disease to develop. With 1 mutated copy present genetically, the probability of acquiring a mutation of the second gene (potentially from exposure to environmental mutagens) is much greater than the probability of acquiring independent mutations in both copies of the gene as would be necessary in people with 2 normal Rb alleles. (See Chap. 8 for additional discussion of tumor suppressor genes.)

As our understanding of the human genome increases, more “susceptibility” genes will be discovered, and it is likely that the etiology of many chronic diseases will be shown to be related to a combination of genetics and environment. Simple blood tests may ultimately be developed that allow an individual to learn whether he or she may be particularly susceptible to specific drugs or environmental pollutants. Although the public health significance of this type of information could be immense, the disclosure of such information raises many important ethical and legal issues that must be addressed before wide use of such tests.

The study of “gene–environment” interactions, or “ecogenetics” (Costa and Eaton, 2006), is a rapidly developing field of substantial relevance to toxicology. It is likely that the majority of chronic diseases develop as a result of the complex interplay between multiple genes and the myriad of environmental factors, including diet, lifestyle, and occupational and/or environmental exposures to toxic substances. The growing field of epigenetics, discussed in more detail later in this chapter, is likely to have an equally great impact on the science of toxicology, as it is likely that many xenobiotics will be found to exert many of their chronic adverse effects through subtle effects on gene expression.

## DESCRIPTIVE ANIMAL TOXICITY TESTS

Two main principles underlie all descriptive animal toxicity testing. The first is that the effects produced by a compound in laboratory animals, when properly qualified, are applicable to humans. This premise applies to all of experimental biology and medicine. Most, if not all, known chemical carcinogens in humans are carcinogenic in some species, but not necessarily in all species of laboratory animals. It has become increasingly evident that the converse—that all chemicals identified as carcinogenic in laboratory animals are also carcinogenic in humans—is not true (Dybing and Sanner, 1999; Grisham, 1997; Hengstler *et al.*, 1999). However, for regulatory

and risk assessment purposes, positive carcinogenicity tests in animals are usually interpreted as indicative of potential human carcinogenicity. If a clear understanding of the mechanism of action of the carcinogen indicates that a positive response in animals is not relevant to humans, a positive animal bioassay may be considered irrelevant for human risk assessment (see Chap. 4). This species variation in carcinogenic response appears to be due in many instances to differences in biotransformation of the procarcinogen to the ultimate carcinogen (see Chap. 6).

The second principle is that exposure of experimental animals to chemicals in high doses 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. Practical considerations in the design of experimental model systems require that the number of animals used in toxicology experiments always be small compared with the size of human populations at risk. Obtaining statistically valid results from such small groups of animals 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. Thus, for example, it has been shown that bladder tumors observed in rats fed very high doses of saccharin will not occur at the much lower doses of saccharin encountered in the human diet. At the high concentrations fed to rats, saccharin forms an insoluble precipitate in the bladder that subsequently results in chronic irritation of bladder epithelium, enhanced cell proliferation, and ultimately bladder tumors (Cohen, 1998, 1999). In vitro studies have shown that precipitation of saccharin in human urine will not occur at the concentrations

that could be obtained from even extraordinary consumption of this artificial sweetener. As noted above and shown in Fig. 2-8, even for mutagenic chemicals that form DNA adducts, the response at high doses, as seen for DBC, may not be linear at low doses, although for another DNA-reactive carcinogen, AFB<sub>1</sub>, the high-dose data were reflective of low-dose response in an approximately linear fashion. Examples such as these illustrate the importance of considering the molecular, biochemical, and cellular mechanisms responsible for toxicological responses when extrapolating from high to low dose and across species.

Toxicity tests are not designed to demonstrate that a chemical is safe but to characterize the toxic effects a chemical can produce. Although there are no set toxicology tests that have to be performed on every chemical intended for commerce, a tiered approach typical of many hazard assessment programs is illustrated in Fig. 2-13. 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. The FDA, EPA, and Organization for Economic Cooperation and Development (OECD) have written good laboratory practice (GLP) standards and other guidance that stipulate that procedure must be defined and accountability documented. These guidelines are expected to be followed when toxicity tests are conducted in support of the introduction of a chemical to the market.

The following sections provide an overview of basic toxicity testing procedures in use today. For a detailed description of these tests, the reader is referred to several authoritative texts on this subject (Barile, 2010; Hayes, 2008; Jacobson-Kram and Keller, 2006; Eaton and Gallagher, 2010).

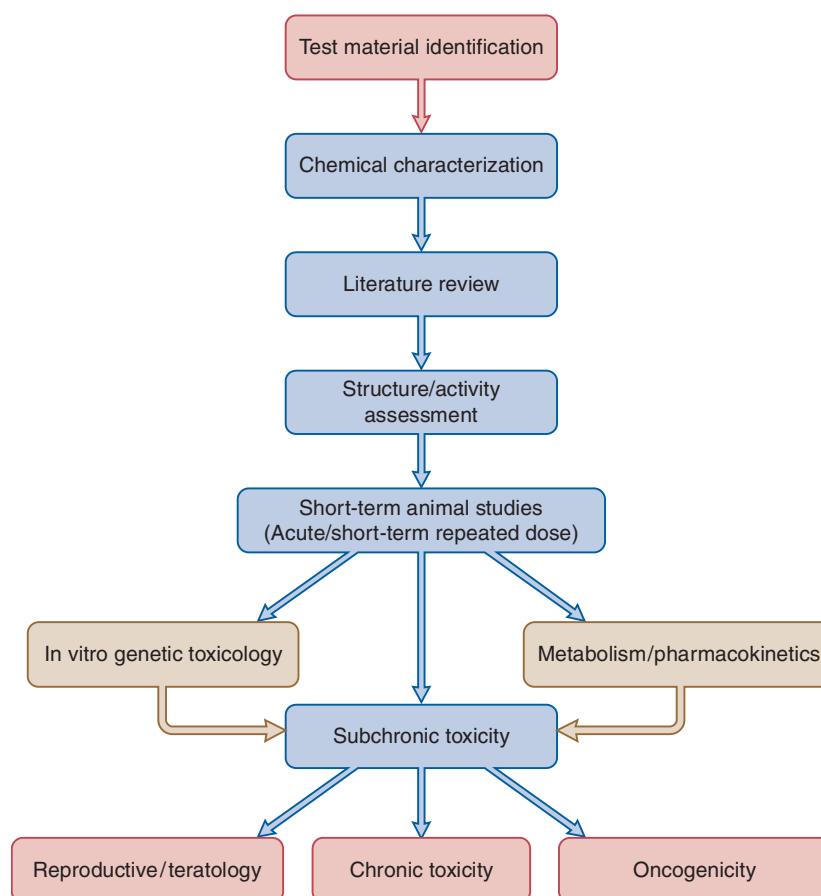


Figure 2-13. Typical tiered testing scheme for the toxicological evaluation of new chemicals. (From Wilson *et al.* 2008, Fig. 19-1, p. 918.)

Table 2-3

## International Conference on Harmonization (ICH) Codification of “Safety” Protocols

**Carcinogenicity studies**

S1A	Need for Carcinogenicity Studies of Pharmaceuticals
S1B	Testing for Carcinogenicity of Pharmaceuticals
S1C(R1)	Dose Selection for Carcinogenicity Studies of Pharmaceuticals & Limit Dose

**Genotoxicity studies**

S2A	Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals
S2B	Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals

**Toxicokinetics and pharmacokinetics**

S3A	Note for Guidance on Toxicokinetics: The Assessment of Systemic Exposure in Toxicity Studies
S3B	Pharmacokinetics: Guidance for Repeated Dose Tissue Distribution Studies

**Toxicity testing**

	Single Dose Toxicity Tests
S4	Duration of Chronic Toxicity Testing in Animals (Rodent and Non Rodent Toxicity Testing)

**Reproductive toxicology**

S5(R2)	Detection of Toxicity to Reproduction for Medicinal Products & Toxicity to Male Fertility
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**Biotechnological products**

S6	Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals
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**Pharmacology studies**

S7A	Safety Pharmacology Studies for Human Pharmaceuticals
S7B	The Non-Clinical Evaluation of the Potential for Delayed Ventricular Repolarization (QT Interval Prolongation) by Human Pharmaceuticals

**Immunotoxicology studies**

S8	Immunotoxicity Studies for Human Pharmaceuticals
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**Joint safety/efficacy (multidisciplinary) topic**

M3(R1)	Non-Clinical Safety Studies for the Conduct of Human Clinical Trials for Pharmaceuticals
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*Titles and abbreviations adopted in November 2005. Data from [http://www.ich.org/fileadmin/Public\\_Web\\_Site/ICH\\_Products/Guidelines/Guidelines\\_Index.pdf](http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Guidelines_Index.pdf).*

Although different countries have often had 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 Harmonization (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use includes regulatory authorities from Europe, Japan, and the United States (primarily the FDA), as well as experts from the pharmaceutical industry in the 3 regions, who worked together to develop internationally recognized scientific and technical approaches to pharmaceutical product registration. ICH has adopted guidelines for most areas of toxicity testing (Table 2-3). In addition to safety assessment (ICH guidelines designated with an “S”), ICH has also established guidelines on quality (Q), efficacy (E), and multidisciplinary (M) topics. (See <http://www.ich.org/products/guidelines.html> for a description of current ICH guidelines and reviews by Pugsley *et al.* (2008, 2011) for a detailed discussion of in vitro and in vivo approaches to safety pharmacology that has been informed by the ICH regulatory guidance document for preclinical safety testing of drugs.)

Typically, a tiered approach is used, with subsequent tests dependent on results of initial studies. A general framework for how new chemicals are evaluated for toxicity is shown in Fig 2-13. Early studies require careful chemical evaluation of the compound

or mixture to assess purity, stability, solubility, and other physicochemical factors that could impact the ability of the test compound to be delivered effectively to animals. Once this information is obtained, the chemical structure of the test compound is compared with similar chemicals for which toxicological information is already available. Structure–activity relationships may be derived from a review of existing toxicological literature, and can provide additional guidance on design of acute and repeated-dose experiments, and what 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.

Because of increased societal pressure to reduce or eliminate the use of animals in toxicity testing, while also ensuring that new chemicals do not represent unreasonable risks to human health or the environment, regulatory agencies have been encouraging new approaches to descriptive toxicity tests that do not rely on laboratory animals. For example, the European Union (EU) promulgated an important regulatory initiative for the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). The implementation of REACH “will have significant impact on applied toxicology and exposure assessment by stimulating innovation in sampling and analysis, toxicology testing, exposure modeling, alternative toxicity testing, and risk assessment practices”

(Williams *et al.*, 2009b). Alternative, in vitro approaches to toxicity assessment are likely to transform the way that product safety evaluation is done in the future, although the standard approaches to hazard evaluation described in this section are likely to continue as the mainstay of toxicity evaluation for the next decade, irrespective of the fact that some areas, such as acute toxicity testing and eye irritation, are likely to be largely replaced by in vitro tests in the next decade (Ukelis *et al.*, 2008).

The development of new “omics” technologies (discussed later in this section) may have profound implications for toxicity testing in the future (NAS/NRC, 2007). The recognition that many of the existing chemicals in commercial use today, as well as new chemicals being introduced into commerce, have little toxicological information about them has prompted calls for new “high-throughput” approaches to toxicity testing that will allow at least basic hazard characterization for the thousands of untested chemicals currently in the marketplace, as well as the many new chemicals that are introduced each year. A report from the National Academy of Sciences/National Research Council in 2007 called for a “paradigm shift” in how toxicity testing is done (NAS/NRC, 2007). A key component of this new vision on toxicity testing is the use of an extensive battery of in vitro tests to evaluate “pathways” of toxicity (NAS/NRC, 2010). The hope is that new technologies in genomics, transcriptomics, proteomics, metabolomics, and bioinformatics (discussed later in this chapter) can be 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 end points (eg, 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 (NAS/NRC, 2010). However, it is also recognized that validation of such tests is critically important to the reliable use of such screening technologies, and that the traditional in vivo studies described in the following section will continue to serve an important role in hazard evaluations for years to come, especially as a means of validating new high-throughput screening approaches.

## Acute Toxicity Testing

Generally, the first toxicity test performed on a new chemical is acute toxicity, determined from the administration of a single exposure. The objectives of acute toxicity testing are to: (1) provide an estimate of the intrinsic toxicity of the substance, often times expressed as an approximate LD (eg, LD<sub>50</sub>), (2) provide information on target organs and other clinical manifestations of toxicity, (3) identify species differences and susceptible species, (4) establish the reversibility of the toxic response, and (5) provide information that will assist in the design and dose selection for longer-term (subchronic, chronic) studies. It should be noted that the ICH recommended in 1991 (D’Arcy and Harron, 1992) the elimination of LD<sub>50</sub> determinations for pharmaceuticals, although other regulatory requirements, for example, pesticide registration, may still require determinations of LD<sub>50</sub>s.

The LD<sub>50</sub> and other acute toxic effects are determined after 1 or more routes of administration (1 route being oral or the intended route of exposure) in 1 or more species. The species most often used are the mouse and rat. Studies are performed in both adult male and female animals. Food is often withheld the night before dosing. The number of animals that die in a 14-day period after a single dosage is tabulated. In addition to mortality and weight, daily examination of test animals should be conducted for signs

of intoxication, lethargy, behavioral modifications, morbidity, food consumption, and so on.

Determination of the LD<sub>50</sub> has become a public issue because of increasing concern for the welfare and protection of laboratory animals. The LD<sub>50</sub> is not a biological constant. Many factors influence toxicity and thus may alter the estimation of the LD<sub>50</sub> in any particular study. 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. These and other factors have been discussed in detail in earlier editions of this textbook (Doull, 1980). Because of this inherent variability in LD<sub>50</sub> estimates, it is now recognized that for most purposes it is only necessary to characterize the LD<sub>50</sub> within an order of magnitude range such as 5 to 50 mg/kg, 50 to 500 mg/kg, and so on.

There are several traditional approaches to determining the LD<sub>50</sub> and its 95% confidence limit as well as the slope of the probit line. The reader is referred to the classic works of Litchfield and Wilcoxon (1949), Bliss (1957), and Finney (1971) for a description of the mechanics of these procedures. Other statistical techniques that require fewer animals, such as the “moving averages” method of Thompson and Weill (Weil, 1952), are available but do not provide confidence limits for the LD<sub>50</sub> and the slope of the probit line. Finney (1985) has succinctly summarized the advantages and deficiencies of many of the traditional methods. For most circumstances, an adequate estimate of the LD<sub>50</sub> and an approximation of the 95% confidence intervals can be obtained with as few as 6 to 9 animals, using the “up-and-down” method as modified by Bruce (1985). When this method was compared with traditional methods that typically utilize 40 to 50 animals, excellent agreement was obtained for all 10 compounds tested (Bruce, 1987). In mice and rats the LD<sub>50</sub> is usually determined as described above, but in the larger species only an approximation of the LD<sub>50</sub> is obtained by increasing the dose in the same animal until serious toxic effects are evident.

Alternative in vitro approaches to estimating the LD<sub>50</sub> have been proposed. For example, the *Registry of Cytotoxicity* (RC), originally published in German in 1998 (Halle, 2003), was developed by linear regression analysis of the mean IC<sub>50</sub> values determined in mammalian cells in culture and the LD<sub>50</sub> values reported in the literature from various laboratory species. Using this approach, the authors predicted (within a reasonable dose range) the acute oral LD<sub>50</sub> for 252 of 347 xenobiotics, and the intravenous LD<sub>50</sub> for rats and/or mice for 117 of 150 xenobiotics (Halle, 2003). Of course, such in vitro approaches do not fully account for dispositional effects that could result in large species differences in acute toxicity, but do provide a rapid first approximation of acute toxicity without the use of experimental animals.

If there is a reasonable likelihood of substantial exposure to the material by dermal or inhalation exposure, acute dermal and acute inhalation studies are performed. When animals are exposed acutely to chemicals in the air they breathe or the water they (fish) live in, the dose the animals receive is usually not known. For these situations, the lethal concentration 50 (LC<sub>50</sub>) is usually determined, that is, the concentration of chemical in the air or water that causes death to 50% of the animals. In reporting an LC<sub>50</sub>, it is imperative that the time of exposure be indicated. The acute dermal toxicity test is usually performed in rabbits. The site of application is shaved. The test substance is kept in contact with the skin for 24 hours by wrapping the skin with an impervious plastic material. At the end of the exposure period, the wrapping is removed and the skin is wiped to remove any test substance still remaining. Animals are observed at various intervals for 14 days, and the LD<sub>50</sub> is calculated. If no



toxicity is evident at 2 g/kg, further acute dermal toxicity testing is usually not performed. Acute inhalation studies are performed that are similar to other acute toxicity studies except that the route of exposure is inhalation. Most often, the length of exposure is 4 hours.

By themselves  $LD_{50}$  and  $LC_{50}$  values are of limited significance given the growing sophistication of target organ toxicity end points and mechanistic analysis. The most meaningful scientific information derived from acute toxicity tests comes from clinical observations and post-mortem examination of animals rather than from the specific  $LD_{50}$  value.

## Skin and Eye Irritations

The ability of a chemical to irritate the skin and eye after an acute exposure is usually determined in rabbits. For the dermal irritation test (Draize test), rabbits are prepared by removal of fur on a section of the back by electric clippers. The chemical is applied to the skin (0.5 mL of liquid or 0.5 g of solid) under 4 covered gauze patches (1 in square; 1 intact and 2 abraded skin sites on each animal) and usually kept in contact for 4 hours. The nature of the covering patches depends on whether occlusive, semioclusive, or nonocclusive tests are desired. For occlusive testing, the test material is covered with an impervious plastic sheet; for semioclusive tests, a gauze dressing may be used. Occasionally, studies may require that the material be applied to abraded skin. The degree of skin irritation is scored for erythema (redness), eschar (scab), and edema (swelling) formation, and corrosive action. These dermal irritation observations are repeated at various intervals after the covered patch has been removed. To determine the degree of ocular irritation, the chemical is instilled into 1 eye (0.1 mL of liquid or 100 mg of solid) of each test rabbit. The contralateral eye is used as the control. The eyes of the rabbits are then examined at various times after application.

Controversy over this test has led to the development of alternative in vitro models for evaluating cutaneous and ocular toxicity of substances. The various in vitro methods that have been evaluated for this purpose include epidermal keratinocyte and corneal epithelial cell culture models. Several commercially available “reconstructed human epidermis” models have been developed explicitly for the purposes of in vitro skin irritation and corrosion tests (Netzlaff *et al.*, 2005).

## Sensitization

Information about the potential of a chemical to sensitize skin is needed in addition to irritation testing for all materials that may repeatedly come into contact with the skin. Numerous procedures have been developed to determine the potential of substances to induce a sensitization reaction in humans (delayed hypersensitivity reaction), including the Draize test, the open epicutaneous test, the Buehler test, Freund’s complete adjuvant test, the optimization test, the split adjuvant test, and the guinea pig maximization test (Hayes *et al.*, 2008; Rush *et al.*, 1995). Although they differ in regard to route and frequency of duration, they all utilize the guinea pig as the preferred test species. In general, the test chemical is administered to the shaved skin topically, intradermally, or both and may include the use of adjuvant to enhance the sensitivity of the assay. Multiple administrations of the test substance are generally given over a period of 2 to 4 weeks. Depending on the specific protocol, the treated area may be occluded. Approximately 2 to 3 weeks after the last treatment, the animals are challenged with a nonirritating concentration of the test substance and the development of erythema is evaluated.

## Subacute (Repeated-Dose Study)

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 3 to 4 different dosages of the chemicals to the animals by mixing it in their feed. For rats, 10 animals per sex per dose are often used; for dogs, 3 dosages and 3 to 4 animals per sex are used. Clinical chemistry and histopathology are performed after either 14 or 28 days of exposure, as described in the section “Subchronic.”

## Subchronic

The toxicity of a chemical after subchronic exposure is then determined. Subchronic exposure can last for different periods of time, but 90 days is the most common test duration. The principal goals of the subchronic study are to establish a NOAEL and to further identify and characterize the specific organ or organs affected by the test compound after repeated administration. One may also obtain a “lowest observed adverse effect level” (LOAEL) as well as the NOAEL for the species tested. The numbers obtained for NOAEL and LOAEL will depend on how closely the dosages are spaced and the number of animals examined. Determinations of NOAELs and LOAELs have numerous regulatory implications. For example, the EPA utilizes the NOAEL to calculate the *reference dose* (RfD), which may be used to establish regulatory values for “acceptable” pollutant levels (Barnes and Dourson, 1988) (see Chap. 4). An alternative to the NOAEL approach referred to as the *benchmark dose* uses all the experimental data to fit 1 or more dose–response curves (Crump, 1984). These curves are then used to estimate a benchmark dose that is defined as “the statistical lower bound on a dose corresponding to a specified level of risk” (Allen *et al.*, 1994a). Although subchronic studies are frequently the primary or sole source of experimental data to determine both the NOAEL and the benchmark dose, these concepts can be applied to other types of toxicity testing protocols, such as that for chronic toxicity or developmental toxicity (Allen *et al.*, 1994a,b; Faustman *et al.*, 1994) (see also Chap. 4 for a complete discussion of the derivation and use of NOAELs, RfDs, and benchmark doses). If chronic studies have been completed, these data are generally used for NOAEL and LOAEL estimates in preference to data from subchronic studies.

A subchronic study is usually conducted in 2 species (usually rat and dog for FDA, and mouse for EPA) by the route of intended exposure (usually oral). At least 3 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. Each animal should be uniquely identified with permanent markings such as ear tags, tattoos, or electronically coded microchip implants. Only healthy animals should be used, and each animal should be housed individually in an adequately controlled environment. 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 12–14 weeks) to maintain a constant intake of material based on food consumption and rate of change in body weight (Wilson *et al.*, 2008). 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 as soon as possible. Severely moribund animals should be terminated immediately to preserve tissues and reduce unnecessary suffering.

At the end of the 90-day study, all the remaining animals should be terminated and blood and tissues should be collected for further analysis. The gross and microscopic condition of the organs and tissues (about 15–20) and the weight of the major organs (about 12) are recorded and evaluated. Hematology and blood chemistry measurements are usually done before, in the middle of, and at the termination of exposure. 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, ALT, serum AST, gamma-glutamyltranspeptidase (GGT), sorbitol dehydrogenase, 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 osmolality, 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. 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.

For chemicals that are to be registered as drugs, acute and subchronic studies (and potentially additional special tests if a chemical has unusual toxic effects or therapeutic purposes) must be completed before the company can file an Investigational New Drug (IND) application with the FDA. If the application is approved, clinical trials can commence. At the same time phase I, phase II, and phase III clinical trials are performed, chronic exposure of the animals to the test compound can be carried out in laboratory animals, along with additional specialized tests.

## Chronic

Long-term or chronic exposure studies are performed similarly to subchronic studies except that the period of exposure is longer than 3 months. In rodents, chronic exposures are usually for 6 months to 2 years. Chronic studies in nonrodent species are usually for 1 year but may be longer. The length of exposure is somewhat dependent on the intended period of exposure in humans. For example, for pharmaceuticals, the ICH S4 guidance calls for studies of 6 months in duration in rodents, and 9 months in nonrodents. However, if the chemical is a food additive with the potential for lifetime exposure in humans, a chronic study up to 2 years in duration is likely to be required.

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”). This is generally derived from subchronic studies, but additional longer studies (eg, 6 months) may be necessary if delayed effects or extensive cumulative toxicity are indicated in the 90-day subchronic study. The MTD has had various definitions (Haseman, 1985). It has been defined by some regulatory agencies as the dose that suppresses body weight gain slightly (ie, 10%) in a 90-day subchronic study (Reno, 1997). However, regulatory agencies may also consider the use of parameters other than weight gain, such as physiological and pharmacokinetic considerations and urinary metabolite profiles, as indicators

of an appropriate MTD (Reno, 1997). Generally, 1 or 2 additional doses, usually fractions of the MTD (eg, one-half and one-quarter MTD), and a control group are tested.

Chronic toxicity tests may include a consideration of the carcinogenic potential of chemicals so that a separate lifetime feeding study that addresses carcinogenicity does not have to be performed. However, specific chronic studies designed to assess the carcinogenic potential of a substance may be required (see below).

## Developmental and Reproductive Toxicity

The effects of chemicals on reproduction and development also need to be determined. *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 Chap. 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 Chap. 20).

Several types of animal tests are utilized to examine the potential of an agent to alter development and reproduction. (For a detailed description of reproductive and developmental toxicity testing procedures, see Christian [2008].) General fertility and reproductive performance (segment I) tests are usually performed in rats with 2 or 3 doses (20 rats per sex per dose) of the test chemical (neither produces maternal toxicity). Males are given the chemical 60 days and females 14 days before mating. The animals are given the chemical throughout gestation and lactation. Typical observations made include the percentage of females that become pregnant, the number of stillborn and live offspring, and the weight, growth, survival, and general condition of the offspring during the first 3 weeks of life.

The potential of chemicals to disrupt normal embryonic and/or fetal development (teratogenic effects) is also determined in laboratory animals. Current guidelines for these segment II studies call for the use of 2 species, including 1 nonrodent species (usually rabbits). Teratogens are most effective when administered during the first trimester, the period of organogenesis. Thus, the animals (usually 12 rabbits and 24 rats or mice per group) are usually exposed to 1 of 3 dosages during organogenesis (days 7–17 in rodents and 7–19 in rabbits), and the fetuses are removed by cesarean section a day before the estimated time of delivery (gestational days 29 for rabbit, 20 for rat, and 18 for mouse). The uterus is excised and weighed and then examined for the number of live, dead, and resorbed fetuses. Live fetuses are weighed; half of each litter is examined for skeletal abnormalities and the remaining half for soft tissue anomalies.

The perinatal and postnatal toxicities of chemicals also are often examined (segment III). This test is performed by administering the test compound to rats from the 15th day of gestation throughout delivery and lactation and determining its effect on the birth weight, survival, and growth of the offspring during the first 3 weeks of life.

In some instances a multigenerational study may be chosen, often in place of segment III studies, to determine the effects of chemicals on the reproductive system. At least 3 dosage levels are given to groups of 25 female and 25 male rats shortly after weaning (30–40 days of age). These rats are referred to as the  $F_0$  generation. Dosing continues throughout breeding (about 140 days of age), gestation, and lactation. The offspring ( $F_1$  generation) have

thus been exposed to the chemical in utero, via lactation, and in the feed thereafter. When the  $F_1$  generation is about 140 days old, about 25 females and 25 males are bred to produce the  $F_2$  generation, and administration of the chemical is continued. The  $F_2$  generation is thus also exposed to the chemical in utero and via lactation. The  $F_1$  and  $F_2$  litters are examined as soon as possible after delivery. The percentage of  $F_0$  and  $F_1$  females that get pregnant, the number of pregnancies that go to full term, the litter size, the number of still-born, and the number of live births are recorded. Viability counts and pup weights are recorded at birth and at 4, 7, 14, and 21 days of age. The fertility index (percentage of mating resulting in pregnancy), gestation index (percentage of pregnancies resulting in live litters), viability index (percentage of animals that survive 4 days or longer), and lactation index (percentage of animals alive at 4 days that survived the 21-day lactation period) are then calculated. Gross necropsy and histopathology are performed on some of the parents ( $F_0$  and  $F_1$ ), with the greatest attention being paid to the reproductive organs, and gross necropsy is performed on all weanlings.

The International Conference on Harmonization (ICH) guidelines provide for flexible guidelines that address 6 “ICH stages” of development: pre mating and conception (stage A), conception to implantation (stage B), implantation to closure of the hard palate (stage C), closure of the hard palate to end of pregnancy (stage D), birth and weaning (stage E), and weaning to sexual maturity (stage F). All of these stages are covered in the segment I to segment III studies described above (Christian, 2008).

Numerous short-term tests for teratogenicity have been developed (Faustman, 1988). These tests utilize whole-embryo culture, organ culture, and primary and established cell cultures to examine developmental processes and estimate the potential teratogenic risks of chemicals. Many of these in utero test systems are under evaluation for use in screening new chemicals for teratogenic effects. These systems vary in their ability to identify specific teratogenic events and alterations in cell growth and differentiation. In general, the available assays cannot identify functional or behavioral teratogens (Faustman, 1988).

## Mutagenicity

Mutagenesis is the ability of chemicals to cause changes in the genetic material in the nucleus of cells in ways that allow the changes to be transmitted during cell division. Mutations can occur in either of 2 cell types, with substantially different consequences. Germinal mutations damage DNA in sperm and ova, which can undergo meiotic division and therefore have the potential for transmission of the mutations to future generations. If mutations are present at the time of fertilization in either the egg or the sperm, the resulting combination of genetic material may not be viable, and the death may occur in the early stages of embryonic cell division. Alternatively, the mutation in the genetic material may not affect early embryogenesis but may result in the death of the fetus at a later developmental period, resulting in abortion. Congenital abnormalities may also result from mutations. Somatic mutations refer to mutations in all other cell types and are not heritable but may result in cell death or transmission of a genetic defect to other cells in the same tissue through mitotic division. Because the initiating event of chemical carcinogenesis is thought to be a mutagenic one, 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. Some genetic alterations are visible with the light microscope. In this case, cytogenetic analysis of bone marrow smears is used after the animals have been exposed to the test agent. Because some mutations are

incompatible with normal development, the mutagenic potential of a chemical can also be evaluated by the dominant lethal test. This test is usually performed in rodents. The male is exposed to a single dose of the test compound and then is mated with 2 untreated females weekly for 8 weeks. The females are killed before term, and the number of live embryos and the number of corpora lutea are determined.

The test for mutagens that has received the widest attention is the *Salmonella*/microsome test developed by Ames *et al.* (1975). This test uses several mutant strains of *Salmonella typhimurium* that lack the enzyme phosphoribosyl ATP synthetase, which is required for histidine synthesis. These strains are unable to grow in a histidine-deficient medium unless a reverse or back mutation to the wild type has occurred. Other mutations in these bacteria have been introduced to enhance the sensitivity of the strains to mutagenesis. The 2 most significant additional mutations enhance penetration of substances into the bacteria and decrease the ability of the bacteria to repair DNA damage. Because many chemicals are not mutagenic or carcinogenic unless they are biotransformed to a toxic product by enzymes in the endoplasmic reticulum (microsomes), rat liver microsomes are usually added to the medium containing the mutant strain and the test chemical. The number of reverse mutations is then quantified by the number of bacterial colonies that grow in a histidine-deficient medium.

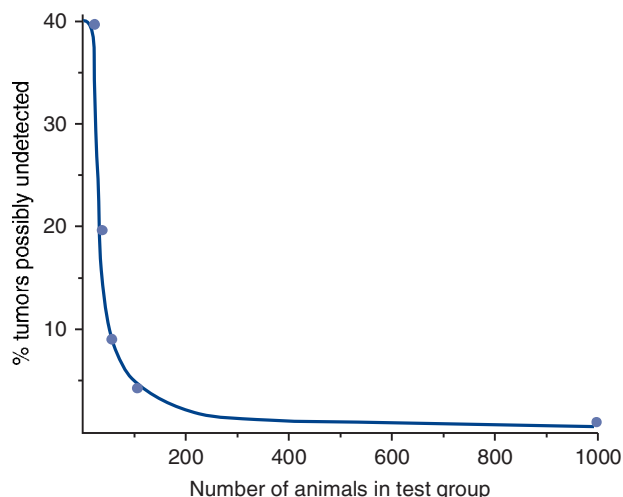
Strains of yeast have recently been developed that detect genetic alterations arising during cell division after exposure to nongenotoxic carcinogens as well as mutations that arise directly from genotoxic carcinogens. This test identifies deletions of genetic material that occur during recombination events in cell division that may result from oxidative damage to DNA, direct mutagenic effects, alterations in fidelity of DNA repair, and/or changes in cell cycle regulation (Galli and Schiestl, 1999). Mutagenicity is discussed in detail in Chap. 9.

With the advent of techniques that readily allow manipulation of the mouse genome, transgenic animals have been developed that allow for in vivo assessment of mutagenicity of compounds. For example, 2 commercially available mouse strains, the “MutaMouse” and “Big Blue,” contain the *lac* operon of *E. coli* that has been inserted into genomic DNA using a lambda phage to DNA to produce a recoverable shuttle vector. Stable, homozygous strains of these transgenic animals (both mice and rats have been engineered) can be exposed to potential mutagenic agents. Following in vivo exposure, the target *lac* genes can be recovered from virtually any cell type or organ and analyzed for mutations (Brusick *et al.*, 2008).

## Oncogenicity Bioassays

Oncogenicity studies are both time consuming and expensive, and are usually only done when there is reason to suspect that a chemical may be carcinogenic, or when there may be wide spread, long-term exposures to humans (eg, widely used food additives, drinking water contaminants, or pharmaceuticals that are likely to be administered repeatedly for long periods of time). Chemicals that test positive in several mutagenicity assays are likely to be carcinogenic, and thus are frequent candidates for oncogenicity bioassay assessment. In the United States, the National Toxicology Program (NTP) has the primary responsibility for evaluating non-drug chemicals for carcinogenic potential. For pharmaceuticals, the FDA may require the manufacturer to conduct oncogenicity studies as part of the preclinical assessment, depending on the intended use of the drug, and the results of mutagenicity assays and other toxicological data.





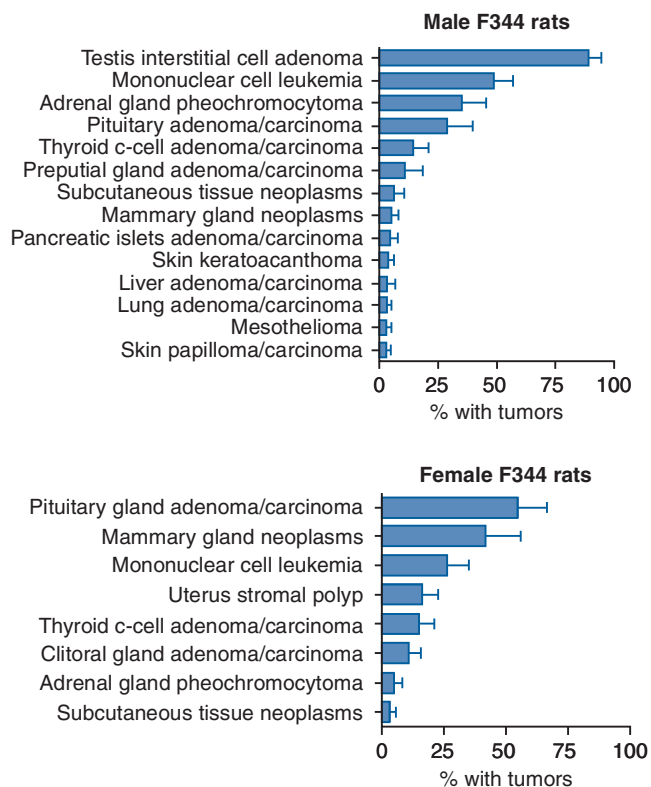
**Figure 2-14.** Statistical limitations in the power of experimental animal studies to detect tumorigenic effects.

Studies to evaluate the oncogenic (carcinogenic) 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, 2–2.5 years for rats). To ensure that 30 rats per dose survive the 2-year study, 60 rats per group per sex are often started in the study. Both gross and microscopic pathological examinations are made not only on animals that survive the chronic exposure but also on those that die prematurely. The use of the MTD in carcinogenicity has been the subject of controversy. The premise that high doses are necessary for testing the carcinogenic potential of chemicals is derived from the statistical and experimental design limitations of chronic bioassays. Consider that a 0.5% increase in cancer incidence in the United States would result in over 1 million additional cancer deaths each year—clearly an unacceptably high risk. However, identifying with statistical confidence a 0.5% incidence of cancer in a group of experimental animals would require a minimum of 1000 test animals, and this assumes that no tumors were present in the absence of exposure (zero background incidence).

Fig. 2-14 shows the statistical relationship between minimum detectable tumor incidence and the number of test animals per group. This curve shows that in a chronic bioassay with 50 animals per test group, a tumor incidence of about 8% could exist even though no animals in the test group had tumors. This example assumes that there are no tumors in the control group. These statistical considerations illustrate why animals are tested at doses higher than those that occur in human exposure. Because it is impractical to use the large number of animals that would be required to test the potential carcinogenicity of a chemical at the doses usually encountered by people, the alternative is to assume that there is a relationship between the administered dose and the tumorigenic response and give animals doses of the chemical that are high enough to produce a measurable tumor response in a reasonable size test group, such as 40 to 50 animals per dose. The limitations of this approach are discussed in Chap. 4. For nonmutagenic pharmaceutical agents, ICH S1C provides the following guidance on dose selection for oncogenicity studies: “The doses selected for rodent bioassays for non-genotoxic pharmaceuticals should provide an exposure to the agent that (1) allow an adequate margin of safety over the human therapeutic exposure, (2) are tolerated without significant chronic physiological dysfunction and are compatible with good survival, (3) are guided by a comprehensive set of animal and human data that focus broadly on the properties of the agent and the suitability of the animal (4) and permit data interpretation in the context of clinical use.”

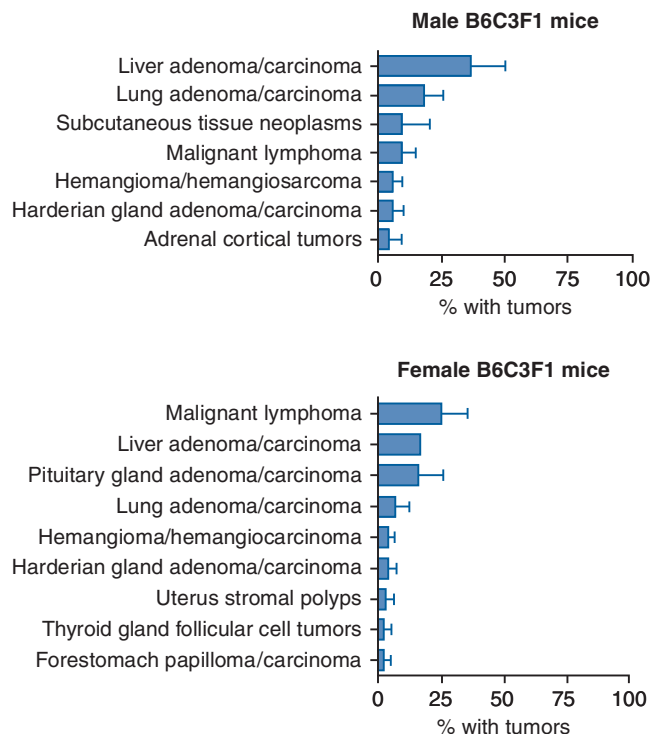
Another approach for establishing maximum doses for use in chronic animal toxicity testing of drugs is often used for substances for which basic human pharmacokinetic data are available (eg, new pharmaceutical agents that have completed phase I clinical trials). For chronic animal studies performed on drugs where single-dose human pharmacokinetic data are available, a daily dose that would provide an area under the curve (AUC) in laboratory animals equivalent to 25 times the AUC in humans given the highest (single) daily dose to be used therapeutically may be used, rather than the MTD. Based on a series of assumptions regarding allometric scaling between rodents and humans (Table 2-2), the ICH noted that it may not be necessary to exceed a dose of 1500 mg/kg per day where there is no evidence of genotoxicity, and where the maximum recommended human dose does not exceed 500 mg per day.

Most regulatory guidelines require that both benign and malignant tumors be reported in oncogenicity bioassays. Statistical increases above the control incidence of tumors (either all tumors or specific tumor types) in the treatment groups are considered indicative of carcinogenic potential of the chemical unless there are qualifying factors that suggest otherwise (lack of a dose response, unusually low incidence of tumors in the control group compared with “historic” controls, etc; Huff, 1999). Thus, the conclusion as to whether a given chronic bioassay is positive or negative for carcinogenic potential of the test substance requires careful consideration of background tumor incidence. Properly designed chronic oncogenicity studies require that a concurrent control group matched for variables such as age, diet, and housing conditions be used. For some tumor types, the “background” incidence of tumors is surprisingly high. Fig. 2-15 shows the background tumor incidence for



**Figure 2-15.** Most frequently occurring tumors in untreated control rats from recent NTP 2-year rodent carcinogenicity studies. The values shown represent the mean  $\pm$  SD of the percentage of animals developing the specified tumor type at the end of the 2-year study. The values were obtained from 27 different studies involving a combined total of between 1319 and 1353 animals per tumor type.





**Figure 2-16.** Most frequently occurring tumors in untreated control mice from recent NTP 2-year rodent carcinogenicity studies. The values shown represent the mean  $\pm$  SD of the percentage of animals developing the specified tumor type at the end of the 2-year study. The values were obtained from 30 different studies involving a total of between 1447 and 1474 animals per tumor type.

various tumors in male and female F344 rats used in 27 NTP 2-year rodent carcinogenicity studies. The data shown represent the percent of animals in control (nonexposed) groups that developed the specified tumor type by the end of the 2-year study. These studies involved more than 1300 rats of each sex. Fig. 2-16 shows similar data for control (nonexposed) male and female B6C3F1 mice from 30 recent NTP 2-year carcinogenicity studies and includes data from over 1400 mice of each sex. There are several key points that can be derived from these summary data:

1. Tumors, both benign and malignant, are not uncommon events in animals even in the absence of exposure to any known carcinogen.
2. There are numerous different tumor types that develop “spontaneously” in both sexes of both rats and mice, but at different rates.
3. Background tumors that are common in 1 species may be uncommon in another (eg, testicular interstitial cell adenomas are very common in male rats but rare in male mice; liver adenomas/carcinomas are about 10 times more prevalent in male mice than in male rats).
4. Even within the same species and strain, large gender differences in background tumor incidence are sometimes observed (eg, adrenal gland pheochromocytomas are about 7 times more prevalent in male F344 rats than in female F344 rats; lung and liver tumors are twice as prevalent in male B6C3F1 mice as in female B6C3F1 mice).
5. Even when the general protocols, diets, environment, strain and source of animals, and other variables are relatively constant, background tumor incidence can vary widely, as shown by the relatively large SDs for some tumor types in the NTP

bioassay program. For example, the range in liver adenoma/carcinoma incidence in 30 different groups of unexposed (control) male B6C3F1 mice went from a low of 10% to a high of 68%. Pituitary gland adenomas/carcinomas ranged from 12% to 60% and 30% to 76% in unexposed male and female F344 rats, respectively, and from 0% to 36% in unexposed female B6C3F1 mice.

Taken together, these data demonstrate the importance of including concurrent control animals in such studies. In addition, comparisons of the concurrent control results to “historic” controls accumulated over years of study may be important in identifying potentially spurious “false-positive” results. The relatively high variability in background tumor incidence among groups of healthy, highly inbred strains of animals maintained on nutritionally balanced and consistent diets in rather sterile environments highlights the dilemma in interpreting the significance of both positive and negative results in regard to the human population, which is genetically diverse, has tremendous variability in diet, nutritional status, and overall health, and lives in an environment full of potentially carcinogenic substances, both natural and human-made.

Finally, it should be noted that both inbred and outbred strains have distinct background tumor patterns and the NTP and most other testing programs select strains based on the particular needs of the agent under study. For example, the NTP used the Wistar rat for chemicals that may have the testis as a target organ, based on acute, subchronic, or other bioassay results. Similarly, the NTP used the Sprague–Dawley strain of rat in studies of estrogenic agents such as genistein because its mammary tumors are responsive to estrogenic stimulation, as are humans’.

## Neurotoxicity Assessment

Neurotoxicity or a neurotoxic effect is defined as an adverse change in the chemistry, structure, or function of the nervous system following exposure to a chemical or physical agent. The structure, function, and development of the nervous system and its vulnerability to chemicals are examined in Chap. 16. When evaluating the potential neurological effects of a compound, effects may be on the central or peripheral nervous system or related to exposure that occurred during development or as an adult. The developing nervous system is particularly sensitive to chemical exposures (see Chap. 10).

In vitro systems often using cell culture techniques are a rapidly developing area of neurotoxicity assessment. Specific cell lines are available to examine effects on neuron or glial cells such as proliferation, migration, apoptosis, synaptogenesis, and other end points. In vitro assays have a number of potential advantages including minimizing the use of animal, lower costs, and adaptable to high-throughput screening. It is also possible to use an in vitro model to examine the interaction of chemicals, such as food additives, on neuronal cells (Lau *et al.*, 2006). The principles and challenges of in vitro neurotoxicity testing are well described (Claudio, 1992; Tiffany-Castiglioni, 2004).

Procedures for the neurobehavioral evaluation of animals were initially developed as part of the scientific investigation of behavioral motivation. Some of these procedures were then used to evaluate the neuropharmacological properties of new drugs. Now animals are commonly used to evaluate the neurotoxic properties of chemicals. A wide range of adult and developmental animal tests are used to access neurobehavioral function. In addition, neuropathological assessment is an important part of the neurotoxicity evaluation and

best practices have been developed for developmental neurotoxicity (DNT) (Bolon *et al.*, 2006). Irwin developed a basic screen for behavioral function in mice (Irwin, 1968), which was subsequently refined to the functional observational battery (FOB) (Moser, 2000). The FOB can also be used in the evaluation of drug safety (Redfern *et al.*, 2005).

The US EPA established a protocol for the evaluation of DNT in laboratory animals (US EPA 870.6300 and OECD 426) (EPA, 1998; OECD, 2004). These protocols include tests of neurobehavioral function, such as auditory startle, learning and memory function, changes in motor activity, and neuropathological examination and morphometric analysis. Methods and procedures for DNT evaluation are well established (Claudio *et al.*, 2000; Cory-Slechta *et al.*, 2001; Dorman *et al.*, 2001; Garman *et al.*, 2001; Miles and Ferenc, 2001). Recent studies examine the neurotoxicity of multiple chemical exposures in animals (Moser *et al.*, 2006). Methods are also available to examine cognitive measures on weanling rodents in DNT studies (Ehman and Moser, 2006). Nonhuman primates have been invaluable in evaluating the effects of neurotoxicants and the risk assessment process (Burbacher and Grant, 2000). Sophisticated assessment of operant behavior, and learning and memory assessment of rodents, has been used to evaluate the effects of lead (Cory-Slechta, 1995, 1996, 2003). Monkeys can also be used to evaluate the low-level effects of neurotoxicants such as mercury on vision, auditory function, and vibration sensitivity (Burbacher *et al.*, 2005; Rice and Gilbert, 1982, 1992, 1995). There is remarkable concordance between human and animal neurotoxicity assessment, for example, in lead, mercury, and PCBs (Rice, 1995).

Human testing for the neurological effects of occupational exposures to chemicals (Anger, 2003; Farahat *et al.*, 2003; Kamel *et al.*, 2003; McCauley *et al.*, 2006), and even the neurotoxic effects of war (Binder *et al.*, 1999, 2001), is advancing rapidly. These methods have also been applied to Hispanic workers (Rohlman *et al.*, 2001b) and populations with limited education or literacy (Rohlman *et al.*, 2003). The WHO has also recommended a test battery for humans (Anger *et al.*, 2000). There are also neurobehavioral test batteries for assessing children (Rohlman *et al.*, 2001a). Evaluation of the childhood neurological effects of lead (Lanphear *et al.*, 2005; Needleman and Bellinger, 1991) and mercury (Myers *et al.*, 2000) has added enormously to our understanding of the health effects of these chemicals and to the methodology of human neurobehavioral testing.

In summary, the neurotoxicological evaluation is an important aspect of developing risk assessments for environmental chemicals and drugs.

## Immunotoxicity Assessment

Under normal conditions, the immune system is responsible for host defense against pathogenic infections and certain cancers. However, environmental exposures can alter immune system development and/or function and lead to hypersensitivity, autoimmunity, or immunosuppression, the outcome of which may be expressed as a pathology in most any organ or tissue (see Chap. 12). Our understanding of the biological processes underlying immune system dysfunction remains incomplete. However, advances in molecular biology (including use of transgenic/knockout mice), analytic methods (including gene expression arrays and multiparameter flow cytometry), animal models (including adoptive transfers in immunocompromised mice and host resistance to viral, bacterial, or tumor cell challenge), and other methods are greatly advancing our knowledge.

From a toxicologist's perspective, evaluation of immune system toxicity represents special challenges. Development of hypersensitivity can take various forms, depending on the mechanism underlying the associated immune response, and standard assumptions regarding dose-response relationships may not necessarily apply. For example, a single or incidental exposure to beryllium has been associated with chronic beryllium disease in some individuals. We are only just beginning to understand the biological basis underlying such individual susceptibility. In the case of chronic beryllium disease, a genetic polymorphism in a gene involved in antigen recognition may be associated with increased susceptibility (see Bartell *et al.*, 2000). Although our ability to predict immunogenicity remains poor, research efforts are continuing to identify aspects of the chemical and the individual that confer immunogenicity and underlie hypersensitivity. For example, the increasing incidence of allergic asthma among preschool-age children in the United States since the 1980s may be associated with exposure to allergens (eg, dust mites, molds, and animal dander), genetic factors, and other factors in the in utero and postnatal environment (see Donovan and Finn, 1999; Armstrong *et al.*, 2005).

Immunosuppression is another form of immune system toxicity, which can result in a failure to respond to pathogenic infection, a prolonged infection period, or expression of a latent infection or cancer. Various chemicals have been associated with immunosuppression. Broad-spectrum and targeted immunosuppressive chemicals are designed and used therapeutically to reduce organ transplant rejection or suppress inflammation. However, a large number of chemicals have been associated with immunosuppression, including organochlorine pesticides, diethylstilbesterol, lead, and halogenated aromatic hydrocarbons (including TCDD), and exposures that occur during critical stages may present special risk to development (Holladay, 2005).

Autoimmunity is a specific immune system disorder in which components of the immune system attack normal (self) tissues. Cases of autoimmunity have been reported for a wide range of chemicals including therapeutic drugs, metals, pesticides, and solvents. As with other forms of immune system toxicity, autoimmunity can present in most any tissue.

Finally, new forms of immunotoxicity are appearing based on novel forms of clinical therapy and immunomodulation. These include the variously classified "tumor lysis syndromes" and "cytokine storms" that arise from massive cytokine dysregulation. A recent example involved 6 healthy volunteers who had enrolled in a phase 1 clinical trial in the United Kingdom who developed a severe cytokine response to an anti-CD28 monoclonal antibody leading to systemic organ failures (Bhagal and Combes, 2006). Such cases are stark reminders of the challenges we face in understanding how the immune system is regulated, developing reliable test systems for identifying such risks prior to human use, and developing safe means for testing these agents in humans.

As described in Chap. 12, current practice for evaluating potential toxic effects of xenobiotic exposures on the immune system involves a tiered approach to immunotoxicity screening (Luster *et al.*, 2003). This tiered approach is generally accepted worldwide in the registration of novel chemical and therapeutic products. Most recently, final guidance to the pharmaceutical industry was published in April 2006 by the ICH of Technical Requirements for Registration of Pharmaceuticals for Human Use (Table 2-3). This guidance, which applies to the nonclinical (animal) testing of human pharmaceuticals, is the accepted standard in the United States, EU, and Japan, and demonstrates the continued commitment by these regulatory bodies to understand the potential risks posed by novel therapeutics.

Tiered testing relies on the concept that standard toxicity studies can provide good evidence for immunotoxicity when considered with known biological properties of the chemical, including structural similarities to known immunomodulators, disposition, and other clinical information, such as increased occurrence of infections or tumors. Evaluation of hematological changes, including differential effects on white blood cells and immunoglobulin changes, and alterations in lymphoid organ weights or histology, can provide strong evidence of potential effects to the immune system. Should such evaluations indicate a potential effect on immune system function, more detailed evaluations may be considered, including the evaluation of functional effects (eg, T-cell-dependent antibody response or natural killer cell activity), flow cytometric immunophenotyping, or host resistance studies. Thus, as with other areas of toxicology, the evaluation of immune system toxicity requires the toxicologist to be vigilant in observing early indications from a variety of sources in developing a weight-of-evidence assessment regarding potential injury/dysfunction.

## Other Descriptive 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 usually are carried out in a dynamic (flowing) chamber rather than in static chambers to avoid particulate settling and exhaled gas complications. Such studies usually require special dispersing and analytic methodologies, depending on whether the agent to be tested is a gas, vapor, or aerosol; additional information on methods, concepts, and problems associated with inhalation toxicology is provided in Chaps. 15 and 28. The duration of exposure for inhalation toxicity tests can be acute, subchronic, or chronic, but acute studies are more common with inhalation toxicology. Other special types of animal toxicity tests include toxicokinetics (absorption, distribution, biotransformation, and excretion), the development of appropriate antidotes and treatment regimens for poisoning, and the development of analytic techniques to detect residues of chemicals in tissues and other biological materials.

## TOXICOGENOMICS

In the past decade, numerous new genome-based technologies have become available that allow for the large-scale analysis of biological responses to external stimuli. Traditional scientific approaches to elucidate the biochemical and molecular effects of toxic substances focused largely on examining biochemical pathways that were logically connected to observed responses identified through gross pathology, histology, blood chemistry, or behavioral observations. Such “hypothesis-driven” research into understanding mechanism of action remains a mainstay of current scientific investigations in toxicology. However, technologies now available allow one to examine the entire “universe” of biological responses to a toxic substance (Fig. 2-17). These new “hypothesis-generating” technologies include genomics (characterization of much or all of the genome of an organism), transcriptomics (characterization of most or all of the messenger RNAs [mRNAs], or transcriptome, expressed in a given cell/tissue), proteomics (characterization of most or all of the proteins expressed in a given cell/tissue), and metabonomics (characterization of most or all of the small molecules in a cell or tissue, including substrates, products, and cofactors of enzyme reactions). Other “omics” approaches (eg, “lipidomics,”

“nutrigenomics”) are being devised to look broadly at the biological response of an organism to change. The integration of all of these levels of molecular function (genomics, transcriptomics, proteomics, metabonomics, etc) to the understanding of how a living organism functions at the cellular level is sometimes referred to as “systems biology” (Weston and Hood, 2004). Because each level of analysis generates a very large quantity of data, the collection, organization, evaluation, and statistical analysis is in itself an enormous undertaking. The field of “bioinformatics” has been developed to address the many computational and statistical challenges of “omics” data. In the field of toxicology, the term “toxicogenomics” is used to define the area of research that “combines transcript, protein and metabolite profiling with conventional toxicology to investigate the interaction between genes and environmental stress in disease causation” (Waters and Fostel, 2004). A conceptual model for how the various new “omics” technologies can be incorporated into toxicological evaluation is shown in Fig. 2-17.

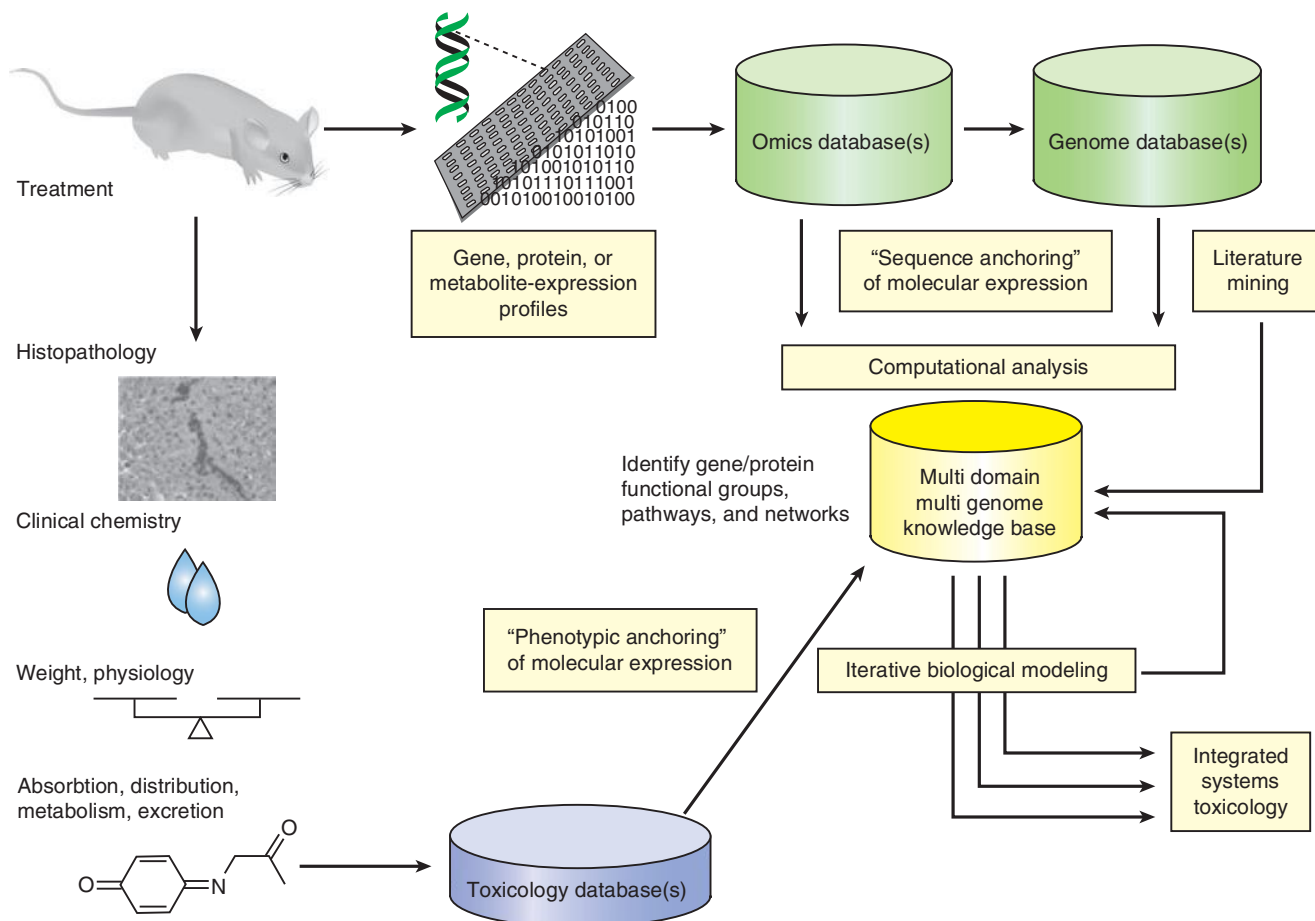
## Genomics

The genome of an organism represents the full complement of genes that are determined at fertilization by the combination of the parental DNA. Thus, each cell of an organism has the same genome, characterized by the nucleotide sequences inherited from its parents. The human genome consists of approximately 3 billion base pairs of deoxyribonucleotides. Within the human genome, there is, on average, about 0.1% variability in DNA sequence between any 2 individuals, and it is these differences that contribute to the uniqueness of each person. Most of this variability exists as “SNPs,” although larger segments of DNA may be variable between individuals, including the duplication or loss of entire genes. The identification of particular genetic variants, such as the GSTM1 polymorphism, which might contribute to interindividual differences in susceptibility to chemicals or other environmental factors discussed previously, represents a relatively new and growing area of study that aims to understand the complex interactions between the human genome and the environment (Costa and Eaton, 2006).

Although the genome provides the blueprint for biological function, in order for the genomic information to be utilized in a cell, it must be expressed. Expression of the genome occurs when the coding sequence of DNA is converted to mRNA. For any given cell, transcription of the genomic information contained in that cell is only partial. It is the differential expression of genes in a given cell that is largely responsible for the diverse function of the thousands of different cells, tissues, and organs that constitute an individual organism. Thus, understanding which genes are expressed in a given tissue, at what level, and how toxicants perturb the “transcriptome” is of great relevance to toxicology. In addition to coding for mRNAs that provide the blueprint for protein synthesis, genomic DNA also generates small interfering RNAs (siRNA, microRNAs) that are biologically active and can participate in the regulation of gene expression.

## Epigenetics/Epigenomics

The expanding research into the relatively new field of epigenomics will have important implications for public health and toxicology. The concept of epigenetics, meaning something acting “above or in addition” to genes, was proposed many decades ago, although the application to the full genome (epigenomics) rather than to single or a few genes (epigenetics) is new. Conrad Hal Waddington first postulated in the 1930s that it was not just the genes that shaped development but also the environment that shapes the genes



**Figure 2-17.** 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 (eg, rats, mice) and the target species of interest (eg, 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). (From Waters and Fostel, 2004, with permission.)

(Holliday, 2006). Understanding a possible mechanism had to wait for a far deeper understanding of DNA and its role in development. Epigenetics has been defined in various ways, with perhaps the strictest definition being “a mitotically or meiotically heritable change in gene expression that occurs independently of an alteration in DNA sequence” (Youngson and Whitelaw, 2008). Typically gene expression is 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 (Fig. 2-18). Epigenetic changes can potentially be transgenerational, as suggested in some animal models, which has important implications for toxicological assessment (Rosenfeld, 2010; Skinner, 2011). Given the growing recognition of epigenetics as a means by which environmental factors can alter biological responses, genomic analyses in toxicology may also include techniques to identify toxicant-induced changes in DNA methylation patterns (Watson and Goodman, 2002; LeBaron *et al.*, 2010).

Although classical approaches to toxicology have thoroughly documented the potential for a variety of environmental toxicants, such as thalidomide, alcohol, lead, mercury, and PCBs, to cause adverse effects on the developing organism, more subtle epigenetic changes, which are not associated with either cytotoxicity or mutations, can also result from environmental exposures and

thus may have important toxicological implications. Epigenetic changes have been demonstrated to occur from exposure to a variety of environmental hazards, including tobacco smoke, metals, alcohol, phthalates, and BPA (Cheng *et al.*, 2012; Perera and Herbstman, 2011; Bernal and Jirtle, 2010; Baccarelli and Bollati, 2009). Furthermore, epigenetic changes can occur through nutrition, methyl content of diet, intake of folic acid and vitamins, or even social and maternal behavior toward the offspring (Cummings *et al.*, 2010). Epigenetic changes have been causally implicated in cancer, neurodevelopment disorders, autoimmune diseases, diabetes and metabolic disorders, asthma, behavioral disorders, and endocrine disorders (Godfrey *et al.*, 2011; Nystrom and Mutanen, 2009; Zhang and Ho, 2011; Attig *et al.*, 2010). There is also concern chemicals in the environment may induce epigenetic changes in wildlife that could be an important consideration in ecotoxicology (Vandegheuchte and Janssen, 2011; Head *et al.*, 2012). Thus, epigenetic changes induced by xenobiotics, dietary factors, and maternal behavior have important implications for safety assessment and risk assessment for xenobiotics (LeBaron *et al.*, 2010; Goodman *et al.*, 2010; Szyf, 2007).

Thus, it is now evident that methylation of DNA is an important determinant of gene expression in cells and tissues, and exogenous chemicals can interfere with transcriptional function



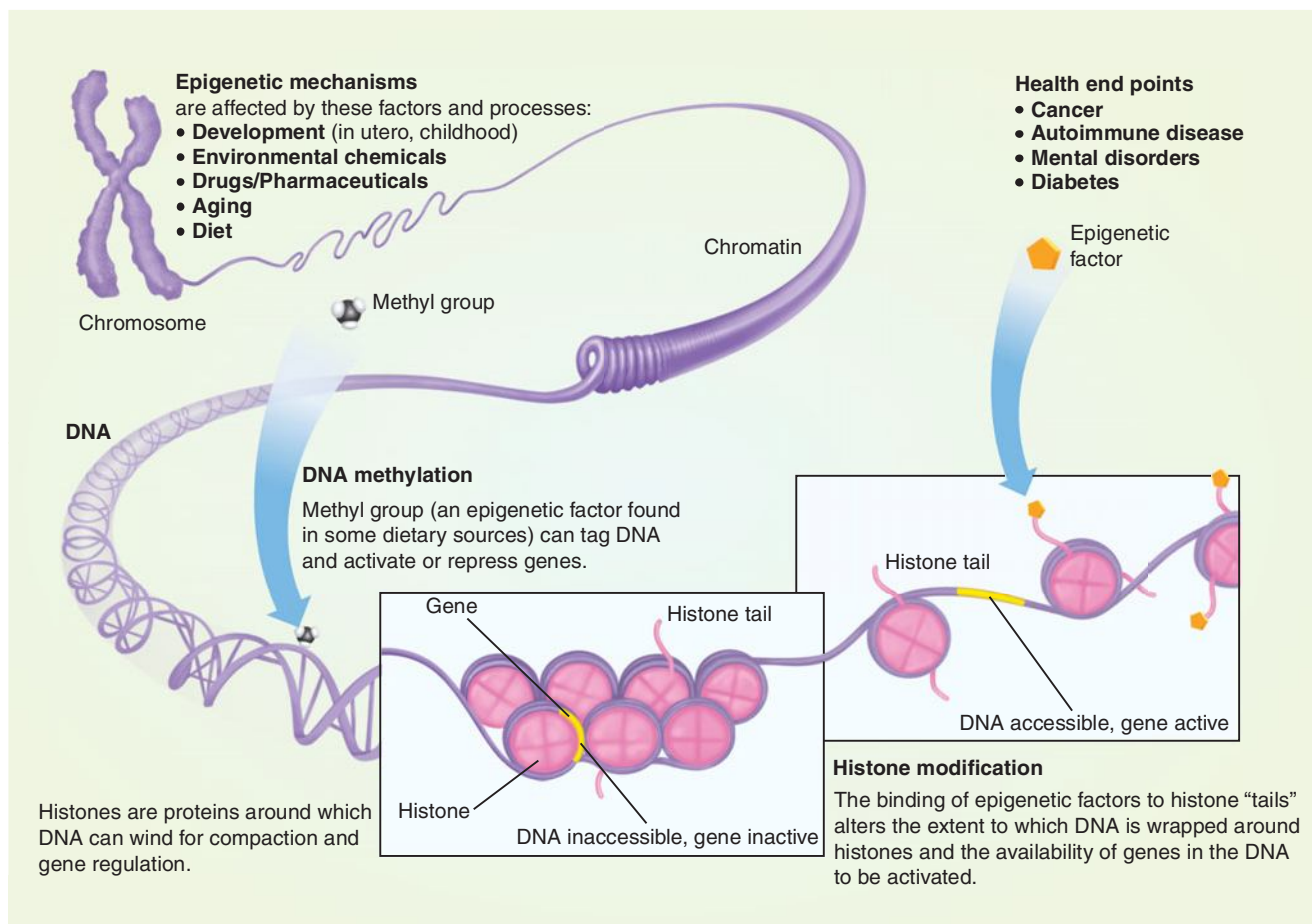


Figure 2-18. Process and consequence of epigenetic regulation of gene expression (National Institute of Health).

via alternating DNA methylation (Watson and Goodman, 2002). Importantly, although such epigenetic changes do not result in the alteration of the genomic sequence, they theoretically can result in heritable phenotypic changes; although proof of multigenerational epigenetic changes from environmental exposures has yet to be demonstrated in humans (Baccarelli and Bollati, 2009), several animal models have demonstrated transgenerational epigenetic changes (Skinner, 2011). Thus, genomic analyses in toxicology may also include techniques to identify toxicant-induced changes in DNA methylation patterns to access epigenetic changes and the potential consequences (Watson and Goodman, 2002; Szyf, 2007).

## Transcriptomics

Among the first changes that a cell will exhibit following exposure to a toxic substance is a change in gene expression. 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. Toxicologists have utilized the so-called Northern blot analysis to assess the level of expression of individual genes in cells or tissues for decades. The “reverse transcriptase polymerase chain reaction” (RT-PCR) allows one to quantitatively measure the relative number of mRNA species in a sample for specific genes. Using general primers, it is also possible to amplify the entire transcriptome quantitatively to make many complete copies of the transcriptome in a test tube. Thus, large amounts of material for analysis can be obtained from a relatively small number of cells. Finally,

using microarray technologies, where tens of thousands of unique oligonucleotides (or cDNAs) are anchored on a solid matrix, toxicologists can now quantitatively assess the expression of thousands of unique mRNAs in a single sample, thus capturing an “expression profile” of the entire transcriptome in 1 analysis.

There is great promise that gene expression profiles may be used to provide signatures of specific types of toxic responses, such as a cellular response to DNA damage or oxidative stress. There is also hope that such signature changes in gene expression could be used to facilitate more accurate cross-species extrapolation, allowing comparison of, for example, toxicant-induced changes in gene expression in rat hepatocytes with that of human hepatocytes under identical experimental conditions. However, 1 of the major challenges in toxicogenomics is the recognition that transcriptional regulation is highly dynamic, and that gene expression profiles can change dramatically with both dose and time. Because microarray experiments are relatively expensive and highly data intensive, it becomes both costly and challenging to conduct and analyze experiments with extensive dose and time course data (although costs are declining). Although changes in gene expression often contribute to, or are reflective of, phenotypic changes that occur in response to a toxic substance, the transcriptome is still somewhat far removed from the ultimate biochemical functions that dictate the actual biological function of the cell. Because the functional expression of a gene generally requires the translation of the mRNA to a protein, there is also great interest in looking at the “proteome”—the entire complement of *proteins* that are present in a cell or tissue at a given point in time.

## Proteomics

Analysis of the proteome of a cell or tissue is much more difficult than analysis of the transcriptome, primarily because it is not yet possible to “amplify” the number of copies of proteins in a cell. Furthermore, unambiguous identification of specific proteins is much more difficult than that for individual mRNAs. Identification of specific proteins is generally done using a combination of separation techniques (eg, 2D gel electrophoresis, high-performance liquid chromatography), followed by tandem mass spectrometry for identification (Aebersold and Mann, 2003). Because of size limitations for accurate mass spectrometry, protein mixtures are usually digested to smaller peptide fragments. The mixture of peptide fragments is resolved into individual components, and the identity of the specific peptides is determined based on high-resolution mass analysis and sequential degradation (sequential loss of single amino acids) of the peptides by various means (Aebersold and Mann, 2003). The large and complex set of peptide mass fragments is then analyzed by computers and compared with a large database of mass fragments of known peptides/proteins. Because as few as 5 amino acid sequences may provide unique identification of a specific protein, the presence and relative abundance of specific proteins in a sample can then be reconstructed through bioinformatic analyses. As with transcriptomics, it is hoped that changes in protein expression can be used as specific biomarkers for particular types of toxic responses. Of course, such conceptual approaches have been used for years, for example, use of serum transaminase proteins as indicators of liver damage, or the presence of prostate-specific antigen (PSA) in serum as a potential biomarker of early stage prostate hyperplasia or cancer. The potential power of proteomics lies in the ability to identify unique patterns of protein expression, or identification of unique proteins or peptides, that are predictive of early toxic response or later development of disease.

## Metabonomics/Metabolomics

These 2 terms are often used interchangeably to describe the analysis of the “universe” of small molecules that serve as substrates, products, and cofactors of the milieu of enzymatic reactions and other metabolic processes that define living cells, and thus the organism. Metabonomics has been defined as “the comprehensive and simultaneous systematic profiling of metabolite levels and their systematic and temporal change through such effects on diet, lifestyle, environment, genetic and pharmaceuticals, both beneficial and adverse, in whole organisms” (Lindon *et al.*, 2003, 2006). The term “metabolomics” has been used principally in studies in plants and in vitro or single-cell systems (Fiehn, 2002). Regardless of the specific term used (metabonomics will be used here), the concept of quantitatively analyzing toxicant-induced changes in the “metabolic profile” (the “metabonome”) of a cell, tissue, or body fluid in some ways represents the “Holy Grail” of toxicogenomics, because the changes in these small molecules must represent a biologically relevant integration of all of the molecular, biochemical, and cellular perturbations that lead to the development of toxicity (Fig. 2-17). In other words, changes in the metabonome should reflect the biologically relevant changes in gene transcription, translation, protein function, and other cellular processes, including temporal and adaptive responses, while ignoring biologically irrelevant changes in these factors. Although conceptually superior to either transcriptomics or proteomics for predictive toxicology, metabonomics lags significantly in technological development of readily accessible tools for thorough analysis of the metabonome.

Two approaches for identifying and measuring hundreds, or even thousands, of small molecules in biological samples have emerged—nuclear magnetic resonance (NMR) and mass spectrometry (Lindon *et al.*, 2003, 2006). Both have their advantages and limitations, and it is likely that the most successful approaches to applying metabonomics to toxicological problems will utilize both techniques (Pan and Raftery, 2007).

## Bioinformatics

One feature in common among all of the various “omics” technologies is the ability to generate very large volumes of data (literally millions of data points from a single experiment). Both the data management and statistical evaluation of toxicogenomics studies represent an enormous challenge. The emerging field of bioinformatics has developed to address these challenges. Numerous commercial platforms for conducting microarray analysis of the transcriptome are available, and sophisticated software is available for both data management and analysis. One of the major challenges in statistical analysis of large data sets is the large number of “false positives” that will result from multiple comparisons. In a typical gene array experiment, it is not uncommon for an investigator to make >20,000 different comparisons. At the typical “95%” statistical confidence limit, one would expect more than 1000 of the noted differences to occur just by chance alone. Thus, more rigorous statistical methods have been developed to reduce the so-called false discovery rate in such experiments (Storey *et al.*, 2005; Gao, 2006).

## Challenges in Using “Omics” Technologies for Predictive Toxicology and Risk Assessment

A conceptual framework for incorporating these new technologies into toxicology, sometimes referred to as “systems toxicology,” is shown in Fig. 2-18. Several key components of such an approach include: (1) large databases of treatment-specific information, such as results of transcriptomic, proteomic, and metabonomic analyses from target tissues and/or body fluids derived from toxicant-treated animals, (2) genomic databases that describe the DNA sequence information from the species of interest, (3) computational tools that extract information from these and other databases and the published literature to identify critical pathways and networks that are altered by the toxicant treatment, and (4) comparison with traditional toxicological end points to ensure that the observed “omics responses” are closely aligned with the toxicant-related pathophysiology in the animal (histopathology, clinical chemistry, etc)—a process called “phenotypic anchoring” (Waters and Fostel, 2004).

Toxicogenomics tools are becoming indispensable for research aimed at identifying the mechanisms and mode of action of toxic substances. However, the incorporation of such approaches into routine toxicity assessment presents numerous challenges. Numerous working group reports and publications have addressed the challenges of incorporating toxicogenomics data into predictive toxicology and risk assessment (Bammler *et al.*, 2005; Maggioli *et al.*, 2006; Boverhof and Zacharewski, 2006).

One of the major challenges to incorporating toxicogenomic data into risk assessment is related to the highly dynamic processes that preceded an observed toxic response. Traditional measure of toxicity, such as histopathological changes in a tissue, tends to be stable or even irreversible, whereas the myriad of molecular, biochemical, and cellular changes that give rise to the toxic response(s) are highly dynamic, frequently changing by the hour. Thus, the

profiles of mRNAs, proteins, and/or metabolites captured at a single point in time may be dramatically different, depending on the specific point in time the sample was collected. Many of the observed changes may be the result of direct effects of the toxicant on specific targets, whereas others will be compensatory or feedback mechanisms invoked in response to the initial damage. Nevertheless, patterns of change in transcript, protein, and/or metabolite profiles are likely to provide informative “signatures” of toxic response that will be of great value in predictive toxicology. Such approaches may be particularly useful in pharmaceutical development, where toxicogenomic profiles may help to accelerate preclinical evaluation of drug candidates by identifying “class prediction” profiles indicative of certain types of desirable (pharmacological efficacy) as well as adverse (eg, DNA damage, oxidative stress) responses.

Finally, it is likely that the introduction of omics technologies to toxicity testing will eventually contribute to the reduction, refinement, and replacement (the “3Rs”) of animals in toxicity testing and product safety evaluations (Kroeger, 2006).

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# chapter 3

## Mechanisms of Toxicity

Zoltán Gregus

### Step 1—Delivery: From the Site of Exposure to the Target

#### Absorption versus Presystemic Elimination

*Absorption*  
*Presystemic Elimination*

#### Distribution To and Away from the Target

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*Excretion*  
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*Toxication*  
*Detoxication*

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*Adaptation by Decreasing the Target Density or Responsiveness*  
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*When Repair Fails*  
*When Adaptation Fails*

#### Toxicity Resulting from Inappropriate Repair and Adaptation

*Tissue Necrosis*  
*Fibrosis*  
*Carcinogenesis*

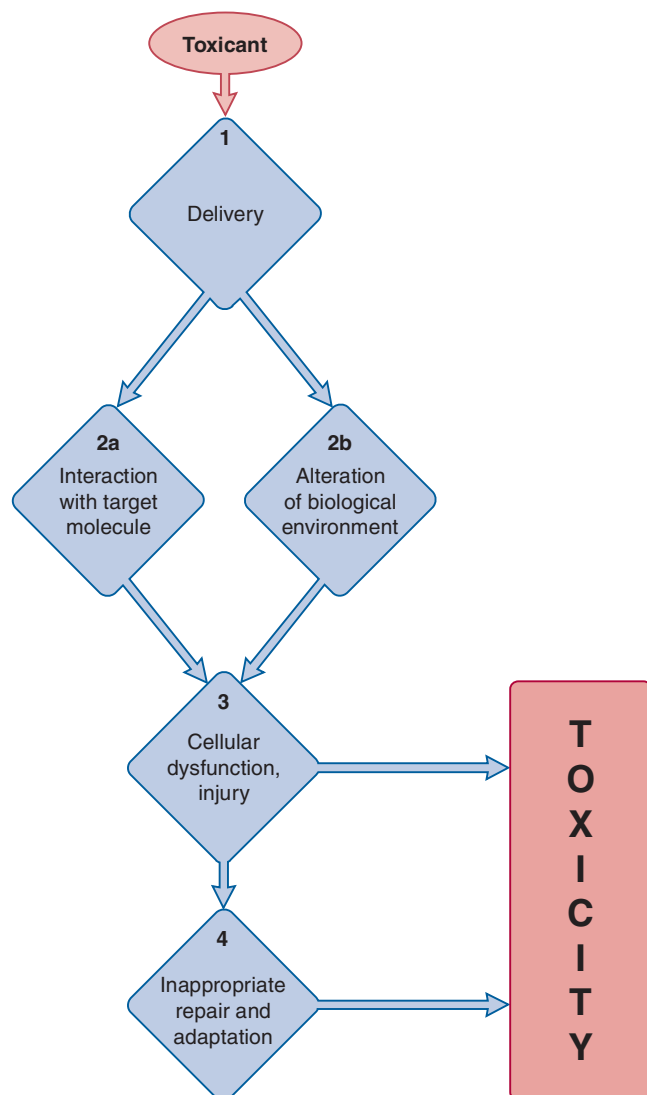
### Conclusions

Depending primarily on the degree and route of exposure, chemicals may adversely affect the function and/or structure of living organisms. The qualitative and quantitative characterization of these harmful or toxic effects is essential for an evaluation of the potential hazard posed by a particular chemical. It is also valuable to understand the mechanisms responsible for the manifestation of toxicity—that is, how a toxicant enters an organism, how it interacts with target molecules, and how the organism deals with the insult.

An understanding of the mechanisms of toxicity is of both practical and theoretical importance. Such information provides a rational basis for interpreting descriptive toxicity data, estimating the probability that a chemical will cause harmful effects, establishing procedures to prevent or antagonize the toxic effects, designing drugs and industrial chemicals that are less hazardous, and developing pesticides that are more selectively toxic for their target organisms. Elucidation of the mechanisms of chemical toxicity has led to a better understanding of fundamental physiologic and biochemical processes ranging from neurotransmission (eg, curare-type arrow poisons) through deoxyribonucleic acid (DNA)

repair (eg, alkylating agents) to transcription, translation, and signal transduction pathways (eg, chemicals acting through transcription factors [TFs], such as the aryl hydrocarbon receptor [AhR]). Pathologic conditions such as cancer and Parkinson disease are better understood because of studies on the mechanism of toxicity of chemical carcinogens and 1,2,3,6-tetrahydro-1-methyl-4-phenylpyridine (MPTP), respectively. Continued research on mechanisms of toxicity will undoubtedly continue to provide such insights.

This chapter reviews the cellular mechanisms that contribute to the manifestation of toxicities. Although such mechanisms are also dealt with elsewhere in this volume, they are discussed in detail in this chapter in an integrated and comprehensive manner. We provide an overview of the mechanisms of chemical toxicity by relating a series of events that begins with exposure, involves a multitude of interactions between the invading toxicant and the organism, and culminates in a toxic effect. This chapter focuses on mechanisms that have been identified definitively or tentatively in humans or animals.



**Figure 3-1.** Potential stages in the development of toxicity after chemical exposure.

As a result of the huge number of potential toxicants and the multitude of biological structures and processes that can be impaired, there are a tremendous number of possible toxic effects. Correspondingly, there are various pathways that may lead to toxicity (Fig. 3-1). A common course is when a toxicant delivered to its target reacts with it, and the resultant cellular dysfunction manifests itself in toxicity. An example of this route to toxicity is that taken by the puffer fish poison, tetrodotoxin. After ingestion, this poison reaches the voltage-gated  $\text{Na}^+$  channels of neurons (step 1). Interaction of tetrodotoxin with this target (step 2a) results in blockade of  $\text{Na}^+$  channels, inhibition of the activity of motor neurons (step 3), and ultimately skeletal muscle paralysis. No repair mechanisms can prevent the onset of such toxicity.

Sometimes a xenobiotic does not react with a specific target molecule but rather adversely influences the biological (micro) environment, causing molecular, organellar, cellular, or organ dysfunction leading to deleterious effects. For example, 2,4-dinitrophenol, after entering the mitochondrial matrix space (step 1), collapses the outwardly directed proton gradient across the inner membrane by its mere presence there (step 2b), causing mitochondrial dysfunction (step 3), which is manifest in toxic

effects such as hyperthermia and seizures. Chemicals that precipitate in renal tubules and block urine formation represent another example for such a course (step 2b).

The most complex path to toxicity involves more steps (Fig. 3-1). First, the toxicant is delivered to its target or targets (step 1), after which the ultimate toxicant interacts with endogenous target molecules (step 2a), triggering perturbations in cell function and/or structure (step 3), which initiate repair mechanisms at the molecular, cellular, and/or tissue levels as well as adaptive mechanisms to diminish delivery, boost repair capacity, and/or compensate for dysfunction (step 4). When the perturbations induced by the toxicant exceed repair and adaptive capacity or when repair and adaptation becomes malfunctional, toxicity occurs. Tissue necrosis, cancer, and fibrosis are examples of chemically induced toxicities whose development follow this 4-step course.

## STEP 1—DELIVERY: FROM THE SITE OF EXPOSURE TO THE TARGET

Theoretically, the intensity of a toxic effect depends primarily on the concentration and persistence of the ultimate toxicant at its site of action. The ultimate toxicant is the chemical species that reacts with the endogenous target molecule (eg, receptor, enzyme, DNA, microfilamental protein, lipid) or critically alters the biological (micro) environment, initiating structural and/or functional alterations that result in toxicity. Often the ultimate toxicant is the original chemical to which the organism is exposed (parent compound). In other cases, the ultimate toxicant is a metabolite of the parent compound or a reactive oxygen or nitrogen species (ROS or RNS) generated during the biotransformation of the toxicant. Occasionally, the ultimate toxicant is an unchanged or altered endogenous molecule (Table 3-1).

The concentration of the ultimate toxicant at the target molecule depends on the relative effectiveness of the processes that increase or decrease its concentration at the target site (Fig. 3-2). The accumulation of the ultimate toxicant at its target is facilitated by its absorption, distribution to the site of action, reabsorption, and toxication (metabolic activation). Conversely, presystemic elimination, distribution away from the site of action, excretion, and detoxication oppose these processes and work against the accumulation of the ultimate toxicant at the target molecule.

## Absorption versus Presystemic Elimination

**Absorption** Absorption is the transfer of a chemical from the site of exposure, usually an external or internal body surface (eg, skin, mucosa of the alimentary and respiratory tracts), into the systemic circulation. Whereas transporters may contribute to the gastrointestinal (GI) absorption of some chemicals (eg, salicylate and valproate by monocarboxylate transporters, some  $\beta$ -lactam antibiotics and ACE inhibitor drugs by peptide transporters (PEPT),  $\text{Fe}^{2+}$ ,  $\text{Cd}^{2+}$ , as well as some other divalent metal ions by the divalent metal ion transporter, and arsenate by phosphate transporters), the vast majority of toxicants traverse epithelial barriers and reach the blood capillaries by diffusing through cells. The rate of absorption is related to the concentration of the chemical at the absorbing surface, which depends on the rate of exposure and the dissolution of the chemical. It is also related to the area of the exposed site, the characteristics of the epithelial layer through which absorption takes place (eg, the thickness of the stratum corneum in the skin), the intensity of the subepithelial microcirculation, and the physicochemical properties of the toxicant. Lipid solubility is usually the most important



Table 3-1

## Types of Ultimate Toxicants and Their Sources

## Parent xenobiotics as ultimate toxicants

Pb ions  
Tetrodotoxin  
TCDD  
Methylisocyanate  
HCN  
CO

## Xenobiotic metabolites as ultimate toxicants

Amygdalin	→	HCN
Arsenate	→	Arsenite
Fluoroacetate	→	Fluorocitrate
Ethylene glycol	→	Oxalic acid
Hexane	→	2,5-Hexanedione
Acetaminophen	→	<i>N</i> -Acetyl- <i>p</i> -benzoquinoneimine
CCl <sub>4</sub>	→	CCl <sub>3</sub> OO•
Benzo[ <i>a</i> ]pyrene (BP)	→	BP-7,8-diol-9,10-epoxide
Benzo[ <i>a</i> ]pyrene (BP)	→	BP-radical cation

## Reactive oxygen or nitrogen species as ultimate toxicants

Hydrogen peroxide	}	→	Hydroxyl radical (HO•)
Diquat, doxorubicin, nitrofurantoin			
Cr(V), Fe(II), Mn(II), Ni(II)			
Paraquat → O <sub>2</sub> <sup>•</sup> + NO•		→	Peroxynitrite (ONOO <sup>-</sup> )

## Endogenous compounds as ultimate toxicants

Sulfonamides → albumin-bound bilirubin	→	Bilirubin
CCl <sub>3</sub> OO• → unsaturated fatty acids	→	Lipid peroxyl radicals
CCl <sub>3</sub> OO• → unsaturated fatty acids	→	Lipid alkoxyl radicals
CCl <sub>3</sub> OO• → unsaturated fatty acids	→	4-Hydroxynon-2-enal
HO• → proteins	→	Protein carbonyls

property influencing absorption. In general, lipid-soluble chemicals are absorbed more readily than are water-soluble substances.

**Presystemic Elimination** During transfer from the site of exposure to the systemic circulation, toxicants may be eliminated. This is not unusual for chemicals absorbed from the GI tract because they must first pass through the GI mucosal cells, liver, and lung before being distributed to the rest of the body by the systemic circulation. The GI mucosa and the liver may eliminate a significant fraction of a toxicant during its passage through these tissues, decreasing its systemic availability. For example, ethanol is oxidized by alcohol dehydrogenase in the gastric mucosa (Lim *et al.*, 1993), cyclosporine is returned from the enterocyte into the intestinal lumen by multidrug resistance protein (MDR1, also known as P-glycoprotein, an ATP-dependent xenobiotic transporter) and is also hydroxylated by cytochrome P450 (CYP3A4) in these cells (Lin *et al.*, 1999), morphine is glucuronidated in intestinal mucosa and liver, and manganese is taken up from the portal blood into liver and excreted into bile. Such processes may prevent a considerable quantity of chemicals from reaching the systemic blood. Thus, presystemic or first-pass elimination reduces the toxic effects of chemicals that reach their target sites by way of the systemic circulation. In contrast, the processes involved in presystemic elimination may contribute to injury of the digestive mucosa, liver, and lungs by chemicals such as ethanol, iron salts,  $\alpha$ -amanitin, and paraquat because these processes promote their delivery to those sites.

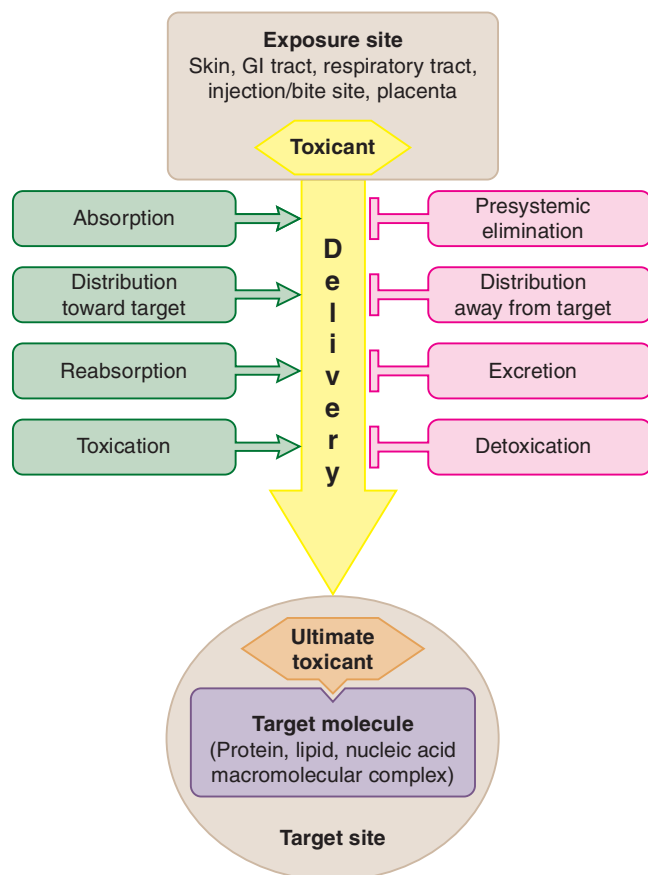
## Distribution To and Away from the Target

Toxicants exit the blood during the distribution phase, enter the extracellular space, and may penetrate into cells. Chemicals dissolved in plasma water may diffuse through the capillary endothelium via aqueous intercellular spaces and transcellular pores called fenestrae and/or across the cell membrane. Lipid-soluble compounds move readily into cells by diffusion. In contrast, highly ionized and hydrophilic xenobiotics (eg, tubocurarine and aminoglycosides) are largely restricted to the extracellular space unless specialized membrane carrier systems are available to transport them.

During distribution, toxicants reach their site or sites of action, usually a macromolecule on either the surface or the interior of a particular type of cell. Chemicals also may be distributed to the site or sites of toxication, usually an intracellular enzyme, where the ultimate toxicant is formed. Some mechanisms facilitate, whereas others delay, the distribution of toxicants to their targets.

**Mechanisms Facilitating Distribution to a Target** Distribution of toxicants to specific target sites may be enhanced by (1) the porosity of the capillary endothelium, (2) specialized membrane transport, (3) accumulation in cell organelles, and (4) reversible intracellular binding.

**Porosity of the Capillary Endothelium** Endothelial cells in the hepatic sinusoids and in the renal peritubular capillaries have larger



**Figure 3-2.** The process of toxicant delivery is the first step in the development of toxicity.

fenestrae (50–150 nm in diameter) that permit passage of even protein-bound xenobiotics. This favors the accumulation of chemicals in the liver and kidneys.

### Specialized Transport Across the Plasma Membrane

Specialized ion channels and membrane transporters can contribute to the delivery of toxicants to intracellular targets. For example, aquaglyceroporin channels may mediate influx of arsenite, which is present at physiologic pH as uncharged  $\text{As}(\text{OH})_3$ , voltage-gated  $\text{Ca}^{2+}$  channels permit the entry of cations such as lead or barium ions into excitable cells, and  $\text{Na}^+, \text{K}^+$ -ATPase promotes intracellular accumulation of thallous ion. By mimicking  $\text{Na}^+$ ,  $\text{Li}^+$  may enter excitable cells through voltage-gated  $\text{Na}^+$  channels and, from the tubular fluid, into the principal cells of the renal collecting duct via the epithelial  $\text{Na}^+$  channels. Paraquat enters into pneumocytes via hitherto unspecified transporters and an MPTP metabolite ( $\text{MPP}^+$ ) is taken up into extrapyramidal dopaminergic neurons by the dopamine transporter. Organic cation transporters (OCT) mediate the uptake of small-molecular-weight organic cations (eg, metformin, cimetidine, and cisplatin) from blood into liver cells (by OCT1) and into renal proximal tubular cells (by OCT2). The hepatocellular uptake of  $\alpha$ -amanitin is mediated by the Na-dependent bile acid transporter (NTCP). Specific organic anion-transporting polypeptide (OATP) transporters mediate the hepatic uptake and hepatotoxicity of microcystin, a cyclic heptapeptide toxin produced by blue-green algae. In mice, Oatp1b2 (the rodent ortholog of OATP1B1/1B3) is essential, as Oatp1b2-null mice are resistant to microcystin-induced liver injury (Lu *et al.*, 2008). Organic anion transporters such as human OAT1 and OAT3 mediate renal tubular uptake of ochratoxin and mercuric ion (the latter as the

dicysteine conjugate Cys-Hg-Cys), whereas both OAT1 and amino acid transporters can carry methylmercury as its cysteine conjugate  $\text{CH}_3\text{-Hg-Cys}$ , and an MPTP metabolite ( $\text{MPP}^+$ ) enters into extrapyramidal dopaminergic neurons by means of the dopamine transporter. Endocytosis of some toxicant–protein complexes, such as Cd–metallothionein (MT) or hydrocarbons bound to the male rat–specific  $\alpha_{2u}$ -globulin, by renal proximal tubular cells can also occur. Particles, such as asbestos and manufactured nanomaterial, may also enter cells by endocytosis, depending on their size and shape. Lipoprotein receptor–mediated endocytosis contributes to entry of lipoprotein-bound toxicants into cells equipped with such transporters. Membrane recycling can internalize cationic aminoglycosides associated with anionic phospholipids in the brush border membrane of renal tubular cells (Laurent *et al.*, 1990). This process may also contribute to cellular uptake of heavy metal ions. Such uptake mechanisms facilitate the entry of toxicants into specific cells, rendering those cells targets. Thus, carrier-mediated uptake of paraquat by pneumocytes and internalization of aminoglycosides by renal proximal tubular cells expose those cells to toxic concentrations of those chemicals.

### Accumulation in Cell Organelles

Amphipathic xenobiotics with a protonable amine group and lipophilic character accumulate in lysosomes as well as mitochondria and cause adverse effects there. Lysosomal accumulation occurs by pH trapping, that is, diffusion of the amine (eg, amiodarone, amitriptyline, fluoxetine) in unprotonated form into the acidic interior of the organelle, where the amine is protonated, preventing its efflux. The entrapped amine inhibits lysosomal phospholipases, impairing degradation of lysosomal phospholipids and causing phospholipidosis. Mitochondrial accumulation takes place electrophoretically. The amine is protonated in the intermembrane space (to where the mitochondria eject protons). The cation thus formed will then be sucked into the matrix space by the strong negative potential there ( $-220$  mV), where it may impair  $\beta$ -oxidation and oxidative phosphorylation. By such mechanisms, the valued antiarrhythmic drug amiodarone is entrapped in the hepatic lysosomes and mitochondria, causing phospholipidosis (Kodavanti and Mehendale, 1990) and microvesicular steatosis with other liver lesions (Fromenty and Pessayre, 1997), respectively. The cationic metabolite of MPTP ( $\text{MPP}^+$ ) also electrophoretically accumulates in the mitochondria of dopaminergic neurons, causing mitochondrial dysfunction and cell death, whereas highly lipophilic local anesthetics (eg, tetracaine, bupivacaine), when overdosed or inadvertently injected into a blood vessel, accumulate in cardiac mitochondria, compromising mitochondrial energy production and causing cardiac failure. Human equilibrative nucleoside transporter 1 (ENT1) in the mitochondrial inner membrane appears responsible for targeting fialuridine (an already withdrawn thymidine nucleoside analogue antiviral drug) into human mitochondria, where it inhibits mitochondrial DNA synthesis, thereby inducing hepatotoxicity. The fact that ENT1 is not localized in rodent mitochondria may account for the dramatic difference in mitochondrial toxicity of fialuridine between humans and rodents (Lee *et al.*, 2006).

### Reversible Intracellular Binding

Binding to the pigment melanin, an intracellular polyanionic aromatic polymer, is a mechanism by which chemicals, such as organic and inorganic cations and polycyclic aromatic hydrocarbons, can accumulate in melanin-containing cells in retina, substantia nigra, and skin (Larsson, 1993). The release of melanin-bound toxicants is thought to contribute to the retinal toxicity associated with chlorpromazine and chloroquine, injury to substantia nigra neurons by MPTP and manganese, and the induction of melanoma by polycyclic aromatics.

**Keratins** are the major structural proteins in the epidermis and its appendages (nail and hair), constituting up to 85% of fully differentiated keratinocytes (skin cells). As keratins are abundant in cysteine residues, they can sequester thiol-reactive metal ions and metalloids compounds, whose nail and hair contents are indicative of exposure. **Release of keratin-bound arsenic in keratinocytes may adversely affect these cells, leading to dermal lesions common in arsenicism.**

**Mechanisms Opposing Distribution to a Target** Distribution of toxicants to specific sites may be hindered by several processes. The processes include (1) binding to plasma proteins, (2) specialized barriers, (3) distribution to storage sites such as adipose tissue, (4) association with intracellular binding proteins, and (5) export from cells.

**Binding to Plasma Proteins** As long as xenobiotics such as **DDT** (an insecticide) and TCDD (often called dioxin, an environmental pollutant) are bound to high-molecular-weight proteins or lipoproteins in plasma, they cannot leave the capillaries by diffusion. **Even if they exit the bloodstream through fenestrae, they have difficulty permeating cell membranes.** Dissociation from proteins is required for most xenobiotics to leave the blood and enter cells. Therefore, strong binding to plasma proteins delays and prolongs the effects and elimination of toxicants.

**Specialized Barriers** Brain capillaries have very low aqueous porosity because their **endothelial cells lack fenestrae and are joined by extremely tight junctions.** This blood–brain barrier prevents the access of hydrophilic chemicals to the brain except for those that can be actively transported. In the choroid plexus, where the capillaries are fenestrated, the choroidal epithelial cells are sealed together by tight junctions, forming the blood–cerebrospinal fluid barrier. Water-soluble toxicants also have restricted access to reproductive cells, which are separated from capillaries by other cells. The oocyte in the ovary is surrounded by multiple layers of granulosa cells, and the spermatogenic cells are supported by Sertoli cells that are tightly joined in the seminiferous tubules to form the blood–testis barrier (see Chap. 20). Transfer of hydrophilic toxicants across the placenta is also restricted. However, none of these barriers are effective against lipophilic substances.

**Distribution to Storage Sites** Some chemicals accumulate in tissues (ie, storage sites) where they do not exert significant effects. For example, highly lipophilic substances such as chlorinated hydrocarbon insecticides concentrate in adipocytes, whereas lead is deposited in bone by substituting for  $\text{Ca}^{2+}$  in hydroxyapatite. Such storage decreases the availability of these toxicants for their target sites and acts as a temporary protective mechanism. However, insecticides may return to the circulation and be distributed to their target site, the nervous tissue, when there is a rapid lipid loss as a result of fasting. This contributes to the lethality of pesticide-exposed birds during migration or during the winter months, when food is restricted. **The possibility that lead is mobilized from the bone during pregnancy is of concern.**

**Association with Intracellular Binding Proteins** Binding to nontarget intracellular sites also reduces the concentration of toxicants at the target site, at least temporarily. MT, a cysteine-rich cytoplasmic protein, serves such a function in acute cadmium intoxication (Klaassen *et al.*, 1999).

**Export from Cells** Intracellular toxicants may be transported back into the extracellular space. This occurs in brain capillary endothelial cells. In their luminal membrane, these cells contain

ATP-dependent membrane transporters (ATP-binding cassette [ABC] transporters) such as the MDR1, or P-glycoprotein, which extrudes chemicals and contributes to the blood–brain barrier (Schinkel, 1999). Compared with normal mice, mice with disrupted *mdrla* gene exhibit 100-fold higher brain levels of and sensitivity to ivermectin, a neurotoxic pesticide and human antihelmintic drug that is one of many P-glycoprotein substrates (Schinkel, 1999). The oocyte is also equipped with P-glycoprotein that provides protection against chemicals that are substrates for this efflux pump (Elbling *et al.*, 1993). Hematopoietic stem cells (and perhaps other stem cells) are also protected by ABC transporters, such as MDR1, MRP1, and **breast cancer resistance protein (BCRP), of which the latter confers these cells resistance to mitoxantrone.** ABC transporters that export drugs were first identified in tumor cells that often overexpress them, thereby making these cells resistant to antitumor drugs these transporters pump out (see Chap. 5).

## Excretion versus Reabsorption

**Excretion** Excretion is the removal of xenobiotics from the blood and their return to the external environment. **It is a physical mechanism, whereas biotransformation is a chemical mechanism for eliminating the toxicant.**

For nonvolatile chemicals, the major excretory structures in the body are **the renal glomeruli, which hydrostatically filter small molecules (<60 kDa) through their pores, and the proximal renal tubular cells and hepatocytes, which actively transport chemicals from the blood into the renal tubules and bile canaliculi, respectively.** These cells are readily exposed to blood-borne chemicals through the large endothelial fenestrae; **they have transporters of the solute carrier (SLC) family (eg, OAT, OCT, and OATP type) that mediate the basolateral uptake of particular chemicals and transporters of the ABC carrier family (eg, MRP and MDR type) that mediate the luminal export of certain chemicals (see Chap. 5).** Renal transporters have a preferential affinity for smaller (<300 Da), and hepatic transporters for larger (>400 Da) amphiphilic molecules. A less common “excretory” mechanism consists of diffusion and partition into the excreta on the basis of their lipid content (see below) or acidity. **For example, morphine is transferred into milk and amphetamine is transferred into gastric juice by nonionic diffusion. This is facilitated by pH trapping of those organic bases in those fluids, which are acidic relative to plasma (see Chap. 5).**

The route and speed of excretion depend largely on the physicochemical properties of the toxicant. The major excretory organs—kidney and liver—can efficiently remove only highly hydrophilic, usually ionized chemicals such as organic acids and bases. The reasons for this are as follows: (1) in the renal glomeruli, only compounds dissolved in plasma water can be filtered; (2) transporters in hepatocytes and renal proximal tubular cells are specialized for secretion of highly hydrophilic organic acids and bases; (3) only hydrophilic chemicals are freely soluble in the aqueous urine and bile; and (4) lipid-soluble compounds are readily reabsorbed by transcellular diffusion.

**There are no efficient elimination mechanisms for nonvolatile, highly lipophilic chemicals such as polyhalogenated biphenyls and chlorinated hydrocarbon insecticides. If they are resistant to biotransformation, such chemicals are eliminated very slowly and tend to accumulate in the body on repeated exposure. Three rather inefficient processes are available for the elimination of such chemicals: (1) excretion by the mammary gland after the chemical is dissolved in milk lipids; (2) excretion in bile in association with biliary micelles and/or phospholipid vesicles; and (3) intestinal excretion, an incompletely understood transport from blood into the**



intestinal lumen. Volatile, nonreactive toxicants such as gases and volatile liquids diffuse from pulmonary capillaries into the alveoli and are exhaled.

**Reabsorption** Toxicants delivered into the renal tubules may diffuse back across the tubular cells into the peritubular capillaries. This process is facilitated by tubular fluid reabsorption, which increases the intratubular concentration as well as the residence time of the chemical by slowing urine flow. Reabsorption by diffusion is dependent on the lipid solubility of the chemical. For organic acids and bases, diffusion is inversely related to the extent of ionization, because the nonionized molecule is more lipid-soluble. The ionization of weak organic acids, such as salicylic acid and phenobarbital, and bases, such as amphetamine, procainamide, and quinidine, is strongly pH-dependent in the physiologic range. Therefore, their reabsorption is influenced significantly by the pH of the tubular fluid. Acidification of urine favors the excretion of weak organic bases, whereas alkalization favors the elimination of weak organic acids. Some organic compounds may be reabsorbed from the renal tubules by transporters. For example, PEPT can move peptidomimetic drugs, such as some  $\beta$ -lactam antibiotics and angiotensin-converting enzyme inhibitors, across the brush border membrane. Carriers for the physiologic oxyanions mediate the reabsorption of some toxic metal oxyanions in the kidney. Thus, chromate and molybdate are reabsorbed by the sulfate transporter, whereas arsenate is reabsorbed by the phosphate transporter.

Toxicants delivered to the GI tract by biliary, gastric, and intestinal excretion and secretion by salivary glands and the exocrine pancreas may be reabsorbed by diffusion across the intestinal mucosa. Because compounds secreted into bile are usually organic acids, their reabsorption is possible only if they are sufficiently lipophilic or are converted to more lipid-soluble forms in the intestinal lumen. For example, glucuronides of toxicants such as diethylstilbestrol (DES) and glucuronides of the hydroxylated metabolites of polycyclic aromatic hydrocarbons, chlordecone, and halogenated biphenyls are hydrolyzed by the  $\beta$ -glucuronidase of intestinal microorganisms, and the released aglycones are reabsorbed (Gregus and Klaassen, 1986). Glutathione conjugates of hexachlorobutadiene and trichloroethylene are hydrolyzed by intestinal and pancreatic peptidases, yielding the cysteine conjugates, which are reabsorbed and serve as precursors of some nephrotoxic metabolites (Anders, 2004).

## Toxication versus Detoxication

**Toxication** A number of xenobiotics (eg, strong acids and bases, nicotine, aminoglycosides, ethylene oxide, methylisocyanate, heavy metal ions, HCN, CO) are directly toxic, whereas the toxicity of others is due primarily to metabolites. Biotransformation to harmful products is called *toxication or metabolic activation*. With some xenobiotics, toxication confers physicochemical properties that adversely alter the microenvironment of biological processes or structures. For example, oxalic acid formed from ethylene glycol may cause acidosis and hypocalcaemia as well as obstruction of renal tubules by precipitation as calcium oxalate. Occasionally, chemicals acquire structural features and reactivity by biotransformation that allows for a more efficient interaction with specific receptors or enzymes. For example, the organophosphate insecticide parathion is biotransformed to paraoxon, an active cholinesterase inhibitor; the rodenticide fluoroacetate is converted in the citric acid cycle to fluorocitrate, a false substrate that inhibits aconitase; as a result of its CYP2E1-catalyzed oxidation, the general anesthetic methoxyflurane releases fluoride ion that inhibits several enzymes (including enolase in the glycolytic pathway) and that

contributes to renal injury after prolonged anesthesia; some cephalosporin antibiotics (eg, cephoperazone) may cause hemorrhage because they undergo biotransformation with release of 1-methyl-tetrazole-5-thiol that inhibits vitamin K epoxide reductase and thus impairs activation of clotting factors; and fialuridine, an antiviral drug withdrawn from development because it produced lethal hepatotoxicity in patients involved in the clinical trial, is phosphorylated to the triphosphate, which inhibits DNA polymerase- $\gamma$  and thus impairs synthesis of mitochondrial DNA (Lewis *et al.*, 1996). Most often, however, toxication renders xenobiotics and occasionally other molecules in the body, such as oxygen and nitric oxide ( $\text{*NO}$ ), indiscriminately reactive toward endogenous molecules with susceptible functional groups. This increased reactivity may be due to conversion into (1) electrophiles, (2) free radicals, (3) nucleophiles, or (4) redox-active reactants.

**Formation of Electrophiles** Electrophiles are molecules containing an electron-deficient atom with a partial or full positive charge that allows it to react by sharing electron pairs with electron-rich atoms in nucleophiles. The formation of electrophiles is involved in the toxication of numerous chemicals (Table 3-2) (see Chap. 6). Such reactants are often produced by insertion of an oxygen atom, which withdraws electrons from the atom it is attached to, making that electrophilic. This is the case when aldehydes, ketones, epoxides, arene oxides, sulfoxides, nitroso compounds, phosphonates, and acyl halides are formed (Table 3-2). In other instances, conjugated double bonds are formed, which become polarized by the electron-withdrawing effect of an oxygen, making one of the double-bonded carbons electron-deficient (ie, electrophilic). This occurs when  $\alpha,\beta$ -unsaturated aldehydes and ketones as well as quinones, quinoneimines, and quinone methides are produced (Table 3-2). Formation of many of these electrophilic metabolites is catalyzed by cytochrome P450s (Leung *et al.*, 2012).

Cationic electrophiles may be produced as a result of heterolytic bond cleavage. For example, methyl-substituted aromatics, such as 7,12-dimethylbenzanthracene, and aromatic amines (amides), such as 2-acetylaminofluorene, are hydroxylated to form benzylic alcohols and *N*-hydroxy arylamines (amides), respectively (Miller and Surh, 1994). 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 Chap. 6). The antiestrogen tamoxifen undergoes similar activation by hydroxylation and sulfation to form a carbocationic metabolite (Kim *et al.*, 2004). After being C-hydroxylated by CYP2E1, the hepatocarcinogen dimethylnitrosamine undergoes spontaneous decomposition, finally a heterolytic cleavage of the C–N bond to form methyl carbonium cation ( $\text{*CH}_3$ ) (see Chap. 6). In a similar process, the tobacco-specific nicotine-derived nitrosamine ketone (NNK), the most potent carcinogen in tobacco, may generate either methyl carbonium cation or piridylxobutyl carbonium cation, depending on the site of C-hydroxylation that initiates its decomposition. Formally, spontaneous heterolytic cleavage of the C–Cl bond results in formation of reactive episulfonium ion from the vesicant chemical warfare agent sulfur mustard (2,2'-bis-chloroethylsulfide). An episulfonium ion metabolite is also the ultimate toxicant produced from the fumigant 1,2-dibromoethane following its conjugation with glutathione to form *S*-(2-bromoethyl) glutathione conjugate, a so-called half-sulfur mustard (Anders, 2004). The oxidation of mercury ( $\text{Hg}^0$ ) to  $\text{Hg}^{2+}$  by catalase and reduction of  $\text{CrO}_4^{2-}$  into DNA-reactive  $\text{Cr}^{3+}$  by ascorbate (Quievryn *et al.*, 2002) are examples for formation of electrophilic toxicants from inorganic chemicals. Reduction of arsenate (containing  $\text{As}^V$ )

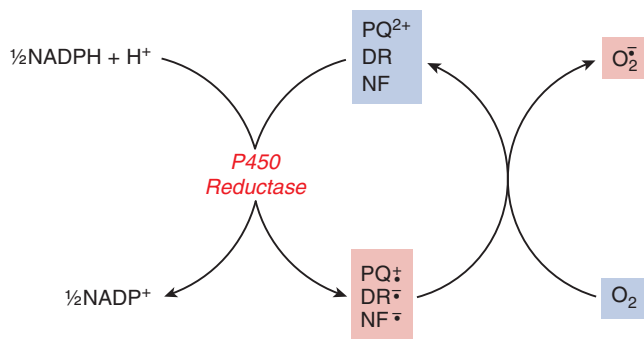


Table 3-2

## Toxication by Formation of Electrophilic Metabolites

ELECTROPHILIC METABOLITE	PARENT TOXICANT	ENZYMES CATALYZING TOXICATION	TOXIC EFFECT
<b>Nonionic electrophiles</b>			
Aldehydes, ketones			
Acetaldehyde	Ethanol	ADH	Hepatic fibrosis (?)
Zomepirac glucuronide	Zomepirac	GT → isomerization	Immune reaction (?)
2,5-Hexanedione	Hexane	CYP	Axonopathy
$\alpha,\beta$ -Unsaturated aldehydes, ketones			
Acrolein	Allyl alcohol	ADH	Hepatic necrosis
Acrolein	Allyl amine	MAO	Vascular injury
Acrolein	Cyclophosphamide	CYP → sp. cl.	Hemorrhagic cystitis
Muconic aldehyde	Benzene	Multiple	Bone marrow injury
Atropaldehyde (2-phenylacrolein)	Felbamate	Esterase → ADH → sp. cl.	Bone marrow and liver injury
4-Hydroxynon-2-enal	Fatty acids	Lipid peroxidation	Cellular injury (?)
Quinones, quinoneimines			
DES-4,4'-quinone	DES	Peroxidases	Carcinogenesis (?)
N-Acetyl-p-benzoquinoneimine	Acetaminophen	CYP, peroxidases	Hepatic necrosis
Epoxides, arene oxides			
Aflatoxin B <sub>1</sub> 8,9-epoxide	Aflatoxin B <sub>1</sub>	CYP	Carcinogenesis
2-Chlorooxirane	Vinyl chloride	CYP	Carcinogenesis
Bromobenzene 3,4-oxide	Bromobenzene	CYP	Hepatic necrosis
Benzo[a]pyrene 7,8-diol 9,10-oxide	Benzo[a]pyrene	CYP	Carcinogenesis
Sulfoxides			
Thioacetamide S-oxide	Thioacetamide	FMO	Hepatic necrosis
Nitroso compounds			
Nitroso-sulfamethoxazole	Sulfamethoxazole	CYP	Immune reaction
Phosphonates			
Paraoxon	Parathion	CYP	ChE inhibition
Acyl halides			
Phosgene	Chloroform	CYP	Hepatic necrosis
Trifluoroacetyl chloride	Halothane	CYP	Immune hepatitis
Thionoacyl halides			
2,3,4,4-Tetrachlorothiobut-3-enoic acid chloride	HCBD	GST → GGT → DP → CC $\beta$ L	Renal tubular necrosis
Thioketenes			
Chloro-1,2,2-trichlorovinyl-thioketene	HCBD	GST → GGT → DP → CC $\beta$ L	Renal tubular necrosis
<b>Cationic electrophiles</b>			
Carbonium ions			
Benzylic carbocation	7,12-DMBA	CYP → ST	Carcinogenesis
Carbonium cation	DENA	CYP → sp. ra.	Carcinogenesis
Nitrenium ions			
Arylnitrenium ion	AAF, DMAB, HAPP	CYP → ST	Carcinogenesis
Sulfonium ions			
Episulfonium ion	1,2-dibromoethane	GST	Carcinogenesis
Metal ions			
Mercury(II) ion	Elemental Hg	Catalase	Brain injury
Diaquo-diamino platinate(II)	Cisplatin	sp. ra.	Renal tubular necrosis

KEY: AAF, 2-acetylaminofluorene; ADH, alcohol dehydrogenase; CC $\beta$ L, cysteine conjugate  $\beta$ -lyase; ChE, cholinesterase; CYP, cytochrome P450; DENA, diethylnitrosamine; DMAB, N,N-dimethyl-4-aminoazobenzene; 7,12-DMBA, 7,12-dimethylbenzanthracene; DES, diethylstilbestrol; DP, dipeptidase; FMO, flavin-containing monooxygenase; GT, UDP-glucuronosyltransferase; GGT, gamma-glutamyltransferase; GST, glutathione S-transferase; HAPP, heterocyclic arylamine pyrolysis products; HCBD, hexachlorobutadiene; ST, sulfotransferase; sp. cl., spontaneous cleavage; sp. ra., spontaneous rearrangement.



**Figure 3-3.** Production of superoxide anion radical ( $O_2^{\bullet-}$ ) by paraquat ( $PQ^{2+}$ ), doxorubicin (DR), and nitrofurantoin (NF). Note that formation of  $O_2^{\bullet-}$  is not the final step in the toxication of these xenobiotics, because  $O_2^{\bullet-}$  can yield the much more reactive hydroxyl radical, as depicted in Fig. 3-4.

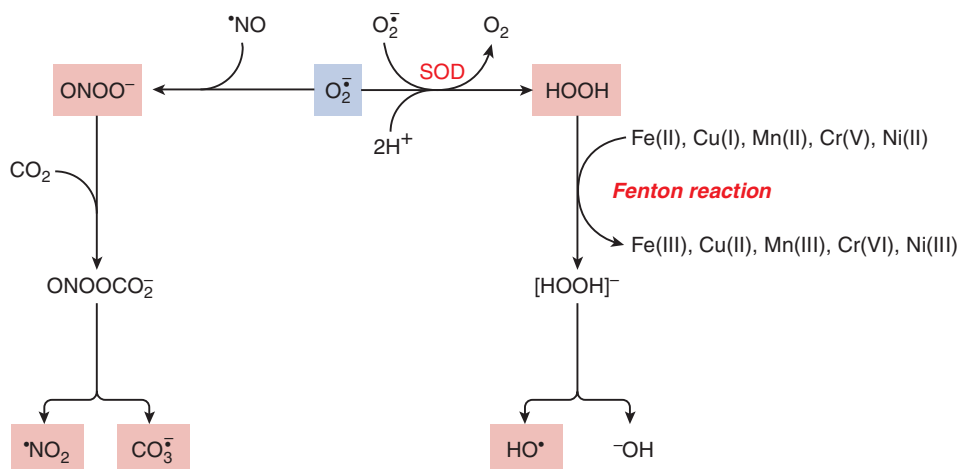
to arsenite (containing As<sup>III</sup>) by glutathione is greatly accelerated via incorporation of arsenate into arsenate esters or anhydrides. These arsenylated metabolites (eg, ribose-1-arsenate, glucose-1-arsenate, ADP-arsenate) can be formed by phosphorolytic enzymes (eg, purine nucleoside phosphorylase and glycogen phosphorylase) and by ATP synthase, which accept arsenate instead of phosphate as their substrate. This is how such enzymes promote reduction of arsenate into the more toxic thiol-reactive arsenite (Gregus *et al.*, 2009; Némethi *et al.*, 2010).

**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 (1) accepting an electron, (2) losing an electron, or (3) homolytic fission of a covalent bond.

1. Xenobiotics such as paraquat, doxorubicin, and nitrofurantoin can accept an electron from reductases to give rise to radicals (Fig. 3-3). These 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 (Kappus, 1986). Through this “redox cycling,”

one electron acceptor xenobiotic molecule can generate many  $O_2^{\bullet-}$  molecules. There are also endogenous sources of  $O_2^{\bullet-}$ . This radical is generated in large quantities by NADPH oxidase (Nox) in activated macrophages and granulocytes during “respiratory burst,” and in smaller quantities by many other cells, such as endothelial cells and vascular smooth muscle cells, which also express Nox in the plasma membrane (Bokoch and Knaus, 2003). Growth factor receptor stimulation in these non-phagocytotic cells is coupled to Nox activation.  $O_2^{\bullet-}$  is also produced by the mitochondrial electron transport chain, especially by complexes I and III. The significance of  $O_2^{\bullet-}$  stems to a large extent from the fact that  $O_2^{\bullet-}$  is a starting compound in two toxication pathways (Fig. 3-4); one leads to formation of hydrogen peroxide (HOOH) and then hydroxyl radical ( $HO^{\bullet}$ ), whereas the other produces peroxynitrite ( $ONOO^-$ ) and ultimately nitrogen dioxide ( $^{\bullet}NO_2$ ), and carbonate anion radical ( $CO_3^{\bullet-}$ ).

2. Nucleophilic xenobiotics such as phenols, hydroquinones, aminophenols, aromatic amines (eg, benzidine), hydrazines, phenothiazines (eg, chlorpromazine; see Fig. 3-6), and thiols are prone to lose an electron and form free radicals in a reaction catalyzed by peroxidases (Aust *et al.*, 1993). Some of these chemicals, such as catechols and hydroquinones, may undergo two sequential one-electron oxidations, producing first semiquinone radicals and then quinones. Quinones are not only reactive electrophiles (Table 3-2) but also electron acceptors with the capacity to initiate redox cycling or oxidation of thiols and NAD(P)H. Polycyclic aromatic hydrocarbons with sufficiently low ionization potential, such as benzo[a]pyrene and 7,12-dimethylbenzanthracene, can be converted via one-electron oxidation by peroxidases or cytochrome P450 to radical cations, which may be the ultimate toxicants for these carcinogens (Cavalieri and Rogan, 1992). Like peroxidases, oxyhemoglobin (Hb-FeII-O<sub>2</sub>) can catalyze the oxidation of aminophenols to semiquinone radicals and quinoneimines. This is another example of toxication, because these products, in turn, oxidize ferrohemeoglobin (Hb-FeII) to methemoglobin (Hb-FeIII), which cannot carry oxygen.



**Figure 3-4.** Two pathways for toxication of superoxide anion radical  $O_2^{\bullet-}$  via nonradical products ( $ONOO^-$  and  $HOOH$ ) to radical products ( $^{\bullet}NO_2$ ,  $CO_3^{\bullet-}$ , and  $HO^{\bullet}$ ). In one pathway, conversion of  $O_2^{\bullet-}$  to  $HOOH$  is spontaneous or is catalyzed by SOD. Homolytic cleavage of  $HOOH$  to hydroxyl radical and hydroxyl ion is called the Fenton reaction and is catalyzed by the transition metal ions shown. Hydroxyl radical formation is the ultimate toxication for xenobiotics that form  $O_2^{\bullet-}$  (see Fig. 3-3) or for  $HOOH$ , the transition metal ions listed, and some chemicals that form complexes with these transition metal ions. 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 is homolytically cleaved to nitrogen dioxide ( $^{\bullet}NO_2$ ) and carbonate anion radical ( $CO_3^{\bullet-}$ ). All 3 radical products indicated in this figure are oxidants, whereas  $^{\bullet}NO_2$  is also a nitrating agent (see Fig. 3-8).

- Free radicals are also formed by homolytic bond fission, which can be induced by electron transfer to the molecule (reductive fission). This mechanism is involved in the conversion of  $\text{CCl}_4$  to the trichloromethyl free radical ( $\text{Cl}_3\text{C}^\bullet$ ) by an electron transfer from cytochrome P450 or the mitochondrial electron transport chain (reductive dehalogenation) (Recknagel *et al.*, 1989). The  $\text{Cl}_3\text{C}^\bullet$  reacts with  $\text{O}_2$  to form the even more reactive trichloromethylperoxy radical ( $\text{Cl}_3\text{COO}^\bullet$ ) (Hippeli and Elstner, 1999).

The hydroxyl radical ( $\text{HO}^\bullet$ ), a free radical of paramount toxicological significance, is also generated by homolytic fission. Such a process yields large amounts of  $\text{HO}^\bullet$  from water on ionizing radiation. Reductive homolytic fission of  $\text{HOOH}$  to the harmful  $\text{HO}^\bullet$  and the harmless  $\text{HO}^-$  (hydroxide anion) is called the Fenton reaction (Fig. 3-4). This is catalyzed by transition metal ions, typically  $\text{Fe(II)}$ ,  $\text{Cu(I)}$ ,  $\text{Cr(V)}$ ,  $\text{Ni(II)}$ , or  $\text{Mn(II)}$ , and is a major toxication mechanism for  $\text{HOOH}$  and its precursor  $\text{O}_2^\bullet$ , as well as for transition metals. Moreover, the toxicity of chemicals, such as nitrotriacetic acid, bleomycin, and orellanin (Hippeli and Elstner, 1999), which chelate transition metal ions is also based on Fenton chemistry, because chelation increases the catalytic efficiency of some transition metal ions. Complexation with some endogenous oligopeptides also increases the catalytic efficiency of weakly Fenton-active metal ions, such as  $\text{Ni(II)}$ . The pulmonary toxicity of inhaled mineral particles such as asbestos and silica is caused, at least in part, by the formation of  $\text{HO}^\bullet$  triggered by Fe ions on the particle surface (Vallyathan *et al.*, 1998; Castranova, 2004).  $\text{HOOH}$  is a direct or indirect by-product of several enzymatic reactions, including monoamine oxidase, acyl-coenzyme A oxidase, xanthine oxidase (Harrison, 2002), aldehyde oxidase, and CYP2E1 (Caro and Cederbaum, 2004). It is produced in large quantities by spontaneous or superoxide dismutase (SOD)-catalyzed dismutation of  $\text{O}_2^\bullet$ .

Homolytic cleavage is also involved in free radical generation from  $\text{ONOO}^-$  (Squadrito and Pryor, 1998) (Fig. 3-4). The facile reaction of  $\text{ONOO}^-$  with the ubiquitous  $\text{CO}_2$  yields nitrosoperoxycarbonate ( $\text{ONOOCO}_2^\bullet$ ), which can spontaneously homolyze into 2 radicals, the oxidant and nitrating agent nitrogen dioxide ( $\text{NO}_2^\bullet$ ) and the oxidant carbonate anion radical ( $\text{CO}_2^\bullet$ ). Thus, formation of  $\text{ONOO}^-$  and the latter radicals represents a toxication mechanism for  $\text{O}_2^\bullet$  and  $\text{NO}^\bullet$ . As  $\text{NO}^\bullet$  is the product of nitric oxide synthase (NOS), this mechanism is especially relevant in and around cells that express NOS constitutively (ie, neurons and endothelial cells) as well as in and around cells that express the inducible form of NOS in response to cytokines. Inside the cell, a predominant site for formation of  $\text{ONOO}^-$  as well as its products  $\text{CO}_2^\bullet$  and  $\text{NO}_2^\bullet$  is the mitochondria, wherein the lipophilic  $\text{NO}^\bullet$  can readily diffuse and where the electron transport chain and the citric acid cycle produce  $\text{O}_2^\bullet$  and  $\text{CO}_2$ , respectively (Denicola and Radi, 2005).

**Formation of Nucleophiles** The formation of nucleophiles is a relatively uncommon mechanism for activating toxicants. Examples include the formation of cyanide from amygdalin, which is catalyzed by bacterial  $\beta$ -glucosidase in the intestine; from acrylonitrile after epoxidation and subsequent glutathione conjugation; and from sodium nitroprusside by thiol-induced decomposition. Carbon monoxide is a toxic metabolite of dihalomethanes that undergo oxidative dehalogenation. Hydrogen selenide, a strong nucleophile and reductant, is formed from selenite by reaction with glutathione or other thiols.

**Formation of Redox-Active Reactants** There are specific mechanisms for the creation of redox-active reactants other than those already mentioned. Examples include the formation of the methemoglobin-producing nitrite from nitrate by bacterial

reduction in the intestine or from esters of nitrous or nitric acids in reaction with glutathione. Dapsone hydroxylamine and 5-hydroxyprimaquine, hydroxylated metabolites of the respective drugs, produce methemoglobin by cooxidation (Fletcher *et al.*, 1988). Reductants such as ascorbic acid and reductases such as NADPH-dependent flavoenzymes reduce  $\text{Cr(VI)}$  to  $\text{Cr(V)}$  (Shi and Dalal, 1990). Xenobiotic radicals formed in redox cycling (eg, those depicted in Fig. 3-3) as well as  $\text{O}_2^\bullet$  can reduce  $\text{Fe(III)}$  bound to ferritin and consequently release it as  $\text{Fe(II)}$ .  $\text{Cr(V)}$  and  $\text{Fe(II)}$  thus formed catalyze  $\text{HO}^\bullet$  formation (Fig. 3-4).

In summary, the most reactive metabolites are electron-deficient molecules and molecular fragments such as electrophiles and neutral or cationic free radicals. Although some nucleophiles are reactive (eg,  $\text{HCN}$ ,  $\text{CO}$ ), many (eg, hydroquinones) are activated by conversion to electrophiles. Similarly, free radicals with an extra electron (eg, those shown in Fig. 3-3) cause damage by giving rise to the neutral  $\text{HO}^\bullet$  radical after the formation and subsequent homolytic cleavage of  $\text{HOOH}$ .

**Detoxication** Biotransformation that eliminates an ultimate toxicant or prevents its formation is called detoxication. In some cases, detoxication may compete with toxication for a chemical. Detoxication can take several pathways, depending on the chemical nature of the toxic substance.

**Detoxication of Toxicants with No Functional Groups** In general, chemicals without functional groups, such as benzene and toluene, are detoxicated in 2 phases. Initially, a functional group such as hydroxyl or carboxyl is introduced into the molecule, most often by cytochrome P450 enzymes. Subsequently, an endogenous acid, such as glucuronic acid, sulfuric acid, or an amino acid, is added to the functional group by a transferase. With some exceptions, the final products are inactive, highly hydrophilic organic acids that are readily excreted. Carbonyl reduction, catalyzed by at least 5 enzymes (eg, 11- $\beta$ -hydroxysteroid dehydrogenase-1, carbonyl reductase, and 3 members of aldo-keto reductase superfamily), initiates detoxication of the potent carcinogen NNK (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone). The formed nicotine-derived nitrosamine alcohol is then readily glucuronidated at its hydroxyl moiety and is excreted into urine (Maser, 2004).

**Detoxication of Nucleophiles** Nucleophiles are generally detoxicated by conjugation at the nucleophilic functional group. Hydroxylated compounds are conjugated by sulfation, glucuronidation, or rarely by 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. An alternative mechanism for the elimination of thiols, amines, and hydrazines is oxidation by flavin-containing monooxygenases (Krueger and Williams, 2005). Some alcohols, such as ethanol, are detoxicated by oxidation to carboxylic acids by alcohol and aldehyde dehydrogenases. A specific detoxication mechanism is the biotransformation of cyanide to thiocyanate by rhodanese or mercaptopyruvate sulfurtransferase.

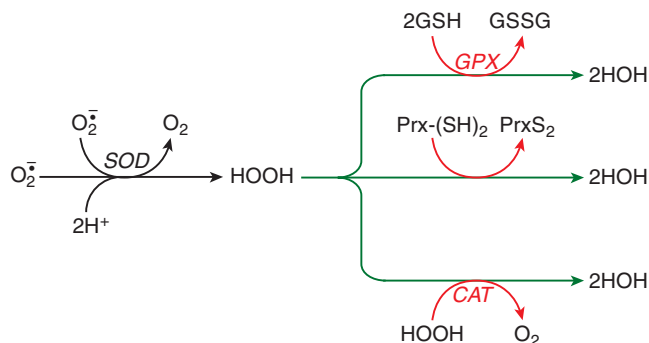
**Detoxication of Electrophiles** A general mechanism for the detoxication of electrophilic toxicants is conjugation with the thiol nucleophile glutathione (Ketterer, 1988). This reaction may occur spontaneously or can be facilitated by glutathione S-transferases. Metal ions—such as  $\text{Ag}^+$ ,  $\text{Cd}^{2+}$ ,  $\text{Hg}^{2+}$ , and  $\text{CH}_3\text{Hg}^+$  ions—readily react with and are detoxicated by glutathione. Specific mechanisms for the detoxication of electrophilic chemicals include epoxide hydrolase-catalyzed biotransformation of epoxides and arene

oxides to diols and dihydrodiols, respectively, and carboxylesterase-catalyzed hydrolysis of organophosphate ester pesticides. Others are two-electron reduction of quinones to hydroquinones by NAD(P)H:quinone oxidoreductase (NQO1) and NRH:quinone oxidoreductase (NQO2), reduction of  $\alpha,\beta$ -unsaturated aldehydes (eg, the lipid peroxidation product 4-oxonon-2-enal) to alcohols or to their saturated derivative by carbonyl reductase (Maser, 2006), or oxidation of  $\alpha,\beta$ -unsaturated aldehydes to acids by aldehyde dehydrogenases. Complex formation of thiol-reactive metal ions by MT and the redox-active ferrous iron by ferritin are special types of detoxications. Covalent binding of electrophiles to proteins can also be regarded as detoxication provided that the 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.

**Detoxication of Free Radicals** Because  $O_2^{\cdot -}$  can be converted into much more reactive compounds (Fig. 3-4), its elimination is an important detoxication mechanism. This is carried out by SOD, high-capacity enzymes located in the cytosol (Cu, Zn-SOD) and the mitochondria (Mn-SOD), which convert  $O_2^{\cdot -}$  to HOOH (Fig. 3-5). Subsequently, HOOH is reduced to water by catalase in the peroxisomes (also in the mitochondria in cardiac muscle), by the selenocysteine-containing glutathione peroxidases in the cytosol and mitochondria, and by peroxiredoxins in the cytosol, mitochondria, and endoplasmic reticulum (EPR) (Fig. 3-5) (Rhee *et al.*, 2005).

No enzyme eliminates  $HO^{\cdot}$ . Whereas some relatively stable radicals, such as peroxyl radicals (see Fig. 3-9), can readily abstract a hydrogen atom from glutathione,  $\alpha$ -tocopherol (vitamin E), or ascorbic acid (vitamin C), thus becoming nonradicals, these antioxidants are generally ineffective in detoxifying  $HO^{\cdot}$  (Sies, 1993). This is due to its extreme reactivity and thus short half-life ( $10^{-9}$  seconds), which provides little time for the  $HO^{\cdot}$  to reach and react with antioxidants. Therefore, the only effective protection against  $HO^{\cdot}$  is to prevent its formation by elimination of its precursor, HOOH, via conversion to water (Fig. 3-5).

$ONOO^-$  (which is not a free radical oxidant) is significantly more stable than  $HO^{\cdot}$  (half-life of about 1 second). Nevertheless, the small biological antioxidant molecules (glutathione, uric acid, ascorbic acid,  $\alpha$ -tocopherol) are relatively inefficient in intercepting it, because  $ONOO^-$  rapidly reacts with  $CO_2$  (Squadrito and Pryor, 1998) to form reactive free radicals (Fig. 3-4). More efficient are the selenocysteine-containing glutathione peroxidase and peroxiredoxins, which can reduce  $ONOO^-$  to nitrite ( $ONO^-$ ) the same way they reduce HOOH to water (Fig. 3-5). Selenoprotein P, which contains 10 selenocysteine residues and coats the surface of endothelial cells, also reduces  $ONOO^-$  and may serve as a protectant against this oxidant in blood. In addition,  $ONOO^-$  reacts with oxyhemoglobin, heme-containing peroxidases, and albumin, all of which

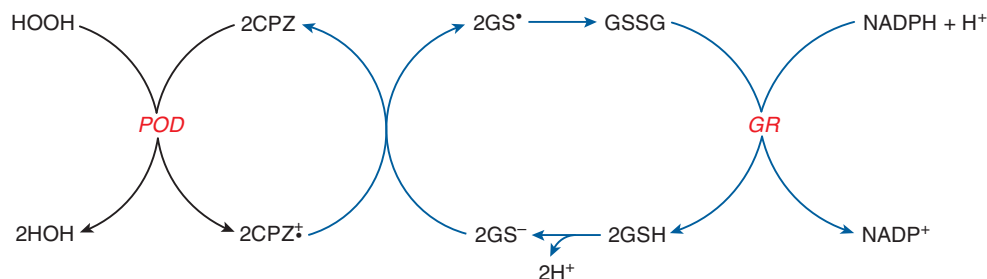


**Figure 3-5.** Detoxication of superoxide anion radical ( $O_2^{\cdot -}$ ) by superoxide dismutase (SOD), and of HOOH by glutathione peroxidase (GPX), peroxiredoxin (Prx-(SH)<sub>2</sub>), and catalase (CAT). When GPX reduces HOOH, it forms glutathione disulfide (GSSG) as a by-product, which is reduced back to GSH by glutathione reductase using NADPH. When Prx-(SH)<sub>2</sub> reduces HOOH, its catalytic thiol group (-R-S-H) becomes first oxidized into a sulfenic acid group (-R-S-OH), which in turn reacts with another SH group of Prx, forming HOH and Prx disulfide (PrxS<sub>2</sub>). The latter can be reduced back into Prx-(SH)<sub>2</sub> by the thioredoxin–thioredoxin reductase–NADPH system, as shown in Fig. 3-22. When HOOH is produced in large excess, the sulfenic acid group (-R-S-OH) in Prx may react with a second molecule of HOOH, causing its overoxidation into a sulfinic acid group (-R-S(O)-OH). This can be reduced back into -R-S-OH group by the ATP-dependent sulfiredoxins, thus reactivating Prx-(SH)<sub>2</sub> (Lowther and Haynes, 2011). Further oxidation into a sulfonic acid form (-R-S(O)(OH)<sub>2</sub>) is irreversible and results in the loss of the peroxidase function of Prx; however, in this form Prx functions as a molecular chaperone (see later). Thus, out of the 3 enzymes eliminating HOOH, the capacity of Prx becomes limited at moderate overload of HOOH, whereas catalase activity is not capacity-limited even at high HOOH overload (Neumann *et al.*, 2009).

could be important sinks for  $ONOO^-$ . Furthermore, elimination of the 2  $ONOO^-$  precursors—that is,  $^{\cdot}NO$  by reaction with oxyhemoglobin (to yield methemoglobin and nitrate) and  $O_2^{\cdot -}$  by SODs (see above)—is a significant mechanism in preventing  $ONOO^-$  buildup (Squadrito and Pryor, 1998).

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. 3-6). Thus, glutathione plays an important role in the detoxication of both electrophiles and free radicals.

**Detoxication of Protein Toxins** Presumably, extracellular and intracellular proteases are involved in the inactivation of toxic polypeptides. Several toxins found in venoms, such as  $\alpha$ - and  $\beta$ -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 (Lozano *et al.*, 1994).



**Figure 3-6.** Detoxication of peroxidase (POD)-generated free radicals such as chlorpromazine free radical (CPZ $^{\cdot +}$ ) by glutathione (GSH). The by-products are glutathione thiyl radical (GS $^{\cdot}$ ) and glutathione disulfide (GSSG), from which GSH is regenerated by glutathione reductase (GR).



**When Detoxication Fails** Detoxication may be insufficient for several reasons:

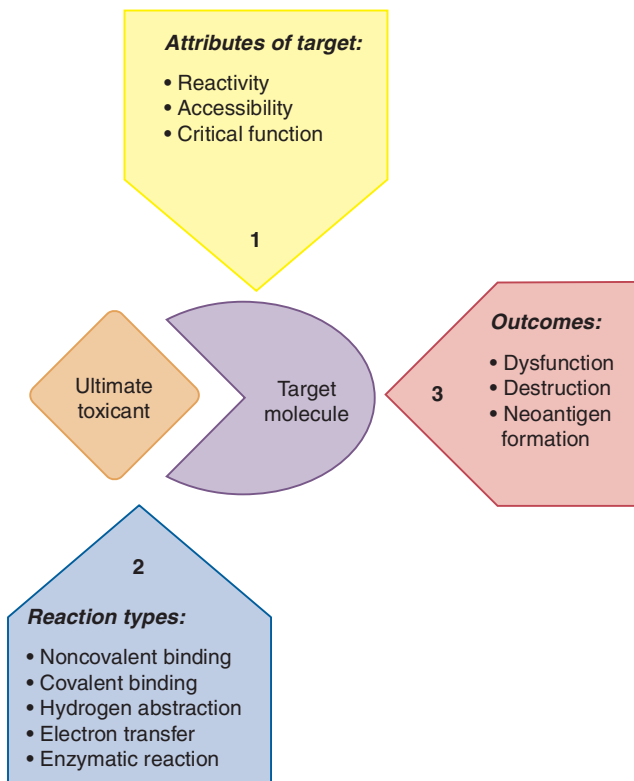
1. Toxicants may overwhelm detoxication processes, leading to saturation of the detoxication enzymes, consumption of the cosubstrates, or depletion of cellular antioxidants such as glutathione, ascorbic acid, and  $\alpha$ -tocopherol. This results in the accumulation of the ultimate toxicant.
2. Occasionally, a reactive toxicant inactivates a detoxicating enzyme. For example, ONOO<sup>-</sup> incapacitates Mn-SOD, which normally would counteract ONOO<sup>-</sup> formation (Murphy, 1999) (see Fig. 3-4).
3. Some conjugation reactions can be reversed. For example, 2-naphthylamine, a bladder carcinogen, is *N*-hydroxylated and glucuronidated in liver, with the glucuronide excreted into urine. While in the bladder, the glucuronide is hydrolyzed, and the released arylhydroxylamine is converted by protonation and dehydration to the reactive electrophilic arylnitrenium ion (Bock and Lilienblum, 1994). Isocyanates and isothiocyanates form labile glutathione conjugates from which they can be released. Thus, methylisocyanate readily forms a glutathione conjugate in the lung after inhalation. From there, the conjugate is distributed to other tissues, where the reactive electrophilic parent compound may be regenerated (Baillie and Kassahun, 1994). Such conjugates are considered transport forms of toxicants.
4. Sometimes detoxication generates potentially harmful by-products, such as the glutathione thiyl radical and glutathione disulfide, which are produced during the detoxication of free radicals (Fig. 3-6). Glutathione disulfide can form mixed disulfides with protein thiols, whereas the thiyl radical (GS<sup>•</sup>), after reacting with thiolate (GS<sup>-</sup>), forms glutathione disulfide radical anion (GSSG<sup>•-</sup>), which can reduce O<sub>2</sub> to O<sub>2</sub><sup>•-</sup>. Conjugation with glutathione may lead to HCN generation in the course of acrylonitrile biotransformation, the first step of which is epoxidation of acrylonitrile (H<sub>2</sub>C=CH-CN). Whereas glutathione conjugation detoxifies this epoxide if it takes place at the carbon adjacent to the nitrile group (-CN), it causes release of this group as HCN if glutathione conjugates with the epoxide at the carbon not linked to the nitrile moiety.

## STEP 2—REACTION OF THE ULTIMATE TOXICANT WITH THE TARGET MOLECULE

Toxicity is typically mediated by a reaction of the ultimate toxicant with a target molecule (step 2a in Fig. 3-1). Subsequently, a series of secondary biochemical events occur, leading to dysfunction or injury that is manifest at various levels of biological organization, such as at the target molecule itself, cell organelles, cells, tissues and organs, and even the whole organism. Because interaction of the ultimate toxicant with the target molecule triggers the toxic effect, consideration is given to (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 (Fig. 3-7). Finally, consideration is given to toxicities that are initiated not by reaction of the ultimate toxicant with target molecules, but rather by alteration of the biological (micro)environment (step 2b in Fig. 3-1) in which critical endogenous molecules, cell organelles, cells, and organs function.

### Attributes of Target Molecules

Practically all endogenous compounds are potential targets for toxicants. The identification and characteristics of the target molecules



**Figure 3-7.** Reaction of the ultimate toxicant with the target molecule; the second step in the development of toxicity.

involved in toxicity constitute a major research priority, but a comprehensive inventory of potential target molecules is impossible. Nevertheless, the most prevalent and toxicologically relevant targets are macromolecules such as nucleic acids (especially DNA) and proteins. Among the small molecules, membrane lipids are frequently involved, whereas cofactors such as coenzyme A and pyridoxal rarely are involved.

To be a target, an endogenous molecule must possess the appropriate reactivity and/or steric configuration to allow the ultimate toxicant to enter into covalent or noncovalent reactions. For these reactions to occur, the target molecule must be accessible to a sufficiently high concentration of the ultimate toxicant. Thus, endogenous molecules that are exposed to reactive chemicals or are adjacent to sites where reactive metabolites are formed are frequently targets. Technical advances in the field of proteomics make it increasingly possible to identify potential protein targets of reactive chemicals as chemical–protein adducts. A compendium of proteins adducted by reactive toxicant metabolites has been established at the University of Kansas (<http://tpdb.medchem.ku.edu/tpdb.html>). The first target for reactive metabolites is often the enzyme that catalyzes their production or the adjacent intracellular structures. For example, thyroperoxidase, the enzyme involved in thyroid hormone synthesis, converts some nucleophilic xenobiotics (such as methimazole, amitrone, and resorcinol) into reactive free radicals that inactivate thyroperoxidase (Engler *et al.*, 1982). This is the basis for the antithyroid as well as the thyroid tumor-inducing effect of these chemicals. Carbon tetrachloride, which is activated by cytochrome P450, destroys this enzyme as well as the neighboring microsomal membranes (Osawa *et al.*, 1995). Several mitochondrial enzymes are convenient targets for nephrotoxic cysteine conjugates such as dichlorovinyl cysteine, because these conjugates are converted to electrophiles by mitochondrial enzymes with cysteine conjugate

$\beta$ -lyase activity (eg, aspartate aminotransferase and branched chain amino acid aminotransferase) that can readily channel their reactive products to neighboring enzymes such as  $\alpha$ -ketoglutarate dehydrogenase (Anders, 2004). Reactive metabolites that are unable to find appropriate endogenous reaction partners in close proximity to their site of formation may diffuse until they encounter such reactants. For example, hard electrophiles such as the aryl nitrenium ion metabolite of *N*-methyl-4-aminoazobenzene react readily with hard nucleophilic atoms in nucleic acids, and thus target DNA in the nucleus, even though the electrophiles are produced in the cytoplasm. Vinyl chloride epoxide, formed in the hepatocytes from vinyl chloride, reaches its DNA targets in the neighboring endothelial cells (which are more sensitive to this genotoxin than the liver cells), initiating hepatic hemangiosarcoma.

Not all targets for chemicals contribute to the harmful effects. Thus, while carbon monoxide causes toxicity by binding to ferrohemoglobin, it also associates with the iron in cytochrome P450 with little or no consequence. Covalent binding of toxicants to various intracellular proteins, including enzymes and structural proteins, has been demonstrated, yet it is often uncertain which protein(s) is/are involved in binding that is toxicologically relevant (Cohen *et al.*, 1997; Pumford and Halmes, 1997; Rombach and Hanzlik, 1999). Arylation of some hepatic mitochondrial proteins by acetaminophen (4'-hydroxyacetanilide) is thought to be causally related to the liver injury induced by this drug because the nonhepatotoxic regioisomer of acetaminophen (3'-hydroxyacetanilide) does not readily bind covalently to these proteins (Cohen *et al.*, 1997; Jaeschke and Bajt, 2006). In contrast, arylation of a number of hepatic cytoplasmic proteins by acetaminophen is likely to be inconsequential because the non-hepatotoxic regioisomer of this drug also arylates those proteins (Nelson and Pearson, 1990). Covalent binding to proteins without adverse consequences may even represent a form of detoxication by sparing toxicologically relevant targets. This principle is best exemplified by covalent binding of organophosphate insecticides to plasma cholinesterase, which is a significant protective mechanism, as it counteracts phosphorylation of acetylcholinesterase, the target molecule. Thus, to conclusively identify a target molecule as being responsible for toxicity, it should be demonstrated that the ultimate toxicant (1) reacts with the target and adversely affects its function, (2) reaches an effective concentration at the target site, and (3) alters the target in a way that is mechanistically related to the observed toxicity.

## Types of Reactions

The ultimate toxicant may bind to the target molecules noncovalently or covalently and may alter it by hydrogen abstraction, electron transfer, or enzymatically.

**Noncovalent Binding** This type of binding can be due to apolar interactions or the formation of hydrogen and ionic bonds and is typically involved in the interaction of toxicants with targets such as membrane receptors, intracellular receptors, ion channels, and some enzymes. For example, such interactions are responsible for the binding of strychnine to the glycine receptor on motor neurons in the spinal cord, TCDD to the AhR, saxitoxin to sodium channels, phorbol esters to protein kinase C (PKC), and warfarin to vitamin K 2,3-epoxide reductase. Such forces also are responsible for the intercalation of chemicals such as acridine yellow and doxorubicin into the double helix of DNA. These chemicals are toxic because the steric arrangement of their atoms allows them to combine with complementary sites on the endogenous molecule more or less as a key fits into a lock. Noncovalent binding usually is reversible because of the comparatively low bonding energy.

**Covalent Binding** Being practically irreversible, covalent binding is of great toxicological importance because it permanently alters endogenous molecules (Boelsterli, 1993). Covalent adduct formation is common with electrophilic toxicants such as nonionic and cationic electrophiles and radical cations. These toxicants react with nucleophilic atoms that are abundant in biological macromolecules, such as proteins and nucleic acids. Electrophilic atoms exhibit some selectivity toward nucleophilic atoms, depending on their charge-to-radius ratio. 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). Examples are presented in Table 3-3. Metal ions such as silver and mercury are also classified as soft electrophiles. These prefer to react covalently with soft nucleophiles (especially thiol groups). Conversely, hard electrophiles such as lithium, calcium, and barium react preferentially as cations with hard nucleophiles (eg, carboxylate and phosphate anions). Metals falling between these 2 extremes, such as chromium, zinc, and lead, exhibit universal reactivity with nucleophiles. The reactivity of an electrophile determines which endogenous nucleophiles can react with it and become a target.

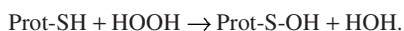
Table 3-3

### Examples of Soft and Hard Electrophiles and Nucleophiles

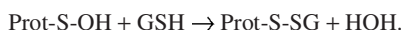
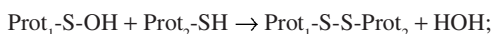
ELECTROPHILES		NUCLEOPHILES
Carbon in polarized double bonds (eg, quinones, $\alpha,\beta$ -unsaturated ketones)	Soft ↑	Sulfur in thiols (eg, cysteinyl residues in proteins and glutathione)
Carbon in epoxides, strained-ring lactones, aryl halides		Sulfur in methionine
Aryl carbonium ions		Nitrogen in primary and secondary amino groups of proteins (eg, lysine $\epsilon$ -amino group)
Benzylic carbonium ions, nitrenium ions		Nitrogen in amino groups in purine bases in nucleic acids
Alkyl carbonium ions	Hard ↓	Oxygen of purines and pyrimidines in nucleic acids
		Phosphate oxygen in nucleic acids

SOURCE: Based on Coles (1984).

A covalent reaction of special biological significance can take place between HOOH (a soft electrophile) and protein thiol groups, preferentially those with low  $pK_a$  value, that is, with a propensity of being in the strongly nucleophilic thiolate anion form (Prot-S<sup>-</sup>) at physiologic pH. This reaction produces protein sulfenic acid (Prot-S-OH):



The S atom in a sulfenic acid is electrophilic as it is made electron-deficient by the electron-withdrawing effect of O. Therefore, a protein-sulfenic acid can react with another thiol group of the same or a different protein or of glutathione (GSH), resulting in, respectively, formation of an intramolecular disulfide, intermolecular disulfide, or a mixed disulfide of the protein and glutathione (ie, glutathionylation of the protein):



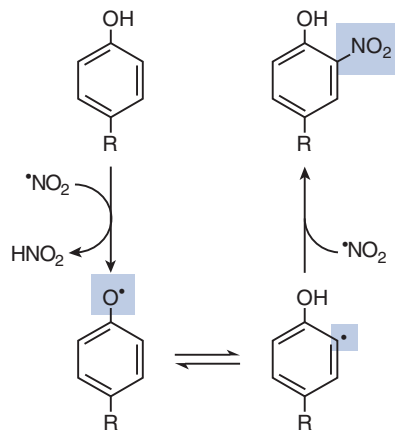
Not only HOOH but also hydroperoxides (eg, lipid hydroperoxides, LOOH; see Fig. 3-9) may enter into such reactions, producing protein-sulfenic acids, protein-disulfides, and glutathionylated proteins, which can be considered as posttranslational modifications with a role in redox signaling (Forman *et al.*, 2004).

Neutral free radicals such as HO•, •NO<sub>2</sub>, and Cl<sub>3</sub>C• can also bind covalently to biomolecules. The addition of Cl<sub>3</sub>C• to double-bonded carbons in lipids or to lipid radicals yields lipids containing chloromethylated fatty acids. The addition of hydroxyl radicals to DNA bases results in the formation of numerous products, including 8-hydroxypurines, 5-hydroxymethylpyrimidines, and thymine and cytosine glycols (Breen and Murphy, 1995).

Nucleophilic toxicants are in principle reactive toward electrophilic endogenous compounds. Such reactions occur infrequently because electrophiles are rare among biomolecules. Examples include the covalent reactions of amines and hydrazides with the aldehyde pyridoxal, a cosubstrate for several enzymes, including glutamate decarboxylase. Carbon monoxide, cyanide, hydrogen sulfide, and azide form coordinate covalent bonds with iron in various heme proteins. Other nucleophiles react with hemoglobin in an electron transfer reaction (see below).

**Hydrogen Abstraction** Neutral free radicals, such as those generated in reactions depicted in Fig. 3-4, can readily abstract H atoms from endogenous compounds, converting those compounds into radicals. Abstraction of hydrogen from thiols (R-SH) creates thiyl radicals (R-S•), which on radical recombination with HO• form sulfenic acids (R-S-OH) that are precursors of disulfides (R-S-S-R) (see above). Radicals can remove hydrogen from CH<sub>2</sub> groups of free amino acids or from amino acid residues in proteins and convert them to carbonyls. These carbonyls react covalently with amines, forming cross-links with DNA or other proteins. Hydrogen abstraction from deoxyribose in DNA yields the C-4' radical, the first step to DNA cleavage (Breen and Murphy, 1995). Abstraction of hydrogen from fatty acids produces lipid radicals and initiates lipid peroxidation. As depicted in Fig. 3-8, nitration of tyrosine residues in proteins purportedly involves H abstraction followed by radical recombination, that is, covalent binding between the resultant tyrosyl radical and •NO<sub>2</sub> (Squadrito and Pryor, 1998).

**Electron Transfer** Chemicals can oxidize Fe(II) in hemoglobin to Fe(III), producing methemoglobinemia. Nitrite can oxidize hemoglobin, whereas N-hydroxyl arylamines (such as dapsone hydroxylamine), phenolic compounds (such as 5-hydroxy



**Figure 3-8.** Formation of 3-nitrotyrosine residues in proteins by reaction with nitrogen dioxide (•NO<sub>2</sub>). •NO<sub>2</sub> is an oxidizing and nitrating species generated from ONOO<sup>-</sup> (Fig. 3-4). In addition, •NO<sub>2</sub> is a contaminant in cigarette smoke, exhaust of gas engines and stoves, as well as the causative agent of “silo-filler’s disease.”

primaquine), and hydrazines (such as phenylhydrazine) are cooxidized with oxyhemoglobin, forming methemoglobin and HOOH (Coleman and Jacobus, 1993).

**Enzymatic Reactions** A few toxins act enzymatically on specific target proteins. For example, the plant toxins ricin and abrin are N-glycosidases; they hydrolyze a specific glycosidic bond in ribosomal RNA, blocking protein synthesis. Botulinum toxin acts as a Zn-protease; it hydrolyzes the fusion proteins that assist in exocytosis of the neurotransmitter acetylcholine in cholinergic neurons, most importantly motor neurons, causing paralysis. The lethal factor component of anthrax toxin is also a Zn-protease, which inactivates mitogen-activated protein kinase kinase (MAPKK), inducing cell death. Other bacterial toxins catalyze the transfer of ADP-ribose from NAD<sup>+</sup> to specific proteins. Through such a mechanism, diphtheria toxin blocks the function of elongation factor 2 in protein synthesis and cholera toxin activates a G protein. Snake venoms contain hydrolytic enzymes that destroy biomolecules.

In summary, most ultimate toxicants act on endogenous molecules on the basis of their chemical reactivity. Those with more than one type of reactivity may react by different mechanisms with various target molecules. For example, quinones may act as electron acceptors and initiate thiol oxidation or free radical reactions that lead to lipid peroxidation, but they may also act as soft electrophiles and bind covalently to protein thiols. The lead ion acts as a soft electrophile when it forms coordinate covalent bonds with critical thiol groups in δ-aminolevulinic acid dehydratase, its major target enzyme in heme synthesis (Goering, 1993). However, it behaves like a hard electrophile or an ion when it binds to PKC or blocks calcium channels, substituting for the natural ligand Ca<sup>2+</sup> at those target sites.

## Effects of Toxicants on Target Molecules

Reaction of the ultimate toxicant with endogenous molecules may cause dysfunction or destruction; in the case of proteins, it may render them foreign (ie, an antigen) to the immune system.

**Dysfunction of Target Molecules** Some toxicants activate protein target molecules, mimicking endogenous ligands. For example, morphine activates opioid receptors, clofibrate is an agonist on the peroxisome proliferator-activated receptor, and phorbol esters and lead ions stimulate PKC.



More commonly, chemicals inhibit the function of target molecules. Several xenobiotics—such as atropine, curare, and strychnine—block neurotransmitter receptors by attaching to the ligand-binding sites, whereas others interfere with the function of ion channels. Tetrodotoxin and saxitoxin, for example, inhibit opening of the voltage-activated sodium channels in the neuronal membrane, whereas DDT and the pyrethroid insecticides inhibit their closure. Some toxicants block ion transporters, others inhibit mitochondrial electron transport complexes, and many inhibit enzymes. Chemicals that bind to tubulin (eg, vinblastine, colchicine, paclitaxel, trivalent arsenic) or actin (eg, cytochalasin B, phalloidin) impair the assembly (polymerization) and/or disassembly (depolymerization) of these cytoskeletal proteins.

Protein function is impaired when conformation or structure is altered by interaction with the toxicant. Many proteins possess critical moieties, especially thiol groups, which are essential for catalytic activity or assembly to macromolecular complexes. Proteins that are sensitive to covalent and/or oxidative modification of their thiol groups include the enzymes glyceraldehyde 3-phosphate dehydrogenase (see Table 3-6) and pyruvate dehydrogenase (see Fig. 3-16), the  $\text{Ca}^{2+}$  pumps (see Fig. 3-17, Table 3-7), the DNA repair enzyme  $O^6$ -methylguanine-DNA-methyltransferase, the DNA-methylating enzyme  $C^5$ -cytosine-DNA-methyltransferase, ubiquitin-activating and -conjugating enzymes (E1 and E2), peroxiredoxins, protein tyrosine phosphatases (PTP, eg, cdc25), the lipid phosphatase PTEN (phosphatase and tensin homologue; see Fig. 3-12) (Rhee *et al.*, 2005), caspases (see Fig. 3-19), the TF AP-1, and the electrophile sensor protein Keap1 (see Fig. 3-27), just to name a few. These and many other proteins are inactivated by thiol-reactive chemicals, causing impaired maintenance of the cell's energy and metabolic homeostasis.

Binding of thiol-reactive chemicals to specific proteins may also initiate a signal. For example, thiol-reactive and oxidant chemicals, such as acrolein, methyl isocyanate, the lacrimator chloroacetophenone, and corrosive gases (eg, phosgene, chloropicrin, and chlorine), react with cysteine thiol groups of the TRPA1 receptor (a cation channel) in the membrane of sensory neurons. Excitation of these neurons located in the cornea and the mucous membranes of the eye and the respiratory tract elicits irritation, pain, lacrimation, bronchial secretion, sneezing, coughing, and bronchospasm (Bessac and Jordt, 2010). If moderate, these responses are alerting and protective, but are incapacitating and detrimental when exaggerated at high exposure. Keap1 may be regarded as an intracellular sensor of similar chemicals, as covalent and/or oxidative modification of thiol groups in Keap1 triggers the adaptive electrophile stress response, which is cytoprotective (see Fig. 3-27; Klaassen and Reisman, 2010). Conversion of the catalytic thiol group (-SH) in PTP and the lipid phosphatase PTEN to sulfenic acid group (-S-OH) on reaction with HOOH or lipid hydroperoxides (see reaction scheme above) inactivates these enzymes. PTPs keep protein kinase-mediated signal transduction pathways under control and PTEN controls the phosphatidylinositol-3-kinase (PI3K)–Akt pathway, both activated by growth factors (see Fig. 3-12). Therefore, inactivation of PTPs and PTEN permits these signalings to surge, thereby initiating, for example, a proliferative response. However, this concept may be an oversimplification because similar inactivation of cdc25 (another PTP) may compromise mitosis, as cdc25 normally dephosphorylates and activates cyclins 1 and 2, which are positive regulators of cell division (see Fig. 3-25). Formation of protein sulfenic acids by the oxidative stress product HOOH is thus considered as an initiating event of redox signaling (Forman *et al.*, 2004). Redox signaling is controlled by the production of HOOH (eg, by Nox activated on growth factor receptor stimulation) and its

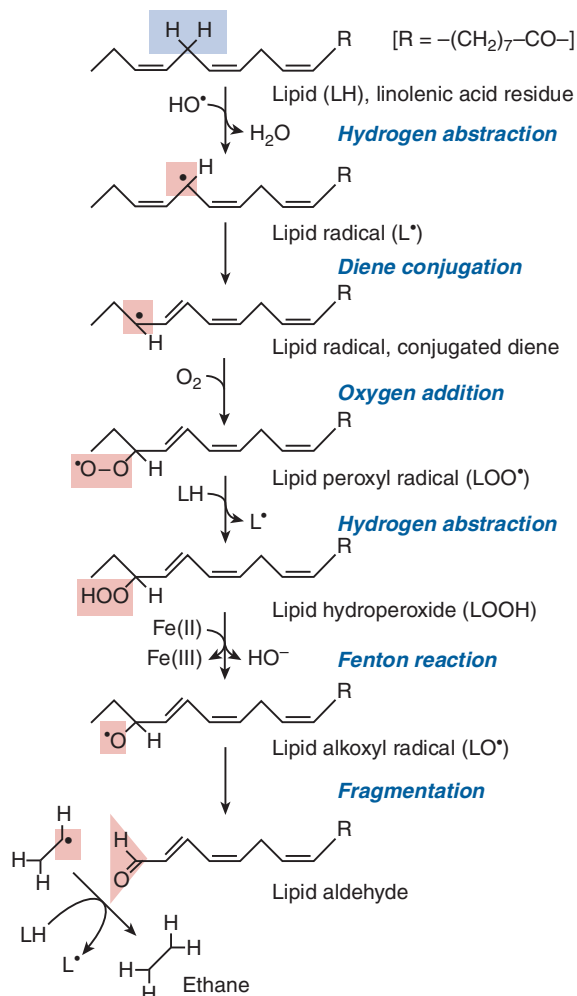
enzymatic elimination. At low level, HOOH is removed effectively by peroxiredoxins (Fig. 3-5); however, these enzymes are incapacitated by overoxidation of their catalytic thiol groups at high HOOH levels (see Fig. 3-5). This modification can be reversed by sulfiredoxins (Lowther and Haynes, 2011). Furthermore, protein sulfenic acid formation is readily reversed by reaction of the sulfenic acid with a thiol (eg, glutathione), resulting in a protein disulfide (eg, protein-S-SG; see the scheme above), which in turn is reduced by thioredoxin or glutaredoxin as shown in Fig. 3-22, thereby regenerating PTPs and PTEN in their active thiol form. Therefore, peroxiredoxins, sulfiredoxins, thioredoxin, thioredoxin reductase, glutaredoxin, glutathione, and NADPH are all important negative regulators of oxidative signaling (Neumann *et al.*, 2009). Protein tyrosine nitration (see Fig. 3-8) may also alter protein function or may interfere with signaling pathways that involve tyrosine kinases and phosphatases.

Toxicants may interfere with the template function of DNA. The covalent binding of chemicals to DNA causes nucleotide mispairing during replication. For example, covalent binding of aflatoxin 8,9-oxide to *N*-7 of guanine results in pairing of the adduct-bearing guanine with adenine rather than cytosine, leading to the formation of an incorrect codon and the insertion of an incorrect amino acid into the protein. Such events are involved in the aflatoxin-induced mutation of the Ras proto-oncogene and the p53 tumor suppressor gene (Eaton and Gallagher, 1994). 8-Hydroxyguanine (8-OH-Gua) and 8-hydroxyadenine are mutagenic bases produced by  $\text{HO}^\bullet$  that can cause mispairing with themselves as well as with neighboring pyrimidines, producing multiple amino acid substitutions (Breen and Murphy, 1995). Cr(III) produced from chromate (Cr(VI)) via reduction by ascorbate, alone or complexed to ascorbate, forms a coordinate covalent bond with phosphate in DNA, thereby inducing a mutation (Quievryn *et al.*, 2002). Chemicals such as doxorubicin, which intercalate between stacked bases in the double-helical DNA, push adjacent base pairs apart, causing an even greater error in the template function of DNA by shifting the reading frame.

**Destruction of Target Molecules** In addition to adduct formation, toxicants alter the primary structure of endogenous molecules by means of cross-linking and fragmentation. Bifunctional electrophiles, such as 2,5-hexanedione, carbon disulfide, acrolein, 4-oxonon-2-enal, 4-hydroxynon-2-enal, and nitrogen mustard alkylating agents, cross-link cytoskeletal proteins, DNA, or DNA with proteins. HOOH and hydroxyl radicals can also induce cross-linking by converting proteins into either reactive electrophiles (eg, protein sulfenic acids and protein carbonyls, respectively), which react with a nucleophilic group (eg, thiol, amine) in another macromolecule. Radicals (eg, those shown in Fig. 3-4) may induce cross-linking of macromolecules by converting them into radicals, which react with each other by radical recombination. Cross-linking imposes both structural and functional constraints on the linked molecules.

Some target molecules are susceptible to spontaneous degradation after chemical attack. Free radicals such as  $\text{Cl}_3\text{COO}^\bullet$  and  $\text{HO}^\bullet$  can initiate peroxidative degradation of lipids by hydrogen abstraction from fatty acids (Recknagel *et al.*, 1989). The lipid radical ( $\text{L}^\bullet$ ) formed is converted successively to lipid peroxy radical ( $\text{LOO}^\bullet$ ) by oxygen fixation, lipid hydroperoxide ( $\text{LOOH}$ ) by hydrogen abstraction, and lipid alkoxyl radical ( $\text{LO}^\bullet$ ) by the  $\text{Fe}^{2+}$ -catalyzed Fenton reaction. Subsequent fragmentation gives rise to hydrocarbons such as ethane and reactive aldehydes such as 4-hydroxynon-2-enal and malondialdehyde (Fig. 3-9). Thus, lipid peroxidation not only destroys lipids in cellular membranes but also generates endogenous toxicants, both free radicals (eg,  $\text{LOO}^\bullet$ ,  $\text{LO}^\bullet$ ) and electrophiles (eg, 4-oxonon-2-enal, 4-hydroxynon-2-enal). These substances can





**Figure 3-9.** Lipid peroxidation initiated by the hydroxyl radical ( $\text{HO}^\bullet$ ). Many of the products, such as the radicals and the  $\alpha,\beta$ -unsaturated aldehydes, are reactive, whereas others, such as ethane, are nonreactive but are indicators of lipid peroxidation.

readily react with adjacent molecules, such as membrane proteins, or diffuse to more distant molecules such as DNA.  $\text{F}_2$ -isoprostanes are stable peroxidation products of arachidonic acid; these are not only sensitive markers of lipid peroxidation but also potent pulmonary and renal vasoconstrictors (Basu, 2004).

Apart from hydrolytic degradation by toxins and radiolysis, toxicant-induced fragmentation of proteins is not well documented. There are, however, examples for destruction of the prosthetic group in enzymes. For instance, cytochrome P450 converts allyl isopropyl acetamide into a reactive metabolite, which alkylates the heme moiety of the enzyme. This leads to loss of the altered heme and to porphyria (De Matteis, 1987). Arsenite ( $\text{AsH}_3$ ) acutely induces heme release from oxyhemoglobin, which may underlie its hemolytic effect. Aconitase is attacked by  $\text{ONOO}^-$  at its  $[\text{4Fe-4S}]^{2+}$  cluster, in which one of the Fe atoms is genuinely labile (as is complexed to inorganic sulfur and not to enzyme-bound cysteines like the others). As a result of the oxidant action of  $\text{ONOO}^-$ , the labile Fe is lost, inactivating the enzyme (Castro *et al.*, 1994) and compromising the citric acid cycle where aconitase functions.

Several forms of DNA fragmentation are caused by toxicants. For instance, attack of DNA bases by  $\text{HO}^\bullet$  can result in the formation of imidazole ring-opened purines or imidazole ring-contracted pyrimidines, which block DNA replication. Formation of a bulky

adduct at guanine *N*-7 destabilizes the *N*-glycosidic bond, inducing depurination. Depurination results in apurinic sites that are mutagenic. Single-strand breaks (SSB) typically are caused by hydroxyl radicals via abstraction of H from desoxyribose in DNA yielding the C-4' radical, followed by  $\text{O}_2^\bullet$  addition, Criegee rearrangement, and cleavage of the phosphodiester bond (Breen and Murphy, 1995). Multiple hydroxyl radical attacks on a short length of DNA, which occur after ionizing radiation, cause double-strand breaks (DSB) that are typically lethal to the affected cell.

**Neoantigen Formation** Whereas the covalent binding of xenobiotics or their metabolites is often inconsequential with respect to the function of the immune system, in some individuals these altered proteins, which carry the xenobiotic adduct as a hapten, evoke an immune response. Some chemicals (eg, dinitrochlorobenzene, penicillin, and nickel ion) are sufficiently reactive to bind to proteins spontaneously. Others may obtain reactivity by auto-oxidation to quinones (eg, urushiols, the allergens in poison ivy) or by enzymatic biotransformation (Park *et al.*, 2005). For example, CYP2E1 biotransforms halothane to an electrophile, trifluoroacetyl chloride, which binds as a hapten to microsomal and cell surface proteins in the liver, inducing a hepatitis-like immune reaction in sensitive patients.

Haptenized proteins released from cells may evoke antibody-mediated (humoral) and/or T-cell-mediated (cellular) immune response. In the humoral response, B cells play the leading role: they bind the complete antigen through their B-cell receptors and associate with T-helper cells ( $\text{CD4}^+$ ). Antigen binding (as signal #1) and cell surface costimulatory molecules on T-helper cells (as signal #2) induce differentiation of B cells into plasma B cells that manufacture and secrete antibody. By binding to the antigen, the antibody assists in destruction of the antigen by phagocytosis; however, harmful consequences may also result. For example, when penicillin-bound proteins as antigens react with IgE-type antibodies on the surface of mast cells, the reaction triggers release of mast cell mediators (eg, histamine, leukotrienes), which in turn may cause bronchoconstriction (asthma), vasodilatation, and plasma exudation (wheal, anaphylactic shock).

In cellular immune response, T cells are the main mediators. In the sensitization phase, T-helper cells become activated by two signals coming from antigen-presenting cells, such as macrophages in internal tissues and dendritic cells in tissues that are in contact with external environment (ie, skin and mucosal lining of the respiratory and GI tract). The APC phagocytoses the haptenized protein and migrates to lymph nodes where most T cells are located. Meanwhile, the APC processes the antigen into appropriate peptides, externalizes, and transfers the processed antigen to the major histocompatibility complex (MHC) on its surface. On reaching the lymph node, the APC presents the MHC-bound peptide to the T-cell receptor (TCR) of T-helper cells (signal #1). In addition, cell surface coactivator molecules (eg,  $\text{CD80}$  and  $\text{CD86}$ ) on APC bind to their counterparts (eg,  $\text{CD28}$ ) on T cells (signal #2). Without signal #2 (which verifies that the antigen detected is foreign) the T cell becomes functionally inert (anergic). On receiving both signals, the helper T cell becomes specific to one particular antigen, expresses the T-cell growth factor IL-2 and its receptor, proliferates, differentiates, and is released into the circulation. On reexposure to the allergen, the elicitation phase commences. Antigen-specific T cells enter the exposure site (eg, the skin) and using IL-2 and other cytokines, they can activate cytotoxic T cells ( $\text{CD8}^+$ ) and induce expression of adhesion molecules at the site of allergen contact. Whereas this response is protective against virus-infected cells and tumor cells, it causes inflammation and cell injury in cells containing xenobiotic

neoantigens. This model describes the response to contact allergens, such as nickel and urushiols, and is the basis of detecting contact allergens in mice with the local lymph node assay.

Besides the above-described classical mechanism of neoantigen-initiated APC-mediated T-cell activation, xenobiotics may cause T-cell activation by more direct ways. For example, Ni ions may not only form coordinate covalent bonds with proteins, making them neoantigens, but also apparently cross-link MHC of APC with the TCR of T cells, activating the latter. Moreover, some drugs (eg, sulfamethoxazole), which can be biotransformed into adduct forming metabolites (eg, nitroso-sulfamethoxazole) that triggers T-cell activation by the classical mechanism, purportedly may also stimulate the TCR of T cells directly, without involvement of APC.

Regarding contact allergens, a new concept has emerged: to elicit allergic contact dermatitis chemicals should not only initiate formation of antigen-specific T cells (as described above) but also evoke a less specific inflammatory reaction (referred to as innate immune signal). For example, nickel alone cannot give a positive response in the local lymph node assay in mice; however, it can when coadministered with lipopolysaccharide (LPS), a bacterial cell wall component. LPS acts through Toll-like receptor 4 (TLR4) on resident skin cells (macrophages, dendritic cells, fibroblasts) to trigger inflammatory gene expression via signaling pathways that involves NF- $\kappa$ B. However, nickel alone can evoke contact dermatitis in sensitive humans, apparently because it activates the human TLR4 that, unlike the mouse receptor, contains 2 nonconserved histidine residues (Schmidt and Goebeler, 2011). The need for an innate immune signal may account for the observation that contact allergy may be facilitated by an injury evoking inflammatory response, such as toxic cell injury by an irritant chemical or microbial infection.

Drug-induced lupus and possibly many cases of drug-induced agranulocytosis are thought to be mediated by immune reactions triggered by drug-protein adducts. The causative chemicals are typically nucleophiles, such as aromatic amines (eg, aminopyrine, clozapine, procainamide, and sulfonamides), hydrazines (eg, hydralazine and isoniazid), and thiols (eg, propylthiouracil, methimazole, and captopril). These substances can be oxidized by myeloperoxidase of granulocytes and monocytes (the precursors of APC) or by the ROS/RNS such cells produce ( $\text{HO}^\bullet$ ,  $\text{ONOO}^-$ , hypochlorous acid [ $\text{HOCl}$ ], see Fig. 3-26) to reactive metabolites that bind to the surface proteins of these cells, making them antigens (Uetrecht, 1992). The reactive metabolites may also activate monocytes and thus promote the immune reaction. Activation of  $\text{CD4}^+$  T cells by inhibition of promoter methylation of specific genes by procainamide and hydralazine as a possible lupus-inducing mechanism is discussed in the section “Dysregulation of Transcription.”

## Toxicity Not Initiated by Reaction with Target Molecules

Some xenobiotics not only interact with a specific endogenous target molecule to induce toxicity but also, instead, alter the biological microenvironment (see step 2b in Fig. 3-1). Included here are (1) chemicals that alter  $\text{H}^+$  ion concentrations in the aqueous bio-phase, such as (a) acids and substances biotransformed to acids, such as methanol and ethylene glycol, (b) protonophoric uncouplers such as 2,4-dinitrophenol and pentachlorophenol, which dissociate their phenolic protons in the mitochondrial matrix, thus dissipating the proton gradient that drives ATP synthesis, and (c) cationic amphiphilic drugs (eg, chloroquine and amiodarone)

that diffuse into lysosomes and accumulate there after being protonated at their amino groups; (2) solvents and detergents that physicochemically alter the lipid phase of cell membranes and destroy transmembrane solute gradients that are essential to cell functions; and (3) other xenobiotics that cause harm merely by occupying a site or space. For example, some chemicals (eg, the ethylene glycol-derived Ca-oxalate, methotrexate, and acyclovir) form water-insoluble precipitates in renal tubules. The poorly water-soluble complex produced in renal tubules from melamine and its derivative cyanuric acid caused the renal injury observed in cats and dogs fed adulterated pet food (Dobson *et al.*, 2008) and is probably also responsible for a similar disorder that occurred in children fed melamine-contaminated infant formula. By occupying bilirubin binding sites on albumin, compounds such as the sulfonamides induce bilirubin toxicity (kernicterus) in neonates. Carbon dioxide displaces oxygen in the pulmonary alveolar space and causes asphyxiation.

## STEP 3—CELLULAR DYSFUNCTION AND RESULTANT TOXICITIES

The reaction of toxicants with a target molecule may result in impaired cellular function as the third step in the development of toxicity (Fig. 3-1). Each cell in a multicellular organism carries out defined programs. Certain programs determine the destiny of cells—that is, whether they undergo division, differentiation (ie, express proteins for specialized functions), or apoptosis. Other programs control the ongoing (momentary) activity of differentiated cells, determining whether they secrete more or less of a substance, whether they contract or relax, and whether they transport and metabolize nutrients at higher or lower rates. For regulation of these cellular programs, cells possess signaling networks (such as those shown in Figs. 3-12 and 3-15) that can be activated and inactivated by external signaling molecules. To execute the programs, cells are equipped with synthetic, metabolic, kinetic, transport, and energy-producing systems as well as structural elements, organized into macromolecular complexes, cell membranes, and organelles, by which they maintain their own integrity (internal functions) and support the maintenance of other cells (external functions).

As outlined in Fig. 3-10, the nature of the primary cellular dysfunction caused by toxicants, but not necessarily the ultimate outcome, depends on the role of the target molecule affected. If the target molecule is involved in cellular regulation (signaling), dysregulation of gene expression and/or dysregulation of momentary cellular function occur primarily. However, if the target molecule is involved predominantly in the cell's internal maintenance, the resultant dysfunction can ultimately compromise the survival of the cell. The reaction of a toxicant with targets serving external functions can influence the operation of other cells and integrated organ systems. The following discussion deals with these consequences.

## Toxicant-Induced Cellular Dysregulation

Cells are regulated by signaling molecules that activate specific cellular receptors linked to signal transducing networks that transmit the signals to the regulatory regions of genes and/or to functional proteins. Receptor activation may ultimately lead to (1) altered gene expression that increases or decreases the quantity of specific proteins and/or (2) a chemical modification of specific proteins, typically by phosphorylation, which activates or inhibits proteins. Programs controlling the destiny of cells primarily affect gene

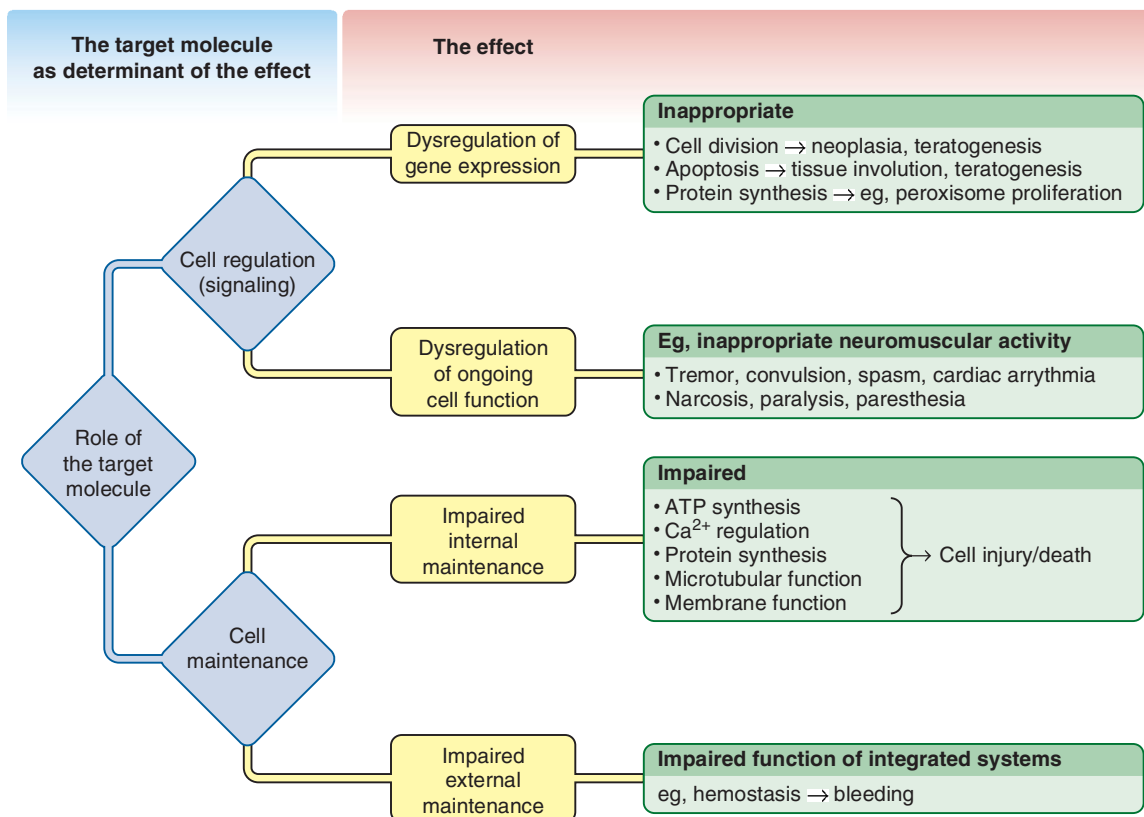


Figure 3-10. The third step in the development of toxicity: alteration of the regulatory or maintenance function of the cell.

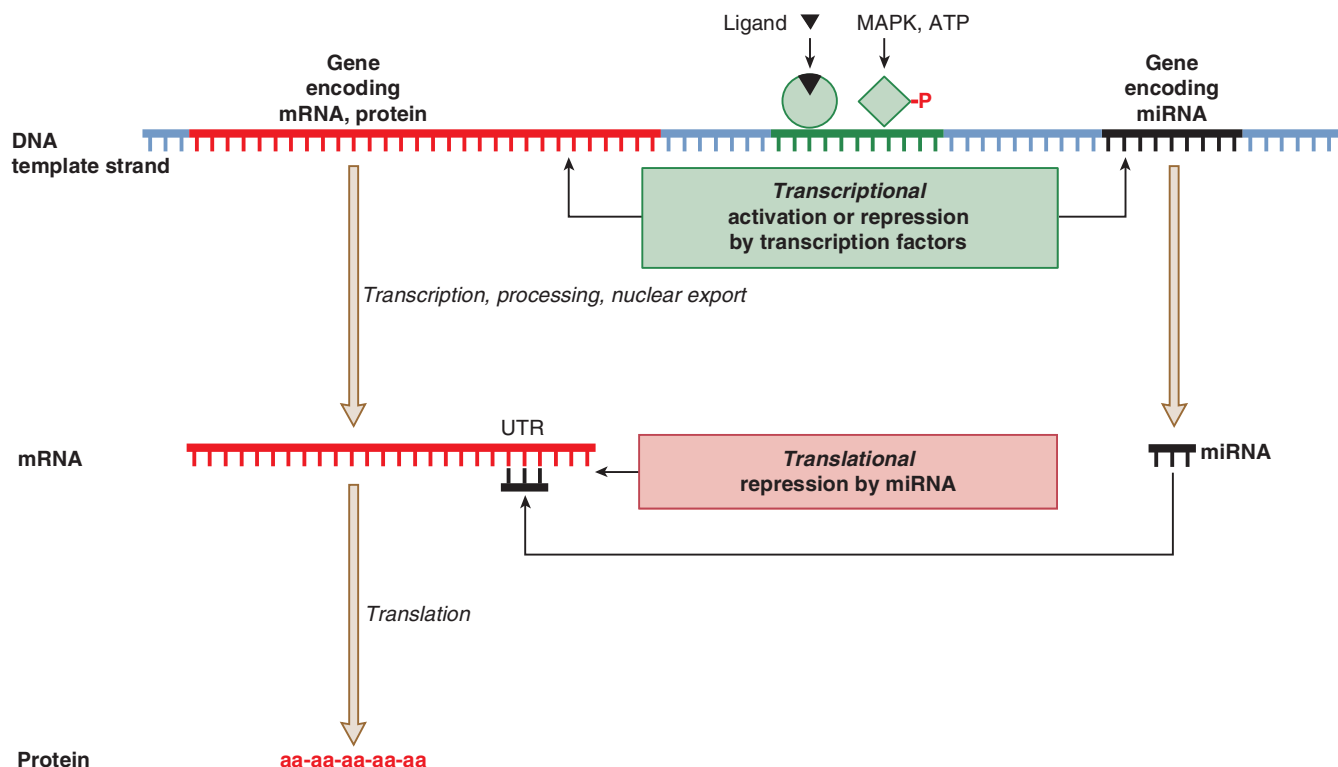
expression, whereas those regulating the ongoing activities primarily influence the activity of functional proteins; however, one signal often evokes both responses because of branching and interconnection of signaling networks.

**Dysregulation of 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 a protein product. The nonprotein-coding genes may be transcribed into other RNAs, among them the recently discovered small silencing RNA or microRNA (miRNA), which can repress translation of mRNA into proteins, thereby regulating protein synthesis posttranscriptionally (Fig. 3-11). Chemicals may dysregulate the expression of genes encoding mRNA, miRNA, or both, in either way causing altered synthesis of specific proteins. Dysregulation of gene expression may occur at elements that are directly responsible for transcription, at components of the intracellular signal transduction pathway, and at the synthesis, storage, or release of the extracellular signaling molecules. Dysregulation of gene expression initiated by chemicals at these 3 levels is discussed below.

**Dysregulation of Transcription** Transcription of genetic information from DNA to mRNA and miRNA is controlled largely by interplay between TFs and the regulatory or promoter region of genes coding for mRNA and miRNA (Fig. 3-11). By binding to distinctive nucleotide sequences in this region, ligand- and signal-activated TFs facilitate or impede the formation of the preinitiation complex, thereby promoting or repressing transcription of the adjacent gene. Xenobiotics may interact with the TFs (or other components of the preinitiation complex) and/or may alter the promoter region of the gene.

**Dysregulation of Transcription by Chemicals Acting Through Ligand-Activated Transcription Factors** Several endogenous compounds, such as hormones (eg, steroids, thyroid hormones) and vitamins (retinoids and vitamin D), influence gene expression by binding to and activating TFs or intracellular receptors (Table 3-4). Xenobiotics may mimic the natural ligands. For example, fibric acid-type lipid-lowering drugs, phthalate esters, and compound Wy-14,643 substitute for polyunsaturated fatty acids as ligands for the peroxisome proliferator-activated receptor (PPAR $\alpha$ ) (Poellinger *et al.*, 1992), and xenoestrogens (eg, DES, DDT, methoxychlor) substitute estradiol, an endogenous ligand for estrogen receptors (ER).

Acting through ligand-activated TFs, natural or xenobiotic ligands when administered at extreme doses or at critical periods during ontogenesis may cause toxicity by inappropriately influencing the fate of cells and inducing cell death or mitosis (Table 3-4). Glucocorticoids such as dexamethasone induce apoptosis of lymphoid cells. Whereas desirable in the treatment of lymphoid malignancies, this is an unwanted response in many other conditions. TCDD, a ligand of the AhR, produces thymic atrophy by causing apoptosis of thymocytes. Estrogens exert mitogenic effects in cells that express the ER, such as those found in female reproductive organs, mammary gland, and liver. ER-mediated proliferation appears to be responsible, at least in part, for formation of tumors in these organs during prolonged estrogen exposure (Green, 1992), as well as for induction of the rare vaginal adenocarcinoma in late puberty after transplacental exposure to the synthetic estrogen DES. Stimulation of these receptors during a critical prenatal period of differentiation reprograms the estrogen target tissue (ie, Mullerian duct), resulting in benign and malignant abnormalities in the reproductive tract later in life (Newbold, 2004). Mice exposed neonatally to DES served



**Figure 3-11.** Basic steps of gene expression—transcription factors regulate transcription, whereas microRNAs regulate (repress) translation. In the process of gene expression, information from a gene is used in the synthesis of a functional gene product. This simplified figure depicts how the synthesis of two important functional gene products, that is, proteins and microRNAs (miRNA, also called small silencing RNA), are interrelated. As described in the text, these normally controlled processes may be dysregulated by endobiotics and xenobiotics as well as during pathologic processes, such as carcinogenesis.

Genetic information in a protein-coding gene (red in the DNA strand) is transcribed into a precursor messenger RNA (mRNA), which is processed into the mature mRNA by capping at the 5' end, splicing (ie, removing noncoding introns) and polyadenylation at the 3' end (not shown). After export from the nucleus, the mRNA associates with the ribosome and serves as a template to determine the amino acid (aa) sequence of the protein being synthesized (translation).

DNA sequences between protein-coding genes (ie, intergenic regions) may code for miRNAs. These are small noncoding RNA molecules (21–25 nucleotides in length) that may control the translation of more than 60% of mRNAs into proteins. The gene for miRNA (black in the DNA strand) codes for an initial transcript, termed pri-miRNA, which may be several kilobases long (not shown; Wahid *et al.*, 2010). The pri-miRNA is then cut shorter in the nucleus by an RNase (Drosha), assisted by its partner protein DiGeorge syndrome critical region in gene 8 (DGCR8), to become pre-miRNA (60–70 nucleotides in length). After export into the cytoplasm, the pre-miRNA is further truncated by another RNase (Dicer) and its partner TRBP to form the mature miRNA. Finally, miRNA associates with an Argonaute protein, thus forming microRNA-induced silencing complex (miRISC). The miRNA guides miRISC to specifically recognize mRNAs, called target mRNAs. The miRNA binds with base pairing to the untranslated region (UTR) of the target mRNA and miRISC downregulates its translation into protein by translational repression and/or mRNA cleavage. Because miRNAs bind to their target mRNAs by partial complementarity over a short sequence, one miRNA may have several hundreds of mRNA targets; therefore, a single miRNA can silence the expression of many proteins. Often a single pri-miRNA is processed into a cluster of miRNAs; therefore, their expression pattern is usually similar. A well-known example is the miR17-92 cluster comprising 7 miRNAs that are often overexpressed in cancer.

Ligand- and signal-activated transcription factors (represented by the round and the rectangular symbols, respectively) can bind to the promoter regions (green in the DNA strand) of the protein-coding genes and the miRNA-coding genes and upregulate or downregulate their transcription, thereby directly influencing the expression of mRNA and/or miRNA and ultimately affecting the synthesis of specific proteins. Transcription factors and miRNAs thus regulate diverse cellular pathways. It is important to note that miRNAs, as a rule, repress the translation of proteins. Therefore, upregulation of miRNA transcription results in downregulation of protein translation from the target mRNAs, whereas downregulation of miRNA causes derepression of protein translation from the target mRNAs, thereby increasing the synthesis of specific proteins.

to model DES-induced late carcinogenesis in the reproductive tract. Compared with the wild-type animals, mice overexpressing estrogen receptor- $\alpha$  (ER $\alpha$ ) were exceedingly sensitive to DES-induced uterine adenocarcinoma, whereas ER $\alpha$ -knockout mice were resistant, indicating the role of ER $\alpha$  in tumorigenesis. It has been speculated that environmental xenoestrogens such as DDT, polychlorinated biphenyls, bisphenol A, and atrazine contribute to an increased incidence of breast cancer. Zearalenone, a mycoestrogen feed contaminant, causes vulval prolapse in swine, an example of an ER-mediated proliferative lesion. The mitogenic and hepatic tumor-promoting effects of peroxisome proliferators in rodents are also receptor-mediated, because they are not observed in PPAR $\alpha$ -null mice (Peters *et al.*, 1998). Humans do not respond with hepatocellular and peroxisomal proliferation to

fibrates and express PPAR $\alpha$  at low levels and often in nonfunctional forms. Humanized mice (ie, PPAR $\alpha$ -null mice transfected with human PPAR $\alpha$ ) also fail to respond with hepatocellular and peroxisomal proliferation to fibrates, indicating that the human receptor cannot signal for proliferation (Gonzalez and Yu, 2006). In addition, pathways downstream of the receptor are also inappropriate to produce such a response in humans, because transfection of rat PPAR $\alpha$  into human hepatocytes failed to make the peroxisomes inducible by PPAR $\alpha$  ligands in the human cells. Chemicals that act on ligand-activated TFs, such as glucocorticoids, TCDD, and retinoids, induce fetal malformations that may be regarded as inappropriate gene expression. Candidate target genes are the homeobox genes that determine the body plan during early ontogenesis.



Table 3-4

## Toxicants Acting on Ligand-Activated Transcription Factors

LIGAND-ACTIVATED TRANSCRIPTION FACTOR	ENDOGENOUS LIGAND	EXOGENOUS LIGAND	EFFECT
Estrogen receptor (ER)	Estradiol	Ethinylestradiol Diethylstilbestrol DDT, methoxychlor Zearalenone	Mammary and hepatic carcinogenesis  Porcine vulval prolapse
Glucocorticoid receptor (GR)	Cortisol	Dexamethasone	Apoptosis of lymphocytes Teratogenesis (cleft palate)
Retinoic acid receptor (RAR, RXR)	All- <i>trans</i> -retinoic acid	13- <i>cis</i> -Retinoic acid	Teratogenesis (craniofacial, cardiac, thymic malformations)
Aryl hydrocarbon receptor (AhR)	Unknown	TCDD PCBs PAHs	Thymic atrophy Wasting syndrome Teratogenesis (cleft palate) Hepatocarcinogenesis in rats Enzyme induction (eg, CYP1A1)
Peroxisome proliferator-activated receptor (PPAR $\alpha$ )	Fatty acids	Fibrate esters (eg, clofibrate) Phthalate esters (eg, DEHP)	Hepatocarcinogenesis in mice Peroxisome proliferation Enzyme induction (eg, CYP4A1, acyl-CoA oxidase)
Constitutive androstane receptor (CAR)	3 $\alpha$ ,5 $\alpha$ -Androstenol 3 $\alpha$ ,5 $\alpha$ -Androstanol (inhibitors)	TCPOBOP, phenobarbital* DDT, PCP, chlorpromazine	Enzyme induction (eg, CYP2B, CYP3A) Hepatocarcinogenesis in mice
Pregnane X receptor (PXR)	Pregnenolone Progesterone	PCN, dexamethasone Spironolactone, cyproterone Rifampicin, zearalenone Lithocholic acid	Enzyme induction (eg, CYP3A) Transporter induction (eg, Oatp1a4, Mdr1, Mrp1, Mrp2, Mrp3)

*TCPOBOP, 1,2-bis[2-(3,5-dichloropyridyloxy)]benzene.*  
*\*Phenobarbital indirectly activates CAR, not as a ligand.*

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 enzymes. For example, the PPAR $\alpha$ -ligand fibrates induce lipoprotein lipase and enzymes involved in fatty acid oxidation. Unlike the hepatic proliferative effects of PPAR $\alpha$  agonists, these effects occur not only in rodents but also in humans, and form the basis of the use of fibrates as lipid-lowering drugs. TCDD, phenobarbital, and pregnenolone 16 $\alpha$ -carbonitrile (PCN) activate AhR, the constitutive androstane receptor (CAR), and the pregnane X receptor (PXR), respectively (Table 3-4), thereby exerting their well-known cytochrome P450-inducing effects. However, rodent, rabbit, and human PXRs and CARs exhibit low degree of sequence conservation in their ligand-binding domains. This accounts for marked species differences in the induction potential of their ligands, such as PCN, rifampicin, and TCPOBOP (Gonzalez and Yu, 2006). Genes of several xenobiotic-metabolizing enzymes may be activated by these chemicals. For example, TCDD increases the expression of cytochrome P450 1A1, UDP-glucuronosyltransferase-1, and several subunits of mouse and rat glutathione *S*-transferase in an AhR-dependent manner. In AhR-null mice, TCDD induces neither these enzymes nor the adverse effects listed in Table 3-4 (Gonzalez and Fernandez-Salguero, 1998). CYP1A1 becomes also strongly expressed in the epithelial cells of the dioxin-induced cystic lesions in the human skin and thus CYP1A1 induction (which is detectable

immunohistochemically in these structures) may serve as a biomarker of human dioxin exposure (Saurat *et al.*, 2012). Chloracne, as this multiple dermal lesion has been called inappropriately, is now identified as hamartoma. Dioxin-induced hamartomas appear in the epidermis and are characterized by (1) nonmalignant proliferation of columnar epithelial cells enclosing cysts with inflammatory signs and (2) involution of sebaceous glands. Whole genome microarray analysis of TCDD-induced hamartomas revealed severe dysregulation of gene expression, with marked overexpression of some genes, such as those coding for CYP1A1, inflammatory proteins, and gremlin 2 (a cytokine that inhibits bone morphogenic protein, a transforming growth factor- $\beta$  [TGF- $\beta$ ]-like growth factor), and repression of many others, such as those encoding several sebaceous lipid synthesis enzymes and solute carriers (Saurat *et al.*, 2012). Mechanistic interpretation of many of these TCDD-induced alterations in gene expression and their role in development of dioxin-induced dermal hamartomas is still uncertain.

The above-mentioned alterations in cell fate and function caused by endobiotics and xenobiotics that act on ligand-activated TFs may in part be mediated by transcriptional upregulation or downregulation of protein-coding genes, that is, genes that are transcribed into mRNA, which in turn is translated into protein (Fig. 3-11). For example, the drug-metabolizing enzymes are induced by TCDD (see above) because the promoter regions of their genes contain a dioxin (or xenobiotic) response element (DRE or

XRE). The TCDD-activated AhR complexed with its nuclear translocator protein Arnt (also called HIF-1 $\beta$ ) binds to this sequence and promotes the transcription of these genes and secondarily the synthesis of enzyme proteins. The cyclooxygenase-2 (COX-2) gene, whose product is involved in the synthesis of inflammatory prostaglandins, also contains DRE/XRE permitting its transactivation by TCDD-activated AhR. Similarly, the ligand-activated PPAR $\alpha$ , after dimerizing with RXR, upregulates the transcription of genes encoding enzymes involved in fatty acid transport and  $\beta$ -oxidation by interacting with the peroxisome proliferator response element (PPRE) located in the promoter of these genes.

As discovered recently, the effects of endobiotics and xenobiotics that act on ligand-activated TFs may also be mediated by transcriptional upregulation or downregulation of genes coding for miRNAs. Because miRNAs repress the translation of target mRNAs into proteins, upregulation of miRNA transcription results in increased repression of protein translation, whereas downregulation of miRNA causes derepression of protein translation from the target mRNAs, and in turn increases the synthesis of specific proteins (Fig. 3-11). The mechanism of hepatocellular proliferation in mice exposed to the peroxisome proliferator Wy-14,643 involves transcriptional downregulation by the Wy-14,643-activated mouse PPAR $\alpha$  of the gene coding for let-7c, a miRNA (Gonzalez and Shah, 2008). Let-7c is known to target c-Myc mRNA and represses its translation into c-Myc protein. Therefore, declining levels of let-7c in response to Wy-14,643 result in derepression of c-Myc mRNA and a consequential surge in c-Myc protein synthesis. As c-Myc is a TF signaling for mitosis (see Figs. 3-12 and 3-32), its increased abundance in the liver induces hepatocellular proliferation. Interestingly, the proliferative action of c-Myc also involves miRNA gene regulation: c-Myc upregulates miR-17-92, a cluster of miRNAs, which in turn repress the translation of p21, a cyclin-dependent kinase inhibitor with antimitotic action (see Fig. 3-32), and Bim, a proapoptotic protein (see Fig. 3-15), thereby permitting mitosis and opposing apoptosis. In contrast to normal mice, PPAR $\alpha$ -humanized mice (expressing the human PPAR $\alpha$ ) exhibit neither downregulation of let-7c gene expression nor hepatocellular proliferation on Wy-14,643 treatment, supporting a causative relationship between these events. The mode of action of some other ligand-activated TFs also involves downregulation of miRNA gene transcription with subsequent derepression of the target mRNAs, causing their increased translation into specific proteins. For example, dexamethasone downregulates the expression of the miRNA cluster miR-17-92, which results in elevated levels of Bim, a proapoptotic member of the Bcl family of proteins (see Fig. 3-15). In addition, glucocorticoids can also upregulate the transcription of the Bim gene. Overexpression of the Bim protein, at both transcriptional and translational levels, may be the principal mechanism of glucocorticoid-induced apoptosis in lymphoid cells (Molitoris *et al.*, 2011). Glucocorticoids also cause skeletal muscle wasting, partly because they stimulate the production of myostatin by the muscle. Acting on the muscle in a paracrine manner, this TGF- $\beta$ -like signaling protein negatively regulates muscle mass (see Fig. 3-30). Glucocorticoids decrease the expression of miR-27a and b (which normally repress the translation of myostatin mRNA, as this mRNA contains a recognition sequence for miR-27a and b in its 3'-untranslated region), thereby increasing the synthesis of myostatin protein. Whereas TCDD, acting through the AhR and the XRE in the COX-2 gene promoter, can transcriptionally upregulate COX-2 expression as mentioned above, studies on mice indicate that TCDD can induce COX-2 protein also by downregulating of miR-101a gene, because one of the targets for miR-101a is the mRNA that translates into the COX-2 enzyme (Yoshioka *et al.*, 2011). It appears

that COX-2 induction plays a role in TCDD-induced liver injury in mice. Furthermore, the expression of drug-metabolizing enzymes is regulated not only by TFs (see above and in Table 3-4) but also by miRNAs. For example, the translation of CYP3A4 (which forms the genotoxic metabolites of aflatoxin B<sub>1</sub> and benzo[a]pyrene) and CYP2E1 (which contributes to toxicification of acetaminophen, carbon tetrachloride, and dimethylnitrosamine) is repressed by miR-27b and miR-378, respectively (Yokoi and Nakajima, 2011). The complexity of protein expression regulation is underlined by the recent recognition that while TFs can control the expression of miRNAs at their transcription, miRNAs can control the expression of TFs at their translation (Yokoi and Nakajima, 2011).

Finally, ligand-activated TFs do not act only by regulating gene expression through binding to their cognate response element but also by interacting with other proteins, including other nuclear receptors or their associates. Such interactions underlie, for example, the antiestrogenic effect of TCDD and dioxin-like chemicals, which involves at least two mechanisms. First, the TCDD-ligated AhR can associate with the ER, facilitating its nuclear export and proteasomal degradation. In addition, dimerization of the TCDD-ligated AhR with its partner Arnt reduces the availability of this protein for ER that employs Arnt as a transcriptional coactivator (Denison *et al.*, 2011).

*Dysregulation of Transcription by Chemicals Altering the Regulatory Region of Genes* Xenobiotics may dysregulate transcription by also altering the regulatory gene regions through direct chemical interaction or by changing their methylation pattern. It has been hypothesized that thalidomide (or its hydrolysis product) exerts teratogenic effect in the embryo by intercalating to GGGCGG sequences (also called GC boxes) that are binding sites for Sp1 and Egr-1 TFs (Stephens *et al.*, 2000). Thalidomide would thus impair insulin-like growth factor-1 (IGF-1) and fibroblast growth factor-2 (FGF2) signaling pathways necessary for angiogenesis and limb formation, because both pathways encompass multiple proteins whose genes contain GC boxes in the regulatory region but lack TATA and CCAAT boxes (eg, specific integrins, FGF2, IGF-1, and IGF-1 receptor substrate-1). Another chemical feature of thalidomide that may account for impaired Sp1-mediated transcriptional activation is that the hydrolysis product of this drug is a glutamine analogue and thus mimics the glutamine-rich activation domain of Sp1 protein.

As described in more detail in the section "Carcinogenesis," methylation of cytosines in CpG islands located in the promoter of genes is a major epigenetic mechanism, which together with coupled histone modifications (eg, acetylation, methylation) influences the transcriptional activity of the adjacent gene. Increased promoter methylation (hypermethylation) 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. Nevertheless, promoter methylation is subject to environmental influences, which can thus cause heritable changes in gene regulation. Altered promoter methylation has been implicated in chemically induced systemic lupus erythematosus (SLE), in developmental deficiencies manifested postnatally or even in subsequent generations, and in cancer. The antiarrhythmic drug procainamide and the antihypertensive hydralazine often induce SLE, an autoimmune inflammatory disease. Both drugs inhibit DNA methylation and induce global DNA hypomethylation in CD4<sup>+</sup> T lymphocytes with concomitant overexpression of proteins important in inflammation, such as CD11a (an integrin subunit), CD70 (a B-cell costimulatory molecule), and perforin (a cell-killing molecule) (Ballestar *et al.*, 2006). Idiopathic

lupus also involves global DNA hypomethylation in T cells with overexpression of these proinflammatory proteins. Therefore, promoter hypomethylation and overexpression of critical genes that convert T cells into aggressive inflammatory cells appears fundamental in the pathogenesis of both idiopathic and drug-induced SLE.

DNA methylation is an important process also in genomic imprinting that takes place during formation of germ cells. The vast majority of genes in both germ cells (ie, the sperm and the egg) similarly undergo or avoid epigenetic alterations (eg, methylation and related histone modifications); therefore, the offspring carries equally expressed 2 copies (ie, maternal and paternal alleles) of most genes. However, a relatively few (100–200) genes (called the imprinted genes) are epigenetically altered (generally methylated and thus typically silenced) only in the male or female germ cells; therefore, either the paternal or the maternal gene copy is expressed in the offspring, whereas the other allele is not expressed. For example, the insulin-like growth factor-2 gene, which encodes an autocrine growth factor important in fetal growth, is expressed only from the paternal allele. Because the genomically imprinted genes have only a single active copy, they are especially susceptible for epigenetic dysregulation. Dysregulation of imprinting can range from loss of imprinting, which results in biallelic expression and double amounts of the gene product, to silencing of both alleles.

Although prenatal exposure to chemicals, such as TCDD and ethanol, influences genomic imprinting (Wu *et al.*, 2004; Stouder *et al.*, 2011), mechanistic interpretation of the role of such epigenetic alterations in chemically induced embryonic, early postnatal, or transgenerational defects in somatic or germ cell development is still uncertain due to the complexity of gene regulation.

The genome is especially susceptible to epigenetic alterations during early development, as in the preimplantation mammalian embryo, the genome (except for the imprinted genes) undergoes extensive demethylation, and appropriate patterns of cytosine methylation are reestablished after implantation. Developing mammalian germ cells are also subject to such changes: primordial germ cells undergo genomic demethylation while they migrate into the early gonad, and then they undergo remethylation during sex determination in the gonad, for example, testis development. During this period (embryonic 11–15 days in the rat), the androgen receptor and ER- $\beta$  are expressed in the developing testis, and ligands of these nuclear receptors can influence the methylation process. Indeed, transient exposure of gestating female rats during the period of gonadal sex determination to vinclozolin (an agricultural fungicide with antiandrogenic activity) or methoxychlor (a DDT-type insecticide with estrogenic activity) decreased the spermatogenic capacity (with reduced sperm number and viability) in the adult male offspring and increased the incidence of male infertility (Anway *et al.*, 2005). Importantly, these effects were transferred through the male germ line to nearly all males of the subsequent 3 generations. These adverse transgenerational effects on reproduction correlate with altered DNA methylation patterns in the germ line. Thus, endocrine disruptors have the potential to epigenetically reprogram the germ line and promote transgenerational disease state, although the relevance of these findings under human exposure conditions is still uncertain.

Transcription is also severely dysregulated in cancer cells by altered promoter methylation (ie, global DNA hypomethylation and tumor suppressor gene hypermethylation). DNA methylation, including its biochemistry, its influence on histone modifications (acetylation and methylation), and its role in chemical carcinogenesis, is discussed in this chapter in the section “Epigenetic Mechanisms in Carcinogenesis: Inappropriate Activation or Responsiveness of the Regulatory Region of Critical Genes.”

**Dysregulation of Signal Transduction** Extracellular signaling molecules (such as growth factors, cytokines, hormones, neurotransmitters, and some secreted proteins) engage cell surface receptors and intracellular signal transducing networks and ultimately activate TFs. Fig. 3-12 depicts a simplified scheme for some of these networks and identifies some signal-activated TFs that are turned on by these systems and that in turn control the transcriptional activity of genes that determine the fate of cells by influencing mitosis and/or apoptosis. Among these TFs are c-Fos and c-Jun proteins, which bind in dimeric combinations (called AP-1) to the tetradecanoylphorbol acetate (TPA) response element (TRE), for example, in the promoter of the cyclin D gene whose product promotes the cell division cycle (see Figs. 3-25 and 3-32). Other signal-activated TFs that upregulate the transcription of cyclin D gene by binding to their cognate nucleotide sequences include c-Myc, E2F, NF- $\kappa$ B, CREB, and STAT. Mitogenic signaling molecules thus induce cell proliferation. In contrast, TGF- $\beta$  activates SMADs and induces the expression of cyclin-dependent kinase inhibitor proteins (eg, p15, p21, and p57), which mediate its antimitotic effect (Johnson and Walker, 1999) (see Fig. 3-32), as well as the proapoptotic protein Bim (see Fig. 3-19), which conveys its apoptotic action.

In addition to the proliferatory signaling pathways illustrated in Fig. 3-12, Figs. 3-13 and 3-14 depict 2 other pathways, the activation of which can also promote cell division. These are set in motion by secreted glycoproteins, the Wnt and Hedgehog (Hh) ligands, which act on specific cell surface receptors, ultimately activating the transcriptional coactivator  $\beta$ -catenin and the TF Gli, respectively, which in turn can also activate the transcription of cyclin D gene, among many others. The Wnt- $\beta$ -catenin and Hh-Gli systems are especially important in certain proliferative processes, such as embryogenesis, development of adult stem cells as well as progenitor cells, tissue regeneration after injury, and oncogenesis.

The signal from the cell surface receptors to the TFs is relayed mainly by successive protein–protein interactions and protein phosphorylations at specific serine, threonine, or tyrosine hydroxyl groups. Cell surface growth factor receptors (item 4 in Fig. 3-12) are in fact phosphorylating enzymes (ie, receptor protein tyrosine kinases). Their ligands induce them to phosphorylate themselves, which, in turn, enable these receptors to bind adapter proteins. Through these, the growth factor receptors can initiate proliferative signaling via two important pathways: (1) by activating Ras, they set in motion the mitogen-activated protein kinase (MAPK) cascade (Qi and Elion, 2005), and (2) by activating PI3K, they start the PI3K–Akt pathway. A crucial step in the latter is the PI3K-catalyzed phosphorylation of a plasma membrane lipid to generate phosphatidylinositol-3,4,5-triphosphate (PIP<sub>3</sub>) that recruits protein kinase Akt to the membrane for activation. Because Akt (also called protein kinase B) has numerous substrates, the PI3K–Akt pathway branches (Morgensztern and McLeod, 2005). Fig. 3-12 shows the branch that leads to activation of the TF NF- $\kappa$ B, a prolife pathway. In addition, Akt may also indirectly activate the mammalian target of rapamycin (mTOR), a ubiquitous protein kinase, whose phosphorylated products promote the trophic state of cells by boosting protein synthesis and restraining autophagy (see Fig. 3-29). Furthermore, Akt catalyzes the inactivating phosphorylation of two important signaling proteins. One is glycogen synthase kinase 3 (GSK), a ubiquitous multifunctional constitutively active protein kinase, whose inactivation by Akt (or by some other Ser/Thr kinases) causes numerous effects ranging from promotion of glycogen synthesis from UDP-glucose to suppression of apoptosis (see later), and whose incapacitation by Wnt and Hh signaling permits  $\beta$ -catenin and Gli, respectively, to escape degradation and



act as transcription activators (see Figs. 3-13 and 3-14). The other is the FoxO family of TFs, whose inactivation by Akt-catalyzed phosphorylation prevents protein degradation in the skeletal muscle and promotes proliferation, thereby countering muscle atrophy (see Fig. 3-30).

The intracellular signal transducer proteins are typically but not always activated by phosphorylation, which is catalyzed by protein kinases, and are usually inactivated by dephosphorylation, which is carried out by protein phosphatases. Interestingly, growth factor receptors can amplify their signals by inducing formation of HOOH. As discussed earlier, by attacking protein thiolate groups and converting them into sulfenic acid groups, HOOH reversibly inactivates PTP and PTEN, a lipid phosphatase that eliminates  $\text{PIP}_3$  (see Fig. 3-12; Rhee *et al.*, 2005). The source of HOOH is  $\text{O}_2^{\cdot -}$  produced by the enzyme complex Nox, which is activated by growth factor receptors via the  $\text{PI3K} \rightarrow \text{PIP}_3 \rightarrow \text{Rac}$  (a G-protein that associates with Nox) pathway. Even if once thought to be restricted to phagocytes, Nox occurs in the plasma membrane of many other cells, such as endothelial cells and vascular smooth muscle cells (Bokoch and Knaus, 2003). Although Nox in these cells is much less efficient to produce  $\text{O}_2^{\cdot -}$  than Nox in phagocytes, the nonphagocytotic Nox releases  $\text{O}_2^{\cdot -}$  into the cytoplasm, whereas the phagocytotic Nox delivers it to the external face of the plasma membrane (El-Benna *et al.*, 2005).

Chemicals may cause aberrant signal transduction in a number of ways, most often by altering protein phosphorylation, occasionally by interfering with the GTPase activity of G proteins (eg, Ras), by disrupting normal protein-protein interactions or by establishing abnormal ones, or by altering the synthesis or degradation of signaling proteins. Such interventions may ultimately influence cell cycle progression.

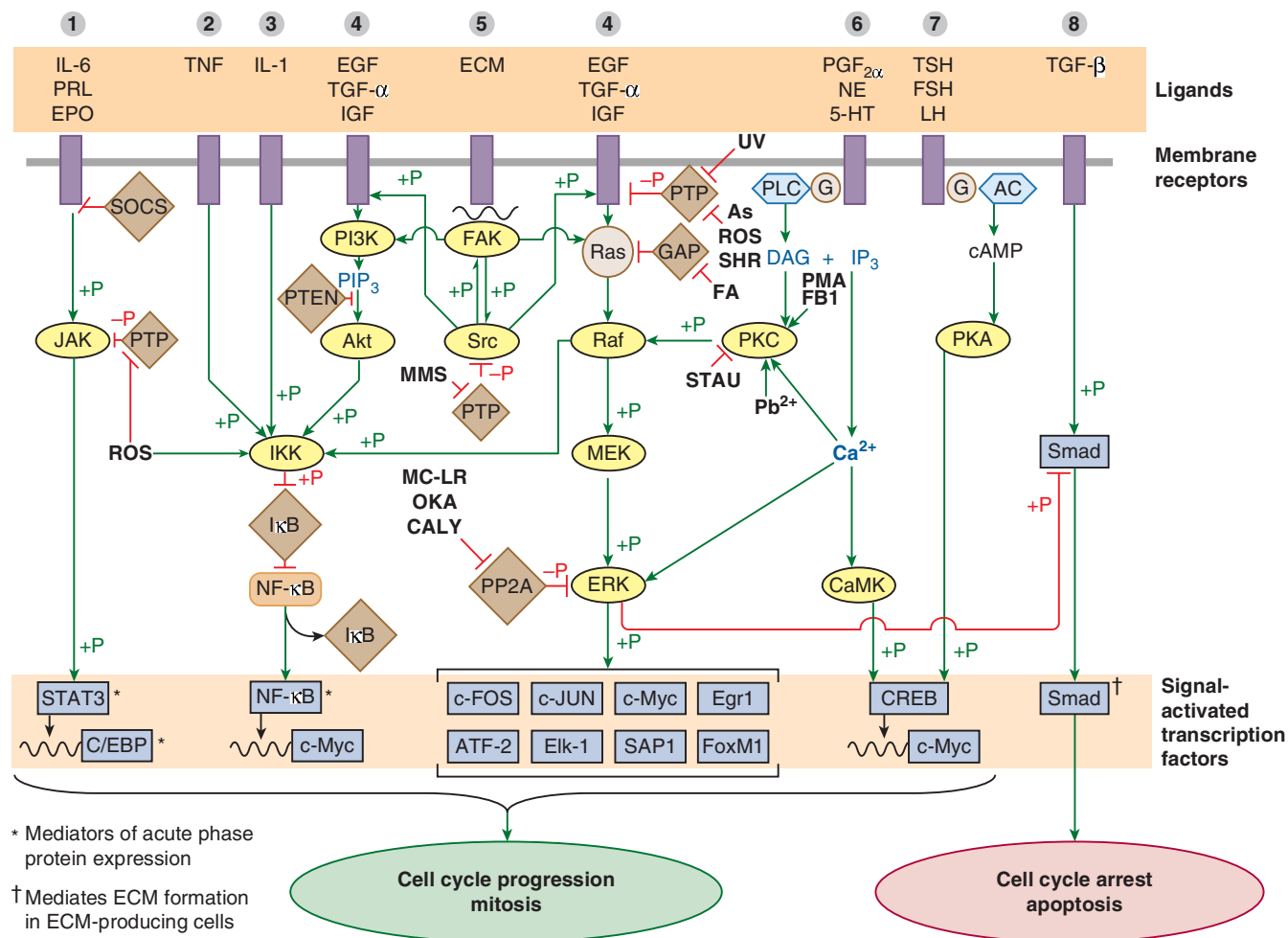
**Chemically Altered Signal Transduction with Proliferative Effect** Xenobiotics that facilitate phosphorylation of signal transducers often promote mitosis and tumor formation. Such are the phorbol esters and fumonisin B that activate PKC. These chemicals functionally mimic diacylglycerol (DAG), one of the physiologic activators of PKC (Fig. 3-12). The other physiologic PKC activator  $\text{Ca}^{2+}$  is mimicked by  $\text{Pb}^{2+}$ , whose effect on  $\text{PKC}\alpha$  is concentration-dependent: stimulatory at picomolar concentration, when  $\text{Pb}^{2+}$  occupies only high-affinity binding sites on PKC, and inhibitory at micromolar concentration, where the low-affinity sites are also occupied (Sun *et al.*, 1999). Lead salts do induce marked hepatocellular proliferation in rats. The activated PKC promotes mitogenic signaling at least in two ways: (1) by phosphorylating Raf, the first protein kinase in the MAPK pathway (Fig. 3-12), and (2) by phosphorylating 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. Protein kinases may also be activated by interacting proteins that had been altered by a xenobiotic. For example, the TCDD-liganded AhR binds to MAPK. This so-called nongenomic effect of TCDD may contribute to overexpression of cyclins and cyclin-dependent kinases in guinea pig liver (Ma and Babbish, 1999).

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 (see Fig. 3-12), appears to be the underlying mechanism of the mitogenic effect of various chemicals, oxidative stress, and ultraviolet (UV) irradiation (Herrlich *et al.*, 1999). PTP and dual-specificity phosphatases (ie, enzymes that remove phosphate from phosphorylated tyrosine as well as serine and threonine residues) as well as PTEN contain a catalytically active cysteine, and are susceptible to inactivation by

HOOH via sulfenic acid formation and by covalent reaction with other SH-reactive chemicals (Forman *et al.*, 2004; Rhee *et al.*, 2005). Indeed, 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 (item 4 in Fig. 3-12) by interfering with the PTP that would dephosphorylate and thus “silence” this receptor (Herrlich *et al.*, 1999; Chen *et al.*, 1998). Arsenite-induced oxidative stress (Lynn *et al.*, 2000; Smith *et al.*, 2001) may in fact result from activation of growth factor receptors. These signal via the  $\text{PI3K} \rightarrow \text{PIP}_3 \rightarrow \text{Rac}$  pathway to the neighboring Nox, which also resides in the plasma membrane. Overproduction of  $\text{O}_2^{\cdot -}$  by the activated Nox in endothelial cells and vascular smooth muscle cells might contribute to the vasculotoxic effects of arsenic. Arsenite may also inactivate the dual-specificity phosphatase that dephosphorylates and “silences” certain MAPKs (c-jun N-terminal kinase [JNK], p38), whereas methylmethane sulfonate (MMS) appears to inhibit a protein phosphatase that inactivates Src, a protein tyrosine kinase (Herrlich *et al.*, 1999). The thiol-oxidizing agent diamide (which increases phosphorylation of MAPKs) and phenolic antioxidants (which form phenoxyl radicals and increase c-Fos and c-Jun expression) (Dalton *et al.*, 1999) may also act by incapacitating PTP. 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 (Goldberg, 1999). PP2A also removes an activating phosphate from a mitosis-triggering protein kinase ( $\text{p}34^{\text{cdc}2}$ ). Several natural toxins are extremely potent inhibitors of PP2A, including the blue-green algae poison microcystin-LR and the dinoflagellate-derived okadaic acid (Toivola and Eriksson, 1999), which are tumor promoters in experimental animals subjected to prolonged low-dose exposure. It is to be noted, however, that acute high-dose exposure to microcystin induces severe liver injury, whereas such exposure to okadaic acid is the underlying cause of the diarrhetic shellfish poisoning. In these conditions, hyperphosphorylation of proteins other than those involved in proliferative signaling (eg, hepatocellular microfilaments in microcystin poisoning) may be primarily responsible for the pathogenesis.

Apart from phosphatases, there are also inhibitory binding proteins that can keep signaling under control. Such is I $\kappa$ B, which binds to NF- $\kappa$ B, preventing its transfer into the nucleus and its function as a TF. On phosphorylation by its designated I $\kappa$ B kinase (IKK), I $\kappa$ B is degraded by the proteasome and NF- $\kappa$ B is set free. IKK can be phosphorylated (activated) by other protein kinases, such as Raf (a member of the MAPK cascade) and Akt (a member of the  $\text{PI3K}$ -Akt pathway) (Fig. 3-12). NF- $\kappa$ B is an important contributor to proliferative and prolife signaling, because via the above pathways, growth factor receptor stimulation can cause I $\kappa$ B phosphorylation and thus the release of NF- $\kappa$ B, and because the released NF- $\kappa$ B transactivates genes whose products accelerate the cell division cycle (eg, cyclin D1 and c-Myc) and inhibit apoptosis (eg, antiapoptotic Bcl proteins and the caspase inhibitor of apoptosis proteins [IAP]) (Karin, 2006). NF- $\kappa$ B activation, via the Akt-mediated IKK phosphorylation, is involved in the proliferative (and possibly carcinogenic) effect of arsenite, nicotine, and the NNK. In addition, because NF- $\kappa$ B also targets the genes of several cytokines (eg, TNF, IL-1 $\beta$ ), chemokines (eg, monocyte chemoattractant protein-1 [MCP-1], cytokine-induced neutrophil chemoattractant [CINC]-1), cell adhesion molecules (eg, ICAM-1, E- and P-selectins), enzymes producing inflammatory lipid mediators (eg, PLA2, COX-2), and acute-phase proteins (eg, C-reactive protein,  $\alpha$ 1-acid glycoprotein), and because such cytokines acting on their receptors (items 2 and 3 in Fig. 3-12) activate

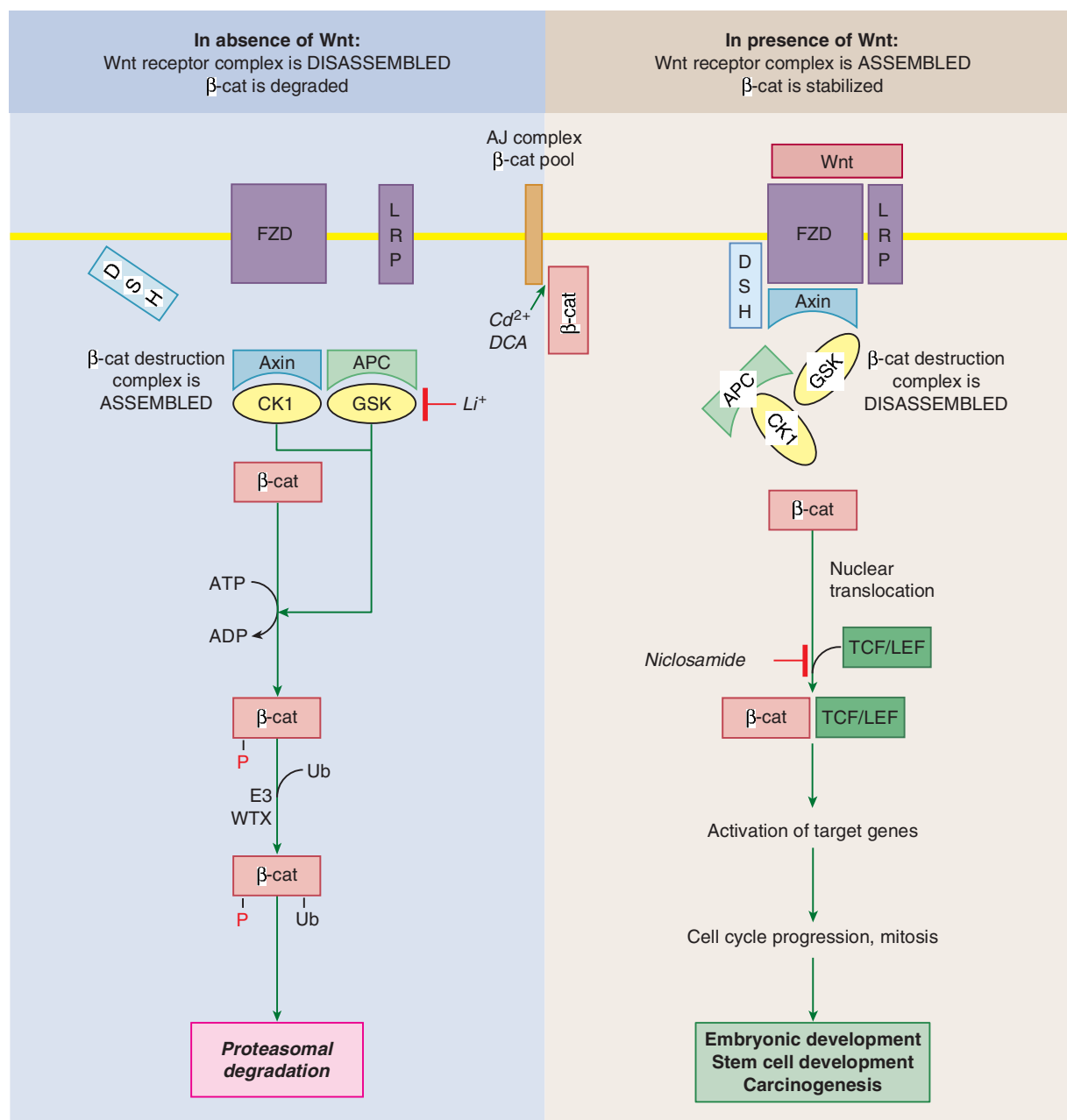




**Figure 3-12.** Some signal transduction pathways from cell membrane receptors to signal-activated nuclear transcription factors that influence transcription of genes involved in cell-cycle regulation. The symbols of cell membrane receptors are numbered 1 to 8 and some of their activating ligands are indicated. 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 I $\kappa$ B and suppressor of cytokine signaling (SOCS). Arrowheads indicate stimulation or formation of second messengers (eg, DAG, IP $_3$ , PIP $_3$ , cAMP, 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 myristate acetate; ROS, reactive oxygen species; SHR, SH-reactive chemicals, such as iodoacetamide; STAU, staurosporin).

Two important proliferative signaling can be evoked by growth factors, such as EGF, acting on their tyrosine kinase receptors (#4), which are duplicated in the figure for clarity. These receptors use adaptor proteins (Shc, Grb2, and SOS; not shown) to activate Ras by converting it from inactive GDP-bound form to active GTP-bound state, which in turn activates the MAP-kinase phosphorylation cascade built up from specific forms of MAPKKK, MAPKK, and MAPK proteins (here Raf, MEK, and ERK, respectively). The phosphorylated MAPK (eg, ERK) moves into the nucleus and phosphorylates transcription factors (eg, c-Fos), thereby enabling them to bind to cognate sequences in the promoter regions of genes (eg, cyclin genes) to facilitate transcription. The same growth factor receptors (#4) can signal through the phosphatidylinositol-3-kinase (PI3K)–Akt–I $\kappa$ B–NF- $\kappa$ B pathway (see the text for more details). There are numerous interconnections between the signal transduction pathways. For example, the G-protein-coupled receptor (#6) can relay signal into the MAPK pathway via phospholipase C (PLC)–catalyzed formation of second messengers that activate protein kinase C (PKC), whereas signals from TNF and IL-1 receptors (#2 and 3) are channeled into the PI3K–Akt–NF- $\kappa$ B pathway by phosphorylating IKK. (Note that TNF may also signal for apoptosis when its receptor recruits an adaptor molecule FADD; see Fig. 3-19.) The integrin receptor (#5), whose ligands are constituents of the extracellular matrix (ECM), can also engage the growth factor receptors and Ras as well as PI3K via focal adhesion kinase (FAK) and the tyrosine kinase Src. The cell cycle acceleration induced by these and other signal transduction pathways is further enhanced by the ERK-catalyzed inhibitory phosphorylation of Smad that blocks the cell-cycle arrest signal from the TGF- $\beta$  receptor (#8). Activation of protein kinases (PKC, CaMK, MAPK) by Ca $^{2+}$  can also trigger mitogenic signaling. Several xenobiotics that are indicated in the figure may dysregulate the signaling network. Some may induce cell proliferation by either activating mitogenic protein kinases (eg, PKC) or inhibiting inactivating proteins, such as protein phosphatases (PTP, PP2A), GAP, or I $\kappa$ B. Others, for example, inhibitors of PKC, oppose mitosis and facilitate apoptosis.

This scheme is oversimplified and tentative in several details. Virtually all components of the signaling network (eg, G proteins, PKCs, MAPKs) are present in multiple, functionally different forms whose distribution may be cell-specific. The pathways depicted are not equally relevant for all cells. In addition, these pathways regulating gene expression not only determine the fate of cells but also control certain aspects of the ongoing cellular activity. For example, NF- $\kappa$ B induces synthesis of acute-phase proteins.

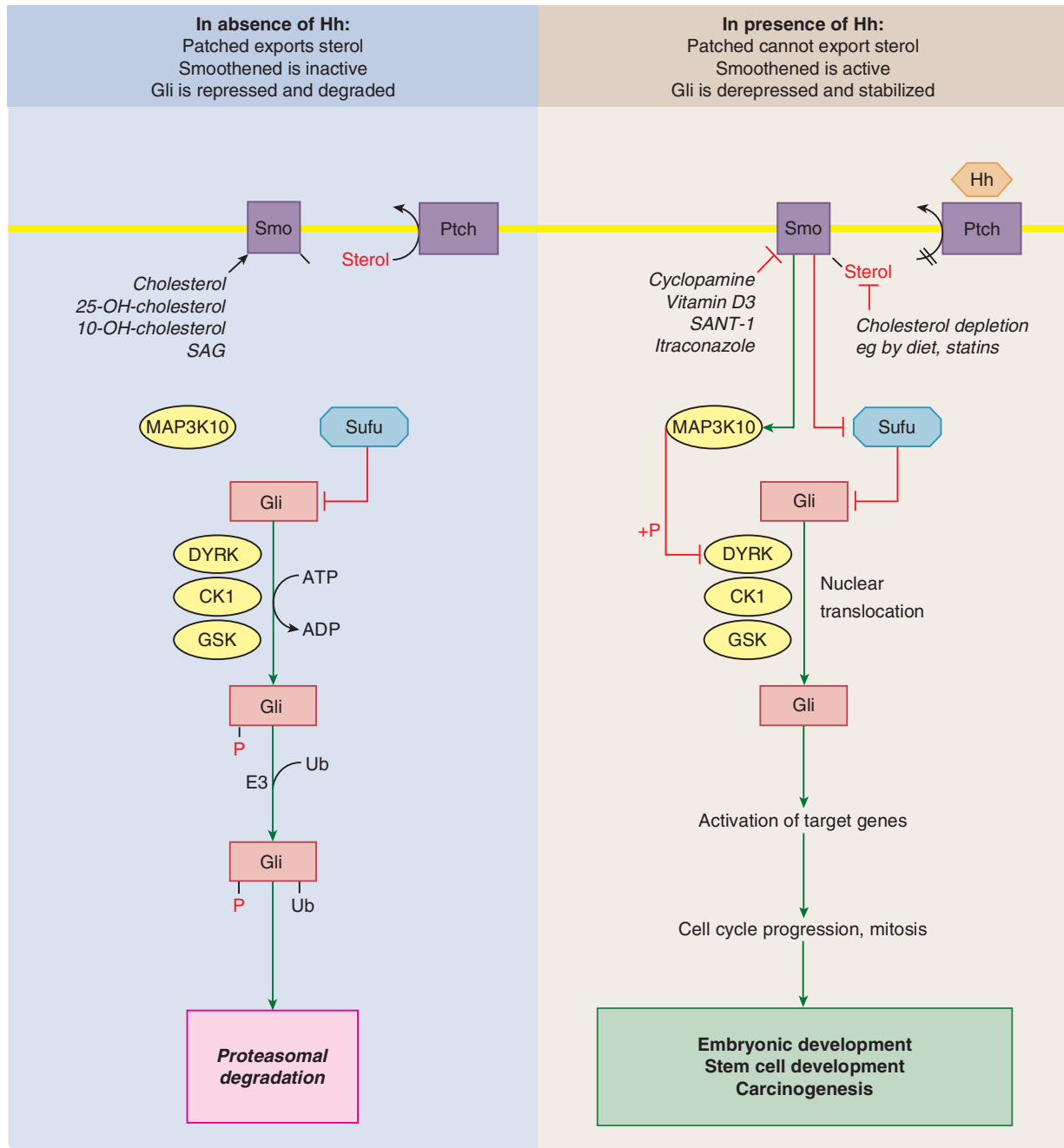


**Figure 3-13.** *The Wnt signaling—a proliferative pathway especially important in embryogenesis, stem cell and progenitor cell development, and carcinogenesis.* Wnt ligands are a secreted proteins covalently modified by palmitoylation and glycosylation. They act in autocrine, paracrine, or endocrine manner through their cell surface receptor frizzled (FZD) and coreceptor lipoprotein receptor–related protein (LRP).

In the absence of Wnt (as in the left side of the figure) intracellular proteins, including Axin, adenomatous polyposis coli (APC), casein kinase 1 (CK1), and glycogen synthase kinase 3 (GSK), form the so-called  $\beta$ -catenin ( $\beta$ -cat) destruction complex. The kinase members of this complex phosphorylate  $\beta$ -cat, thereby targeting it for ubiquitination by an E3 ubiquitin ligase (which is facilitated by WTX) and subsequent proteasomal degradation.

On binding of Wnt to FZD and LRP (as in the right side of the figure) the Wnt receptor complex assembles, and with recruitment of Axin into this complex, the  $\beta$ -cat destruction complex becomes disassembled. Consequently,  $\beta$ -cat escapes phosphorylation, ubiquitination, and degradation. Therefore,  $\beta$ -cat accumulates in the cell, translocates into the nucleus, and is recruited to its target genes by TCF/LEF family of transcription factors. Wnt signaling thus upregulates the transcription of numerous genes, including cyclin-D1, c-Myc, ABCB1 (P-glycoprotein), and S100A4 (a  $Ca^{2+}$ -binding protein involved in cell cycle progression, cell motility, and metastasis development). Interestingly,  $\beta$ -cat is not only a transcriptional coactivator but also a structural protein that associates with E-cadherin at adherens junctions (AJ), thus participating in cell–cell adhesion. The disruption of E-cadherin– $\beta$ -cat complexes causes an increase in  $\beta$ -cat-mediated transcription of Wnt-responsive genes. In contrast, stabilization of the E-cadherin– $\beta$ -cat complex brings about reduced  $\beta$ -cat-mediated Wnt signaling.

Chemicals that influence Wnt signaling may interfere with embryonic development, cell proliferation, and carcinogenesis. Inhibition of GSK activity (eg, by  $Li^+$  ion) can lead to stabilization of  $\beta$ -cat and activation of  $\beta$ -cat-dependent gene transcription.  $Cd^{2+}$  disrupts the E-cadherin– $\beta$ -cat complex of epithelial AJ, and the resultant  $\beta$ -cat-mediated proliferation of renal tubular cells could contribute to cadmium-induced nephrocarcinogenesis (Thévenod and Chakraborty, 2010). By a similar mechanism, deoxycholic acid (DCA) increases the proliferation and invasiveness of colon cancer cells. Inactivating mutations of APC and WTX, negative regulators of Wnt signaling, typically occur in colon cancer and Wilms tumor, respectively. The antihelminthic drug niclosamide inhibits formation of the  $\beta$ -cat-TCF/LEF complex at the promoter of S100A4 gene and thus counters colon cancer metastasis. DSH, disheveled; LEF, lymphoid enhancer-binding factor; TCF, T-cell factor; WTX, Wilms tumor suppressor.



**Figure 3-14.** The hedgehog (Hh) signaling—a proliferative pathway especially important in embryogenesis, stem cell and progenitor cell development, and carcinogenesis. Hh ligands are secreted proteins covalently modified by palmitate and cholesterol. They act in autocrine, paracrine, or endocrine manner through their membrane receptor Patched (Ptch), which is the key inhibitor of Hh signaling in the unligated form (Katoh and Katoh, 2008).

In the absence of Hh (as in the left side of the figure) Ptch impedes the Hh pathway by inhibiting the activity of Smoothened (Smo), a positive regulator of signaling. According to the model presented in the figure, Ptch is a sterol pump that exports sterols (cholesterol or an oxysterol), thereby removing these activator molecules from Smo and making Smo idle. The repression of Smo by Ptch incapacitates the downstream Hh effectors, the glioma-associated family of transcription factors (Gli). This occurs partly because Gli is suppressed by Sufu (suppressor of fused) and partly because protein kinases, such as the dual-specificity tyrosine-(Y)-phosphorylation regulated kinase (DYRK), casein kinase 1 (CK1), and glycogen synthase kinase 3 (GSK), phosphorylate Gli, thereby marking Gli for ubiquitination by a ubiquitin ligase (E3) and subsequent degradation by the proteasome.

Binding of Hh to Ptch (as in the right side of the figure) abrogates its sterol-exporting activity; therefore, the sterol can activate Smo. In a manner incompletely understood, the active Smo suppresses Sufu, thereby permitting Gli activation, and activates mitogen-activated protein kinase kinase kinase 10 (MAP3K10), which in turn phosphorylates and inhibits DYRK, thereby preventing phosphorylation, ubiquitination, and proteasomal degradation of Gli. Thereafter the active Gli accumulates in the cell, translocates into the nucleus, and binds to its target genes. Hh signaling thus upregulates the transcription of numerous genes, including those that encode Ptch and Gli (a positive feedback loop), cyclin-D1 and -D2 (for cell cycle acceleration), several transcription factors such as those of the Forkhead box protein family (for cell fate determination), receptor ligands such as Jagged 2 (a ligand protein that activates Notch and related receptors), and ligand-binding proteins such as the Secreted frizzled-related protein 1 (SFRP1) that binds Wnt protein, thus preventing Wnt signaling (Fig. 3-13).

Chemicals that activate or inhibit Smo (shown in the left and right sides of the figure, respectively) may interfere with embryonic development. For example, the plant alkaloid cyclopamine inhibits Smo and causes cyclopia, a birth defect, in lambs. Smo inhibitors are potentially anticarcinogenic, whereas Smo activators may promote carcinogenesis by increased Hh signaling. Inactivating mutation of Ptch, the main inhibitor of Hh signaling, typically occurs in basal cell carcinoma of the skin that may be induced by UV and ionizing radiation, as well as arsenic exposure (Tang *et al.*, 2007). SAG, Smoothened Agonist; SANT-1, Smoothened Antagonist 1.

NF- $\kappa$ B, this TF plays a leading role also in inflammatory and acute-phase reactions, as well as in cancer caused by chronic inflammation (Karin, 2006; Waddick and Uckun, 1999). Conversely, inactivation of a subtype of NF- $\kappa$ B protein (RelA/p65) by binding to the TCDD-ligated AhR may contribute to the immunosuppressive effect of TCDD (Denison *et al.*, 2011). The proliferative Wnt signaling is also restrained by a set of binding proteins and protein kinases, the so-called  $\beta$ -catenin destruction complex that includes axin, adenomatous polyposis coli (APC), casein kinase 1 (CK1), and glycogen synthase kinase 3 (GSK), which destroy the transcriptional coactivator  $\beta$ -catenin by phosphorylation and ubiquitination (see Fig. 3-13). Impaired function of this complex (eg, by inactivating mutation of APC) can lead to unleashing the transcriptional coactivator  $\beta$ -catenin and in turn to colon cancer.

Another site from which aberrant mitogenic signals may originate is the GTP/GDP-binding protein Ras, which is active in GTP-bound form but inactive in GDP-bound form. The activity of Ras is normally terminated via stimulation of its own GTPase activity by a GTPase-activating protein (GAP) (Fig. 3-12) that returns Ras into its inactive GDP-bound state. Fatty acids, which may accumulate, for example, in response to phospholipase A activation and exposure to peroxisome proliferators (Rose *et al.*, 1999), inhibit GAP and can delay the turning off of Ras. As discussed in more detail later in the chapter, genotoxic carcinogens may mutate Ras, and if the mutation leads to a loss of its GTPase activity, this would result in permanent signaling for the MAPK pathway—a condition that contributes to malignant transformation of the affected cell population.

Finally, increased mobilization of a mitogenic TF from a “silent” intracellular pool may also be a mechanism for chemically altered signal transduction with proliferative effect. This may underlie, at least in part, the nephrocarcinogenicity of cadmium and the colon tumor-promoting action of secondary bile acids, such as deoxycholic acid. By disrupting the E-cadherin/ $\beta$ -catenin complex in the adherens junctions (AJ) of renal and colonic epithelial cells, respectively, these chemicals mobilize  $\beta$ -catenin from AJ (where this protein serves a structural function) into the cytoplasm and the nucleus. There  $\beta$ -catenin functions as a TF of Wnt signaling (Fig. 3-13) and activates the transcription of several genes, including cyclin D1 that promotes the G1/S transition of cells in the cell division cycle.

**Chemically Altered Signal Transduction with Antiproliferative Effect** Turning off the increased proliferative signaling after cell injury may compromise replacement of injured cells. This prediction has been made from a study on cultured Hepa 1-6 cells that exhibited the following, seemingly consequential alterations on exposure to acetaminophen (follow the path in Fig. 3-12): inhibition of Raf  $\rightarrow$  diminished degradation of I $\kappa$ B  $\rightarrow$  diminished binding of NF- $\kappa$ B to DNA  $\rightarrow$  diminished expression of c-Myc mRNA (Boulares *et al.*, 1999). Downregulation of a normal mitogenic signal is a step away from survival and toward apoptosis. Indeed, inhibitors of PKC (staurosporin), PI3K (wortmannin), and I $\kappa$ B degradation (gliotoxin) (Waddick and Uckun, 1999) are apoptosis inducers. TGF- $\beta$  and glucocorticoids increase I $\kappa$ B synthesis and, in turn, decrease NF- $\kappa$ B activation and c-Myc expression (Waddick and Uckun, 1999). These mechanisms may contribute to the apoptotic effect of TGF- $\beta$  and glucocorticoids, the latter in lymphoid cells.

Offsetting the Hh-Gli pathway (Fig. 3-14), a key mitogenic signaling pathway controlling embryonic development, is the mechanism of the teratogenic action of cyclopamine (ie, cyclopia). This severe cephalic malformation was observed in the offspring of sheep grazing in a field where *Veratrum californicum* grew. This plant contains the steroidal alkaloid cyclopamine, which is an inhibitor of Smoothened, a positive regulator of Hh signaling (Chen *et al.*, 2002).

**Dysregulation of Extracellular Signal Production** Hormones of the anterior pituitary exert mitogenic effects on endocrine glands in the periphery by acting on cell surface receptors. Pituitary hormone production is under negative feedback control by hormones of the peripheral glands. Perturbation of this circuit adversely affects pituitary hormone secretion and, in turn, the peripheral gland. For example, xenobiotics that inhibit thyroid hormone production (eg, the herbicide amitrole and the fungicide metabolite ethylenethiourea) or enhance thyroid hormone elimination (eg, phenobarbital) reduce thyroid hormone levels and increase the secretion of thyroid-stimulating hormone (TSH) because of the reduced feedback inhibition. The increased TSH secretion stimulates cell division in the thyroid gland, which is responsible for the goiters or thyroid tumors caused by such toxicants (see Chap. 21). Decreased secretion of pituitary hormone produces the opposite adverse effect, with apoptosis followed by involution of the peripheral target gland. For example, estrogens produce testicular atrophy in males by means of feedback inhibition of gonadotropin secretion. The low sperm count in workers intoxicated with the xenoestrogen chlordecone probably results from such a mechanism.

**Dysregulation of Ongoing Cellular Activity** Ongoing control of specialized cells is exerted by signaling molecules acting on membrane receptors that transduce the signal by regulating  $\text{Ca}^{2+}$  entry into the cytoplasm or stimulating the enzymatic formation of intracellular second messengers. The  $\text{Ca}^{2+}$  or other second messengers ultimately alter the phosphorylation of functional proteins, changing their activity and, in turn, cellular functions almost instantly. Toxicants can adversely affect ongoing cellular activity by disrupting any step in signal coupling.

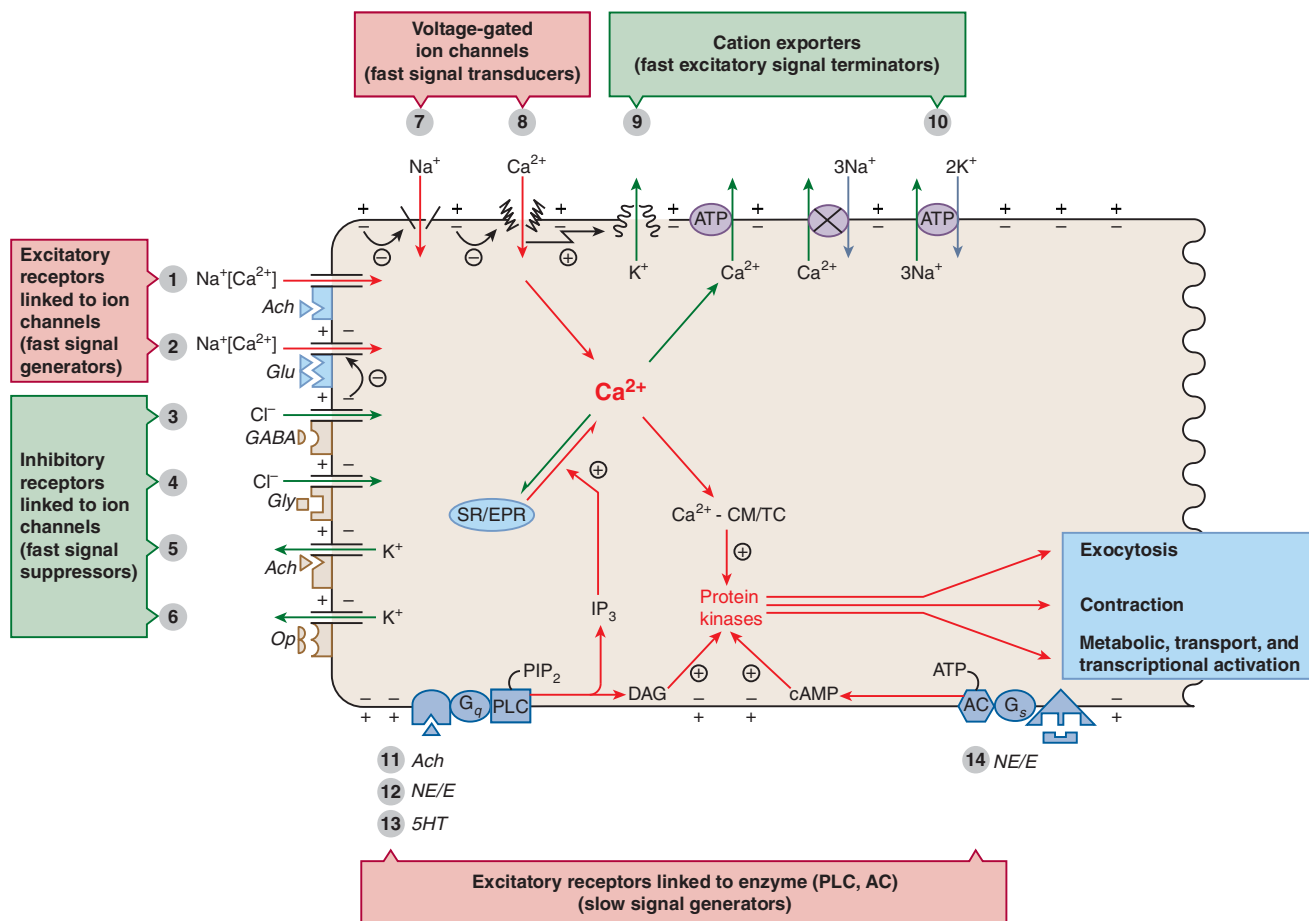
**Dysregulation of Electrically Excitable Cells** Many xenobiotics influence cellular activity in excitable cells, such as neurons, skeletal, cardiac, and smooth muscle cells. Cellular functions such as the release of neurotransmitters and muscle contraction are controlled by transmitters and modulators synthesized and released by adjacent neurons. The major mechanisms that control such cells are shown schematically in Fig. 3-15, and chemicals that interfere with these mechanisms are listed in Table 3-5.

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 toxins (Herken and Hucho, 1992). As neurons are signal-transducing cells, the influence of chemicals on neurons is seen not only on the neuron affected by the toxicant but also on downstream cells influenced by the primary target. Thus, tetrodotoxin, which blocks voltage-gated  $\text{Na}^+$  channels (item 7 in Fig. 3-15) in motor neurons, causes skeletal muscle paralysis. In contrast, cyclodiene insecticides, which block GABA receptors (item 3 in Fig. 3-15) in the central nervous system, induce neuronal excitation and convulsions (Narahashi, 1991).

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.

**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. The convulsive effect of hydrazine and hydrazides (eg, isoniazid) is due to their ability to decrease the synthesis of the inhibitory neurotransmitter GABA (Gale, 1992). Reserpine causes its several adverse effects by inhibiting the neuronal storage of norepinephrine, 5-hydroxytryptamine, and dopamine, thereby depleting these





**Figure 3-15. Signaling mechanisms for neurotransmitters.** This simplified scheme depicts major cellular signaling mechanisms that are operational in many neurons and muscle and exocrine cells. Chemicals acting on the numbered elements are listed in Table 3-5. Fast signaling is initiated by the opening of ligand-gated Na<sup>+</sup>/Ca<sup>2+</sup> channels (1, 2). The resultant cation influx decreases the inside negative potential (ie, evokes depolarization) and thus triggers the opening of the voltage-gated Na<sup>+</sup> and Ca<sup>2+</sup> channels (7, 8). As a second messenger, the influxed Ca<sup>2+</sup> activates intracellular Ca<sup>2+</sup>-binding proteins such as calmodulin (CM) and troponin C (TC), which, in turn, enhance the phosphorylation of specific proteins, causing activation of specific cellular functions. The signal is terminated by channels and transporters (eg, 9, 10) that remove cations from the cells and thus reestablish the inside negative resting potential (ie, cause repolarization) and restore the resting Ca<sup>2+</sup> level. Fast signaling can be suppressed by opening the ligand-activated Cl<sup>-</sup> or K<sup>+</sup> channels (3–6), which increases the inside negativity (ie, induces hyperpolarization) and thus counteracts opening of the voltage-gated Na<sup>+</sup> and Ca<sup>2+</sup> channels (7, 8). Signal transduction from other receptors (11–13) that are coupled to G<sub>q</sub> proteins involves generation of the second messenger inositol 1,4,5-trisphosphate (IP<sub>3</sub>) and diacylglycerol (DAG) by phospholipase C (PLC), whereas signaling from receptor 14 that is coupled to G<sub>s</sub> protein involves production of cyclic AMP (cAMP) by adenylyl cyclase (AC). These second messengers in turn influence cellular activities by mobilizing Ca<sup>2+</sup> from the sarcoplasmic or endoplasmic reticulum (SR and EPR), as IP<sub>3</sub> does, or by activating protein kinases, as cAMP and DAG do, which activate PKA and PKC, respectively. For clarity, this figure does not depict that inhibitory receptors 5 and 6 are G<sub>i</sub> protein-coupled and that besides opening K<sup>+</sup> channels, they also inhibit AC. Ach, acetylcholine; Glu, glutamate; GABA,  $\gamma$ -aminobutyric acid; Gly, glycine; Op, opioid peptides; NE, norepinephrine; E, epinephrine; 5HT, 5-hydroxytryptamine; G, G protein; PIP<sub>2</sub>, phosphatidylinositol 4,5-bisphosphate. Encircled positive and negative signs indicate activation and inhibition, respectively.

transmitters. **Skeletal muscle paralysis caused by botulinum toxin is due to inhibition of acetylcholine release from motor neurons and the lacking stimulation of the acetylcholine receptors at the neuromuscular junction (receptor 1 in Fig. 3-15).** In contrast, **inhibition of acetylcholinesterase by organophosphate or carbamate insecticides or chemical warfare agents (eg, soman) prevents the hydrolysis of acetylcholine, resulting in massive stimulation of cholinergic receptors (receptors 1, 5, and 11 in Fig. 3-15) and a cholinergic crisis (Table 3-5).** Inhibition of the neuronal reuptake of norepinephrine by **cocaine or tricyclic antidepressants** is responsible for overexcitation of  $\alpha_1$ -adrenergic receptors on vascular smooth muscles, resulting in nasal mucosal ulceration and myocardial infarction in heavy cocaine abusers, whereas overstimulation of  $\beta_1$ -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 (Hardman *et al.*, 1995). Concomitant use of drugs that increase the level of serotonin (5-HT) by enhancing its neuronal release and decreasing its neuronal reuptake (eg, **fluoxetine**) or its biotransformation (eg, monoamine oxidase inhibitors) **induce the serotonin syndrome** with cognitive and behavioral changes, autonomic dysfunction, and neuromuscular abnormalities. It is thought that cytotoxic antineoplastic drugs (eg, cisplatin) and radiation cause nausea and emesis, a disturbing reaction to chemotherapy and radiotherapy, by inducing release of 5-HT from enterochromaffin cells of the intestinal mucosa, which stimulates the 5-HT<sub>3</sub> receptors (5-HT-gated cation channel, functionally similar to item 1 in Fig. 3-15) on the adjacent vagal afferent neurons, thereby evoking the vomiting reflex (Endo *et al.*, 2000). Intestinal release of 5-HT and stimulation of vagal afferent neurons are also involved in the

Table 3-5

### Agents Acting on Signaling Systems for Neurotransmitters and Causing Dysregulation of the Momentary Activity of Electrically Excitable Cells such as Neurons and Muscle Cells\*

RECEPTOR/CHANNEL/PUMP		AGONIST/ACTIVATOR		ANTAGONIST/INHIBITOR	
NAME	LOCATION	AGENT	EFFECT	AGENT	EFFECT
1. Acetyl-choline nicotinic receptor	Skeletal muscle	Nicotine Anatoxin-a Cytisine <i>Ind:</i> ChE inhibitors	Muscle fibrillation, and then paralysis	Tubocurarine, lophotoxin $\alpha$ -Bungarotoxin $\alpha$ -Cobrotoxin $\alpha$ -Conotoxin Erabutoxin b <i>Ind:</i> botulinum toxin	Muscle paralysis
	Neurons	See above	Neuronal activation	Pb <sup>2+</sup> , general anesthetics	Neuronal inhibition
2. Glutamate receptor	CNS neurons	<i>N</i> -Methyl-D-aspartate Kainate, domoate Quinolate Quisqualate <i>Ind:</i> hypoxia, HCN → glutamate release	Neuronal activation → convulsion, neuronal injury ("excitotoxicity")	Phencyclidine Ketamine General anesthetics	Neuronal inhibition → anesthesia Protection against "excitotoxicity"
3. GABA <sub>A</sub> 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 Pentylentetrazole Cyclodiene insecticides Lindane, TCAD <i>Ind:</i> isoniazid	Neuronal activation → tremor, convulsion
4. Glycine receptor	CNS neurons, motor neurons	Avermectins (?), General anesthetics	Inhibition of motor neurons → paralysis	Strychnine <i>Ind:</i> tetanus toxin	Disinhibition of motor neurons → tetanic convulsion
5. Acetylcholine M <sub>2</sub> muscarinic receptor	Cardiac muscle	<i>Ind:</i> ChE inhibitors	Decreased heart rate and contractility	Belladonna alkaloids (eg, atropine), atropine-like drugs (eg, TCAD)	Increased heart rate
6. Opioid receptor	CNS neurons, visceral neurons	Morphine and congeners (eg, heroin, meperidine) <i>Ind:</i> clonidine	Neuronal inhibition → analgesia, central respiratory depression, constipation, urine retention	Naloxone	Antidotal effects in opiate intoxication
7. Voltage-gated Na <sup>+</sup> channel	Neurons, muscle cells, etc	Aconitine, veratridine Grayanotoxin Batrachotoxin Scorpion toxins Ciguatoxin DDT, pyrethroids	Neuronal activation → convulsion	Tetrodotoxin, saxitoxin $\mu$ -Conotoxin Local anesthetics Phenytoin Quinidine	Neuronal inhibition → paralysis, anesthesia Anticonvulsive action
8. Voltage-gated Ca <sup>2+</sup> channel	Neurons, muscle cell, etc	Maitotoxin (?) Atrotoxin (?) Latrotoxin (?)	Neuronal/muscular activation, cell injury	$\omega$ -Conotoxin Pb <sup>2+</sup>	Neuronal inhibition → paralysis
9. Voltage/Ca <sup>2+</sup> -activated K <sup>+</sup> channel	Neurons, smooth and skeletal muscle, cardiac muscle	Pb <sup>2+</sup>	Neuronal/muscular inhibition	Ba <sup>2+</sup> , apamin (bee venom), dendrotoxin, 20-HETE, hERG inhibitors (eg, cisapride, terfenadine)	Neuronal/muscular activation → convulsion/spasm vasoconstriction, PMV tachycardia (torsade de pointes)

(continued)

Table 3-5

(Continued)

RECEPTOR/CHANNEL/PUMP		AGONIST/ACTIVATOR		ANTAGONIST/INHIBITOR	
NAME	LOCATION	AGENT	EFFECT	AGENT	EFFECT
10. Na <sup>+</sup> ,K <sup>+</sup> -ATPase	Universal			Digitalis glycosides Oleandrin Chlordecone	Increased cardiac contractility, excitability Increased neuronal excitability → tremor
11. Acetylcholine M <sub>3</sub> muscarinic receptor	Smooth muscle, glands	<i>Ind</i> : ChE inhibitors	Smooth muscle spasm, salivation, lacrimation	Belladonna alkaloids (eg, atropine), atropine-like drugs (eg, TCAD)	Smooth muscle relaxation → intestinal paralysis, decreased salivation, decreased perspiration
Acetylcholine M <sub>1</sub> muscarinic receptor	CNS neurons	Oxotremorine <i>Ind</i> : ChE inhibitors	Neuronal activation → convulsion	See above	
12. Adrenergic α <sub>1</sub> receptor	Vascular smooth muscle	(Nor)epinephrine <i>Ind</i> : cocaine, tyramine, amphetamine, TCAD	Vasoconstriction → ischemia, hypertension	Prazosin	Antidotal effects in intoxication with α <sub>1</sub> -receptor agonists
13. 5-HT <sub>2</sub> receptor	Smooth muscle	Ergot alkaloids (ergotamine, ergonovine)	Vasoconstriction → ischemia, hypertension	Ketanserine	Antidotal effects in ergot intoxication
14. Adrenergic β <sub>1</sub> receptor	Cardiac muscle	(Nor)epinephrine <i>Ind</i> : cocaine, tyramine, amphetamine, TCAD	Increased cardiac contractility and excitability	Atenolol, metoprolol	Antidotal effects in intoxication with β <sub>1</sub> -receptor agonists

CNS, central nervous system; ChE, cholinesterase; *Ind*, indirectly acting (ie, by altering neurotransmitter level); 20-HETE, 20-hydroxy-5,8,11,14-eicosatetraenoic acid; PMV, polymorphic ventricular; TCAD, tricyclic antidepressant.

\*Numbering of the signaling elements in this table corresponds to the numbering of their symbols in Fig. 3-12. This tabulation is simplified and incomplete. Virtually all receptors and channels listed occur in multiple forms with different sensitivity to the agents. The reader should consult the pertinent literature for more detailed information.

emetic effect of ipecac syrup and its alkaloids (cephaelin and emetine). The α<sub>2</sub>-adrenergic receptor agonist clonidine induces release in the brain of β-endorphin, an endogenous peptide that stimulates opioid receptors (item 6 in Fig. 3-15). 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<sub>A</sub> receptor (item 3 in Fig. 3-15), whereas barbiturates, benzodiazepines, general anesthetics, and alcohols are activators (Narahashi, 1991). Thus, all these chemicals cause inhibition of central nervous system activity, resulting in sedation, general anesthesia, coma, and ultimately blockade of 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. Thus, glutamate receptor agonists and muscarinic receptor agonists cause neuronal hyperactivity in the brain and ultimately convulsions, as do inhibitors of GABA<sub>A</sub> receptor. It is also apparent that chemicals acting as agonists/activators on inhibitory receptors and those acting as antagonists/inhibitors on excitatory receptors may exert similar effects. Moreover, general anesthetic solvents induce general anesthesia not only by activating the inhibitory ligand-gated chloride-ion channels (ie, GABA<sub>A</sub> and glycine receptors; see items 3 and 4, respectively, in Fig. 3-15) but also by inhibiting the excitatory ligand-gated cation channels (ie, neuronal nicotinic acetylcholine receptor and glutamate receptors; see items 1 and 2, respectively, in Fig. 3-15) (Franks and Lieb, 1998; Perouansky *et al.*, 1998). Because there are multiple types of receptors for each neurotransmitter, these 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 (Oortgiesen *et al.*, 1993). Other chemicals that produce neurotransmitter receptor-mediated toxicity are listed in Table 3-5.

Some sensory neurons have membrane receptors that are stimulated by noxious chemicals and operate as ligand-gated cation channels. Such are the transient receptor potential (TRP) channels, whose opening cause influx of  $\text{Na}^+$  and  $\text{Ca}^{2+}$  that depolarize and activate the neuron. The TRPV1 receptor is excited by capsaicin, the pungent ingredient of red peppers, and mediates the burning sensation of the tongue and reflex stimulation of the lacrimal gland associated with exposure to pepper spray (also called OC tear gas). The TRPA1 receptor, however, is activated by thiol-reactive and oxidant chemicals, such as the lacrimator compounds in tear gas (eg, chloroacetophenone, chlorobenzalmalonitrile, and dibenzoxazepine), acrolein, methyl isocyanate, corrosive gases (eg, phosgene, chloropicrin, and chlorine) and other irritants. These chemicals evoke sensations (irritation and pain), secretory and respiratory reflexes (lacrimation, bronchial secretion, sneezing, coughing, and bronchospasm), and neurogenic inflammation through the TRPA1 receptor (Bessac and Jordt, 2010).

**Toxicant–Signal Transducer Interactions** Many chemicals alter neuronal and/or muscle activity by acting on signal transduction processes. **Voltage-gated  $\text{Na}^+$  channels** (item 7 in Fig. 3-15), which transduce and amplify excitatory signals generated by ligand-gated cation channels (receptors 1 and 2 in Fig. 3-15), are activated by a number of toxins derived from plants and animals (Table 3-5) as well as by synthetic chemicals such as DDT, resulting in overexcitation (Narahashi, 1992). **In contrast, chemicals that block voltage-gated  $\text{Na}^+$  channels (such as tetrodotoxin and saxitoxin) cause paralysis.** The  $\text{Na}^+$  channels are also important in signal transduction in sensory neurons; therefore,  $\text{Na}^+$ -channel activators evoke sensations and reflexes, whereas  $\text{Na}^+$ -channel inhibitors induce anesthesia. This explains the reflex bradycardia and burning sensation in the mouth that follow the ingestion of monkshood, which contains the  $\text{Na}^+$ -channel activator aconitine, as well as **the use of  $\text{Na}^+$ -channel inhibitors such as procaine and lidocaine for local anesthesia.**

**Toxicant–Signal Terminator Interactions** The cellular signal generated by cation influx is terminated by removal of the cations through channels or by transporters (Fig. 3-15). Inhibition of cation efflux may prolong excitation, as occurs with the blockade of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels (item 9 in Fig. 3-15) by  $\text{Ba}^{2+}$ , which is accompanied by potentially lethal neuroexcitatory and spasmogenic effects. The arachidonic acid metabolite 20-hydroxy-5,8,11,14-eicosatetraenoic acid (20-HETE) also blocks  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels, causing vasoconstriction. Induction by cyclosporine of 20-HETE production in renal proximal tubular cells and the resultant decrease in renal cortical blood flow may underlie the nephrotoxic effect of cyclosporine (Seki *et al.*, 2005). **Blockade of specific voltage-gated  $\text{K}^+$  channels (called hERG channels; item 9 in Fig. 3-15) in the heart delays cardiac repolarization (as indicated by prolonged QT time in ECG) and may evoke polymorphic ventricular tachycardia (termed torsade de pointes), which may cause sudden death if degenerates into ventricular fibrillation (Sanguinetti and Mitcheson, 2005).** Several drugs with such an effect (eg, astemizole, cisapride, grepafloxacin, terfenadine) have been withdrawn from clinical use. Glycosides from digitalis and other plants inhibit  $\text{Na}^+, \text{K}^+$ -ATPase (item 10 in Fig. 3-15) and thus increase the intracellular  $\text{Na}^+$  concentration, which, in turn, decreases  $\text{Ca}^{2+}$  export by  $\text{Ca}^{2+}/\text{Na}^+$  exchange (Fig. 3-15). The resultant rise in the intracellular concentration of  $\text{Ca}^{2+}$  enhances the contractility and excitability of cardiac muscle. Inhibition of brain  $\text{Na}^+, \text{K}^+$ -ATPase by chlordecone may be responsible for the tremor observed in chlordecone-exposed workers (Desai, 1982). Lithium salts, although used therapeutically, have the potential to produce hyperreflexia, tremor, convulsions, diarrhea, and cardiac arrhythmias (Hardman *et al.*, 1995).

Lithium also markedly potentiates cholinergic-mediated seizures. Besides inhibition of  $\text{Li}^+$ -sensitive enzymes (eg, inositol monophosphatase, bisphosphate 3'-nucleotidase, and GSK), another possible reason for these toxic effects is inefficient repolarization of neurons and muscle cells in the presence of  $\text{Li}^+$ . Whereas  $\text{Li}^+$  readily enters these cells through  $\text{Na}^+$  channels, contributing to the signal-induced depolarization, it is not a substrate for the  $\text{Na}^+, \text{K}^+$  pump. Therefore, the cells fail to repolarize properly if a fraction of intracellular  $\text{Na}^+$  is replaced by  $\text{Li}^+$ .

Failure of the  $\text{Na}^+, \text{K}^+$  pump is also believed to contribute to the neuronal damage resulting from hypoxia, hypoglycemia, and cyanide intoxication. Inasmuch as **70% of the ATP produced in neurons is used to drive the  $\text{Na}^+, \text{K}^+$  pump, cessation of ATP synthesis causes a cell to become or remain depolarized.** The depolarization-induced release of neurotransmitters such as glutamate from such neurons is thought to be responsible for the hypoxic seizures and further amplification of neuronal injury by the neurotoxic actions of glutamate (Patel *et al.*, 1993).

**Dysregulation of the Activity of Other Cells** While many signaling mechanisms also operate in **nonexcitable cells**, disturbance of these processes is usually less consequential. For example, rat liver cells possess  $\alpha_1$ -adrenergic receptors (item 12 in Fig. 3-15) whose activation evokes metabolic changes, such as increased glycogenolysis and glutathione export, through elevation of intracellular  $\text{Ca}^{2+}$ , which may have toxicological significance.

Many exocrine secretory cells are controlled by muscarinic acetylcholine receptors (item 11 in Fig. 3-15). **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.** Kupffer cells, resident macrophages in the liver, secrete inflammatory mediators (see Fig. 3-26) that can harm neighboring cells. Because Kupffer cells possess glycine receptors, that is, glycine-gated  $\text{Cl}^-$  channels (item 4 in Fig. 3-15), the secretory function of these macrophages (eg, secretion of inflammatory mediators) can be blocked by administration of glycine, which induces hyperpolarization via influx of  $\text{Cl}^-$ . Such intervention alleviates ethanol-induced liver injury (Yin *et al.*, 1998).

**The discovery that some sulfonamides produce hypoglycemia in experimental animals led to the development of oral hypoglycemic agents for diabetic patients. These drugs inhibit  $\text{K}^+$  channels in pancreatic  $\beta$  cells, inducing sequential depolarization,  $\text{Ca}^{2+}$  influx through voltage-gated  $\text{Ca}^{2+}$  channels, and exocytosis of insulin (Hardman *et al.*, 1995). The antihypertensive diazoxide acts in the opposite fashion on  $\text{K}^+$  channels and impairs insulin secretion. Whereas this effect is generally undesirable, it is exploited in the treatment of inoperable insulin-secreting pancreatic tumors.**

## Toxic Alteration of Cellular Maintenance

Numerous toxicants interfere with cellular maintenance functions. In a multicellular organism, cells must maintain their own structural and functional integrity as well as provide supportive functions for other cells. Execution of these functions may be disrupted by chemicals, resulting in a toxic response.

**Impairment of Internal Cellular Maintenance: Mechanisms of Toxic Cell Death** For survival, all cells must synthesize endogenous molecules; assemble macromolecular complexes, membranes, and cell organelles; maintain the intracellular environment; and produce energy for operation. Chemicals that disrupt these functions, especially the energy-producing function of mitochondria and protein synthesis controlling function of the genome, jeopardize survival and may cause toxic cell death.



There are three critical biochemical disorders that chemicals inflicting cell death may initiate, namely, ATP depletion, sustained rise in intracellular  $\text{Ca}^{2+}$ , and overproduction of ROS and RNS. In the following discussion, these events and the chemicals that may cause them are individually characterized. Then it is pointed out how their concerted action may induce a bioenergetic catastrophe, culminating in necrosis. Finally, there follows a discussion of the circumstances under which the cell can avoid this disordered decay and how it can execute death by activating catabolic processes that bring about an ordered disassembly and removal of the cell, called apoptosis.

### Primary Metabolic Disorders Jeopardizing Cell Survival: ATP Depletion, $\text{Ca}^{2+}$ Accumulation, ROS/RNS Generation

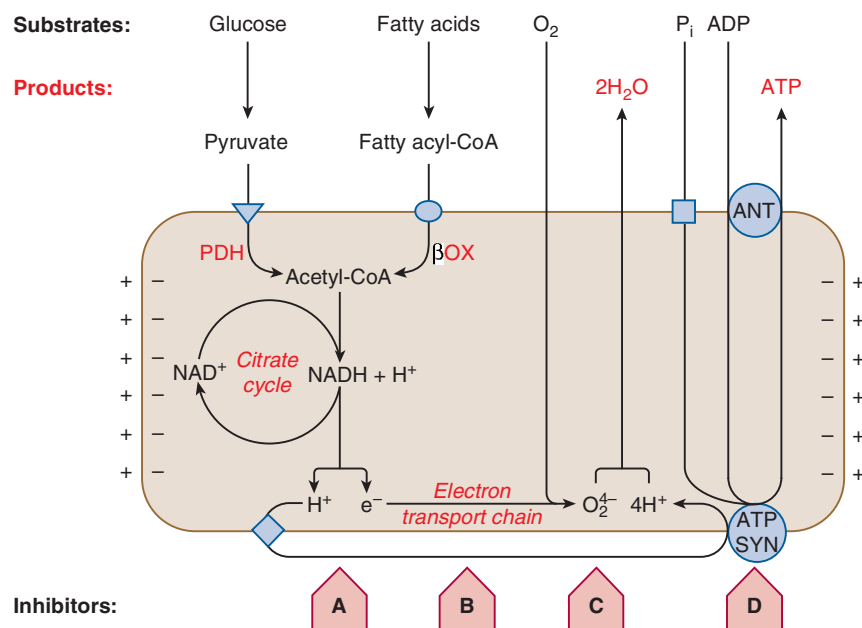
**Depletion of ATP** ATP plays a central role in cellular maintenance both as a chemical for biosynthesis and as the major source of energy. It is utilized in numerous biosynthetic reactions, activating endogenous compounds by phosphorylation and adenylation, and is incorporated into cofactors as well 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 ion transporters, such as the  $\text{Na}^+, \text{K}^+$ -ATPase in the plasma membrane, the  $\text{Ca}^{2+}$ -ATPase in the plasma and the ER membranes, and  $\text{H}^+$ -ATPase in the membrane of lysosomes and neurotransmitter-containing vesicles. These pumps maintain conditions essential for various cell functions. For example, the  $\text{Na}^+$  concentration gradient across the plasma membrane generated by the  $\text{Na}^+, \text{K}^+$  pump drives  $\text{Na}^+$ -glucose and  $\text{Na}^+$ -amino acid cotransporters as well as the  $\text{Na}^+/\text{Ca}^{2+}$  antiporter, facilitating the entry of these nutrients and the removal of  $\text{Ca}^{2+}$ .

Chemical energy is released by hydrolysis of ATP to ADP or AMP. The ADP is rephosphorylated in the mitochondria by ATP synthase (Fig. 3-16). Coupled to oxidation of hydrogen to water, this process is termed *oxidative phosphorylation*. 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  $\text{O}_2$ , 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 (Fig. 3-16).

Several chemicals impede these processes, interfering with mitochondrial ATP synthesis (Commandeur and Vermeulen, 1990; Wallace and Starkov, 2000; Wallace, 2008). These chemicals are divided into 5 groups (Table 3-6). Substances in class A interfere with the delivery of hydrogen to the electron transport chain. For example, fluoroacetate inhibits the citric acid cycle and the production of reduced cofactors. Class B chemicals such as rotenone and cyanide 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. All chemicals that cause hypoxia ultimately act at this site. 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, and thereby impairing synthesis of specific proteins encoded by the mitochondrial genome (eg, subunits of complex I and ATP synthase), are listed in group E. These include the dideoxynucleoside antiviral drugs used against AIDS, such as zidovudine. Table 3-6 lists other chemicals that impair ATP synthesis.

ATP synthase is also present at sites in addition to the mitochondria, such as 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. Unlike its mitochondrial counterpart, the ATP synthase in plasma and myelin



**Figure 3-16. ATP synthesis (oxidative phosphorylation) in mitochondria.** Arrows with letters A to D point to the ultimate sites of action of 4 categories of agents that interfere with oxidative phosphorylation (Table 3-6). 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 3 sites.  $\beta$ OX, beta-oxidation of fatty acids;  $e^-$ , electron;  $\text{P}_i$ , inorganic phosphate; ANT, adenine nucleotide translocator; ATP SYN, ATP synthase ( $\text{F}_0\text{F}_1$ -ATPase).

Table 3-6

**Agents Impairing Mitochondrial ATP Synthesis\*****A. Inhibitors of hydrogen delivery to the electron transport chain acting on/as**

1. Glycolysis (critical in neurons): hypoglycemia; iodoacetate, koniginic acid, and NO<sup>+</sup> at GAPDH
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<sup>-</sup>
  - (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  $\alpha$ -KGDH): ethanol (when chronically consumed)
7. Depletors of 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. Depletors of NADH
  - (a) See group A.V.1. in Table 3-7
  - (b) Activators of poly(ADP-ribose) polymerase: agents causing DNA damage (eg, MNNG, hydrogen peroxide, ONOO<sup>-</sup>)

**B. Inhibitors of electron transport acting on/as**

1. Inhibitors of electron transport complexes
  - (a) NADH-coenzyme Q reductase (complex I): rotenone, amytal, MPP<sup>+</sup>, paraquat
  - (b) Coenzyme Q-cytochrome *c* reductase (complex III): antimycin-A, myxothiazole
  - (c) Cytochrome oxidase (complex IV): cyanide, hydrogen sulfide, azide, formate, \*NO, phosphine (PH<sub>3</sub>)
  - (d) Multisite inhibitors: dinitroaniline and diphenylether herbicides, ONOO<sup>-</sup>
2. Electron acceptors: CCl<sub>4</sub>, doxorubicin, menadione, MPP<sup>+</sup>

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

1. Chemicals causing respiratory paralysis: CNS depressants (eg, opioids), convulsants
2. Chemicals impairing pulmonary gas exchange: CO<sub>2</sub>, "deep pulmonary irritants" (eg, NO<sub>2</sub>, phosgene, perfluoroisobutene)
3. Chemicals inhibiting oxygenation of Hb: carbon monoxide, methemoglobin-forming chemicals
4. Chemicals causing ischemia: ergot alkaloids, cocaine

**D. Inhibitors of ADP phosphorylation acting on/as**

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)

**E. Chemicals causing mitochondrial DNA damage and/or impaired transcription of key mitochondrial proteins**

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

CCCP, carbonyl cyanide *m*-chlorophenylhydrazine; DCVC, dichlorovinyl-cysteine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase;  $\alpha$ -KGDH,  $\alpha$ -ketoglutarate dehydrogenase; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; MPP<sup>+</sup>, 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. 3-13.

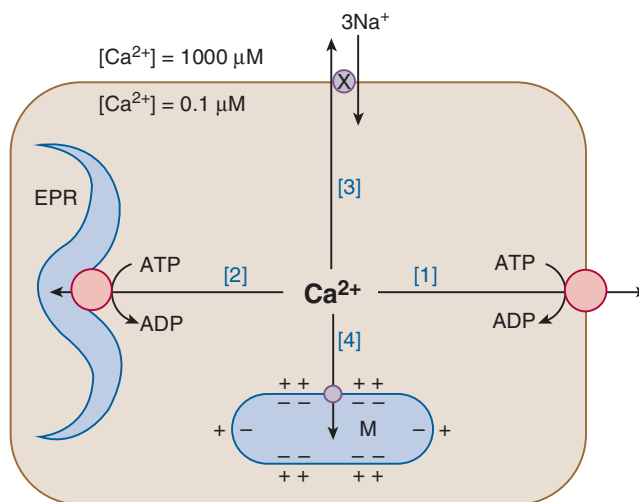
membranes forms ATP on the external membrane face and is called ecto-ATP synthase. It is hypothesized that ATP produced by the myelinic ATP synthase (supported by the colocalized respiratory chain complexes and O<sub>2</sub> well dissolved in the myelin lipids) is delivered into the axon through gap junctions that traverse the myelin membranes and the axolemma (Morelli *et al.*, 2011). Myelinic ATP supply to axons, in which mitochondria are sparse, may explain why demyelinated axons die and why the white matter (composed of myelinated axons) is sensitive to hypoxia. Oxidative phosphorylation of ADP in isolated myelin vesicles (which are devoid of mitochondria) is sensitive to chemicals listed in Table 3-6 as mitochondrial

electron transport chain inhibitors (eg, antimycin and cyanide), ATP synthase inhibitors (eg, oligomycin), and uncouplers (eg, CCCP and valinomycin). If the trophic role of myelin in axonal maintenance under in vivo conditions becomes validated, it will be necessary to evaluate whether the ATP-synthesizing machinery of myelin is a relevant target for known neurotoxicants, such as lead, methyl mercury, hexachlorophene, and the methanol-derived formic acid.

Impairment of oxidative phosphorylation is detrimental to cells because failure of ADP rephosphorylation results in the accumulation of ADP and its breakdown products as well as depletion of ATP. Accordingly, hepatocytes exposed to KCN and iodoacetate

exhibit a rapid rise in cytosolic  $H^+$  and  $Mg^{2+}$  as a result of the hydrolysis of adenosine diphosphates and triphosphates (existing as  $Mg$  salts) and the release of phosphoric acid and  $Mg^{2+}$  (Herman *et al.*, 1990). The increased conversion of pyruvate to lactate also may contribute to the acidosis. The lack of ATP compromises the operation of ATP-requiring ion pumps, leading to the loss of ionic and volume-regulatory controls (Buja *et al.*, 1993). Shortly after intracellular acidosis and hypermagnesemia, liver cells exposed to KCN and iodoacetate exhibit a rise in intracellular  $Na^+$ , probably as a result of the failing  $Na^+$  pump, after which plasma membrane blebs appear. The intracellular phosphoric acidosis is beneficial for the cells presumably because the released phosphoric acid forms insoluble calcium phosphate, preventing the rise of cytosolic  $Ca^{2+}$ , with its deleterious consequences (see below). In addition, a low pH also directly decreases the activity of phospholipases and inhibits mitochondrial permeability transition (MPT; see later). Terminally, the intracellular pH rises, increasing phospholipase activity, and this contributes to irreversible membrane damage (ie, rupture of the blebs) not only by degrading phospholipids but also by generating endogenous detergents such as lysophospholipids and free fatty acids. The lack of ATP aggravates this condition because the reacylation of lysophospholipids with fatty acids is impaired.

**Sustained Rise of Intracellular  $Ca^{2+}$**  Intracellular  $Ca^{2+}$  levels are highly regulated (Fig. 3-17). The 10,000-fold difference between extracellular and cytosolic  $Ca^{2+}$  concentration is maintained by the impermeability of the plasma membrane to  $Ca^{2+}$  and by transport mechanisms that remove  $Ca^{2+}$  from the cytoplasm (Richter and Kass, 1991).  $Ca^{2+}$  is actively pumped from the cytosol across the plasma membrane and is sequestered in the ER and mitochondria (Fig. 3-17). Because mitochondria are equipped with a low-affinity



**Figure 3-17.** Four mechanisms for the elimination of  $Ca^{2+}$  from the cytoplasm:  $Ca^{2+}$ -ATPase-mediated pumping into (1) the extracellular space as well as (2) the endoplasmic reticulum (EPR) and ion-gradient-driven transport into (3) the extracellular space (by the  $Ca^{2+}/Na^+$  exchanger) as well as (4) the mitochondria (M; by the  $Ca^{2+}$  uniporter). Some chemicals that inhibit these mechanisms are listed in Table 3-7, group B.

$Ca^{2+}$  transporter, they play a significant role in  $Ca^{2+}$  sequestration only when the cytoplasmic levels rise into the micromolar range. Under such conditions, a large amount of  $Ca^{2+}$  accumulates in the mitochondria, where it is deposited as calcium phosphate.

Toxicants induce elevation of cytoplasmic  $Ca^{2+}$  levels by promoting  $Ca^{2+}$  influx into or inhibiting  $Ca^{2+}$  efflux from the cytoplasm (Table 3-7). Opening of the ligand- or voltage-gated  $Ca^{2+}$  channels

**Table 3-7**

### 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, resiniferatoxin
  3. TRPA1 receptor agonists: SH-reactive electrophiles, such as lacrimators (eg, chlorobenzalmalonitrile), acrolein, methyl isocyanate, phosgene, chloropicrin
- II. Via voltage-gated channels: maitotoxin (?),  $HO^+$
- 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:  $\gamma$ -HCH (lindane),  $IP_3$  formed during "excitotoxicity"
  2. Ryanodine receptor activators:  $\delta$ -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,  $O_2^-$ , and  $HOOH$  generators (eg, menadione, diquat)
- III. Others: vanadate,  $Cd^{2+}$ , thapsigargin (specific SERCA inhibitor)
- IV. Chemicals impairing mitochondrial ATP synthesis (see Table 3-6)

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

or damage to the plasma membrane causes  $\text{Ca}^{2+}$  to move down its concentration gradient from extracellular fluid to the cytoplasm. Toxicants also may increase cytosolic  $\text{Ca}^{2+}$  by inducing its leakage from the mitochondria or the EPR. They also may diminish  $\text{Ca}^{2+}$  efflux through inhibition of  $\text{Ca}^{2+}$  transporters or depletion of their driving forces. Several chemicals that can cause a sustained rise in cytoplasmic  $\text{Ca}^{2+}$  levels are listed in Table 3-7.

Sustained elevation of intracellular  $\text{Ca}^{2+}$  is harmful because it can result in (1) depletion of energy reserves, (2) dysfunction of microfilaments, (3) activation of hydrolytic enzymes, and (4) generation of ROS and RNS. There are at least 3 mechanisms by which sustained elevations in intracellular  $\text{Ca}^{2+}$  unfavorably influence the cellular energy balance. First, high cytoplasmic  $\text{Ca}^{2+}$  concentrations cause increased mitochondrial  $\text{Ca}^{2+}$  uptake by the  $\text{Ca}^{2+}$  “uniporter,” which, like ATP synthase, utilizes the inside negative mitochondrial membrane potential ( $\Delta\Psi_m$ ) as the driving force. Consequently, mitochondrial  $\text{Ca}^{2+}$  uptake dissipates  $\Delta\Psi_m$  and inhibits the synthesis of ATP. Moreover, chemicals that oxidize mitochondrial NADH activate a transporter that extrudes  $\text{Ca}^{2+}$  from the matrix space (Richter and Kass, 1991). The ensuing continuous  $\text{Ca}^{2+}$  uptake and export (“ $\text{Ca}^{2+}$  cycling”) by the mitochondria further compromise oxidative phosphorylation. Second,  $\text{Ca}^{2+}$  may also impair ATP synthesis by causing oxidative injury to the inner membrane by mechanisms described later. Third, a sustained rise in cytoplasmic  $\text{Ca}^{2+}$  not only impairs ATP synthesis but also increases ATP consumption by the  $\text{Ca}^{2+}$ -ATPases working to eliminate the excess  $\text{Ca}^{2+}$ .

A second mechanism by which an uncontrolled rise in cytoplasmic  $\text{Ca}^{2+}$  causes cell injury is microfilament dissociation (Nicotera *et al.*, 1992; Leist and Nicotera, 1997). 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  $\text{Ca}^{2+}$  causes dissociation of actin filaments from  $\alpha$ -actinin and fodrin, proteins involved in anchoring the filaments to the plasma membrane. This represents a mechanism leading to plasma membrane blebbing, a condition that predisposes the membrane to rupture.

A third event whereby high  $\text{Ca}^{2+}$  concentrations are deleterious to cells is activation of hydrolytic enzymes that degrade proteins, phospholipids, and nucleic acids (Nicotera *et al.*, 1992; Leist and Nicotera, 1997). Many integral membrane proteins are targets for  $\text{Ca}^{2+}$ -activated neutral proteases, or calpains (Liu *et al.*, 2004). Calpain-mediated hydrolysis of actin-binding proteins may also cause membrane blebbing. Indiscriminate activation of phospholipases by  $\text{Ca}^{2+}$  causes membrane breakdown directly and by the generation of detergents. Activation of a  $\text{Ca}^{2+}$ - $\text{Mg}^{2+}$ -dependent endonuclease causes fragmentation of chromatin. Elevated levels of  $\text{Ca}^{2+}$  can lock topoisomerase II in a form that cleaves but does not religate DNA. In summary, intracellular hypercalcemia activates several processes that interfere with the ability of cells to maintain their structural and functional integrity. The relative importance of these processes in vivo requires further definition.

**Overproduction of ROS and RNS** There are a number of xenobiotics that can directly generate ROS and RNS, such as the redox cyclers (Fig. 3-3) and the transition metals (Fig. 3-4). In addition, overproduction of ROS and RNS can be secondary to the intracellular hypercalcemia, as  $\text{Ca}^{2+}$  activates enzymes that generate ROS and/or RNS in the following ways:

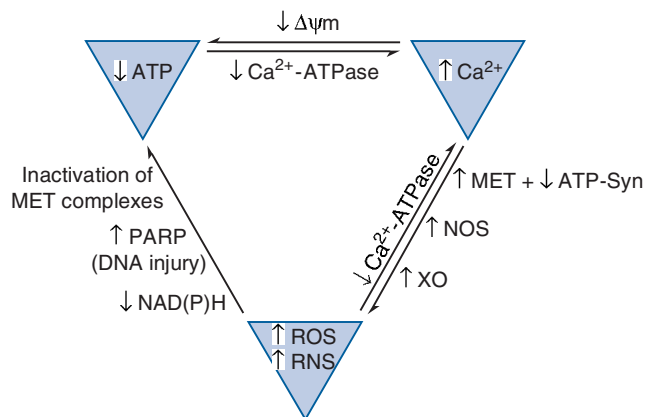
1. Activation of the dehydrogenases in the citric acid cycle by  $\text{Ca}^{2+}$  accelerates the hydrogen output from the citrate cycle and, in turn, the flux of electrons along the electron transport chain (see Fig. 3-16). This, together with the suppressed

ATP-synthase activity (owing to the  $\text{Ca}^{2+}$ -induced uncoupling), increases the formation of  $\text{O}_2^{\bullet}$  by the mitochondrial electron transport chain.

2.  $\text{Ca}^{2+}$ -activated proteases proteolytically convert xanthine dehydrogenase into xanthine oxidase, whose by-products are  $\text{O}_2^{\bullet}$  and  $\text{HOOH}$  (Harrison, 2002).
3. Neurons and endothelial cells constitutively express NOS that is activated by  $\text{Ca}^{2+}$ . Given the extremely high reactivity of  $\cdot\text{NO}$  with  $\text{O}_2^{\bullet}$ , coproduction of these radicals will inevitably lead to formation of  $\text{ONOO}^-$ , a highly reactive oxidant (Murphy, 1999) (Fig. 3-4). Moreover,  $\text{ONOO}^-$  can increase its own formation by incapacitating the highly sensitive Mn-SOD, which would eliminate  $\text{O}_2^{\bullet}$ , a precursor of  $\text{ONOO}^-$ .

**Interplay between the Primary Metabolic Disorders Spells Cellular Disaster** The primary derailments in cellular biochemistry discussed above do not remain isolated but interact and amplify each other in a number of ways (Fig. 3-18):

1. Depletion of cellular ATP reserves deprives the endoplasmic and plasma membrane  $\text{Ca}^{2+}$  pumps of fuel, causing elevation of  $\text{Ca}^{2+}$  in the cytoplasm. With the influx of  $\text{Ca}^{2+}$  into the mitochondria,  $\Delta\Psi_m$  declines, hindering ATP synthase.
2. As stated above, intracellular hypercalcemia facilitates formation of ROS and RNS, which oxidatively inactivate the thiol-dependent  $\text{Ca}^{2+}$  pump, which, in turn, aggravates the hypercalcemia.
3. The ROS and RNS can also drain the ATP reserves.  $\cdot\text{NO}$  is a reversible inhibitor of cytochrome oxidase;  $\text{NO}^+$  (nitrosonium cation, a product of  $\cdot\text{NO}$ ) S-nitrosylates and thus inactivates glyceraldehyde 3-phosphate dehydrogenase, impairing glycolysis, whereas  $\text{ONOO}^-$  irreversibly inactivates respiratory chain complexes I, II, III, and aconitase (by reacting with their Fe-S center) (Murphy, 1999). Therefore,  $\cdot\text{NO}$  and  $\text{ONOO}^-$  inhibit cellular ATP synthesis.
4. Furthermore,  $\text{ONOO}^-$  can induce DNA SSB, which activates poly(ADP-ribose) polymerase (PARP) (Szabó, 1996). As part of the repair strategy, activated PARP transfers multiple ADP-ribose moieties from  $\text{NAD}^+$  to nuclear proteins and PARP itself (D'Amours *et al.*, 1999). Consumption of  $\text{NAD}^+$  severely compromises ATP synthesis (see Fig. 3-16), whereas



**Figure 3-18.** Interrelationship between the primary metabolic disorders (ATP depletion, intracellular hypercalcemia, and overproduction of ROS/RNS) that ultimately cause necrosis or apoptosis. See text for details. ATP-Syn, ATP synthase; MET, mitochondrial electron transport; NOS, nitric oxide synthase; PARP, poly(ADP-ribose) polymerase; ROS, reactive oxygen species; RNS, reactive nitrogen species; XO, xanthine oxidase;  $\Delta\Psi_m$ , mitochondrial membrane potential.



resynthesis of  $\text{NAD}^+$  consumes ATP. Hence, a major consequence of DNA damage by  $\text{ONOO}^-$  is a cellular energy deficit (Murphy, 1999).

The chain of events and their contribution to the worsening metabolic conditions are somewhat cell- and toxicant-specific. For example, cyanide toxicity in neurons is associated with depolarization and glutamate release (Patel *et al.*, 1993), followed by  $\text{Ca}^{2+}$  influx through voltage-gated as well as glutamate-gated channels (see items 8 and 2, respectively, in Fig. 3-15). As they express  $\text{Ca}^{2+}$ -activated NOS, neurons are also prone to generate “nitrosative stress,” which affects not only themselves but also perhaps more significantly the neighboring astrocytes (Szabó, 1996). In contrast, in cyanide- and iodoacetate-poisoned liver cells, the increase in cytoplasmic  $\text{Ca}^{2+}$  is not an early event (Herman *et al.*, 1990). A nitrosative stress critically contributes to propagation of acetaminophen-induced hepatocellular injury, in which the initiating event is covalent binding of *N*-acetyl-*p*-benzoquinoneimine (NAPBQI) to mitochondrial proteins (Jaeschke and Bajt, 2006). This purportedly causes a surge in mitochondrial ( $\text{O}_2^{\cdot -}$ ) generation, followed by in situ formation of  $\text{ONOO}^-$ . Owing to depletion of the protective glutathione by NAPBQI,  $\text{ONOO}^-$  readily nitrates mitochondrial proteins. Concomitantly, covalent binding incapacitates the plasma membrane  $\text{Ca}^{2+}$  pump and the resultant hypercalcemia further deteriorates mitochondrial function and ATP production. It appears that in this and many other cytotoxicities the interplay of ATP depletion, intracellular hypercalcemia, and overproduction of ROS and RNS, involving multiple vicious cycles (Fig. 3-18), can progressively aggravate the biochemical disorder until it becomes a disaster.

**Mitochondrial Permeability Transition and the Worst Outcome: Necrosis** Mitochondrial  $\text{Ca}^{2+}$  uptake, decreased  $\Delta\Psi_m$ , generation of ROS and RNS, depletion of ATP, and consequences of the primary metabolic disorders (eg, accumulation of inorganic phosphate, free fatty acids, and lysophosphatides) are all considered as causative factors of an abrupt increase in the mitochondrial inner-membrane permeability, termed MPT. This is thought to be caused by misfolded proteins from the inner and outer membranes, which aggregate and open a proteinaceous pore (“megachannel”) that spans both mitochondrial membranes (Kroemer *et al.*, 1998; Kim *et al.*, 2003; Rodriguez-Enriquez *et al.*, 2004). As this pore is permeable to solutes of size  $<1500$  Da, its opening permits free influx of protons into the matrix space, causing rapid and complete dissipation of  $\Delta\Psi_m$  and cessation of ATP synthesis as well as osmotic influx of water, resulting in mitochondrial swelling.  $\text{Ca}^{2+}$  that accumulates in the matrix space effluxes through the pore, flooding the cytoplasm. Such mitochondria not only are incapable of synthesizing ATP but also even waste the remaining resources because depolarization of the inner membrane forces the ATP synthase to operate in the reverse mode, as an ATPase, hydrolyzing ATP. Then even glycolysis may become compromised by the insufficient ATP supply to the ATP-requiring glycolytic enzymes (hexokinase, phosphofructokinase). A complete bioenergetic catastrophe ensues in the cell if the metabolic disorders evoked by the toxic chemical (such as ones listed in Tables 3-6 and 3-7) are so extensive that most or all mitochondria in the cell undergo MPT, causing depletion of cellular ATP (see Fig. 3-20). Degradative processes already outlined (eg, oxidative and hydrolytic degradation of macromolecules and membranes as well as disintegration of intracellular solute and volume homeostasis) will go to completion, causing a complete failure in maintenance of cellular structure and functions and culminating in cell lysis or necrosis.

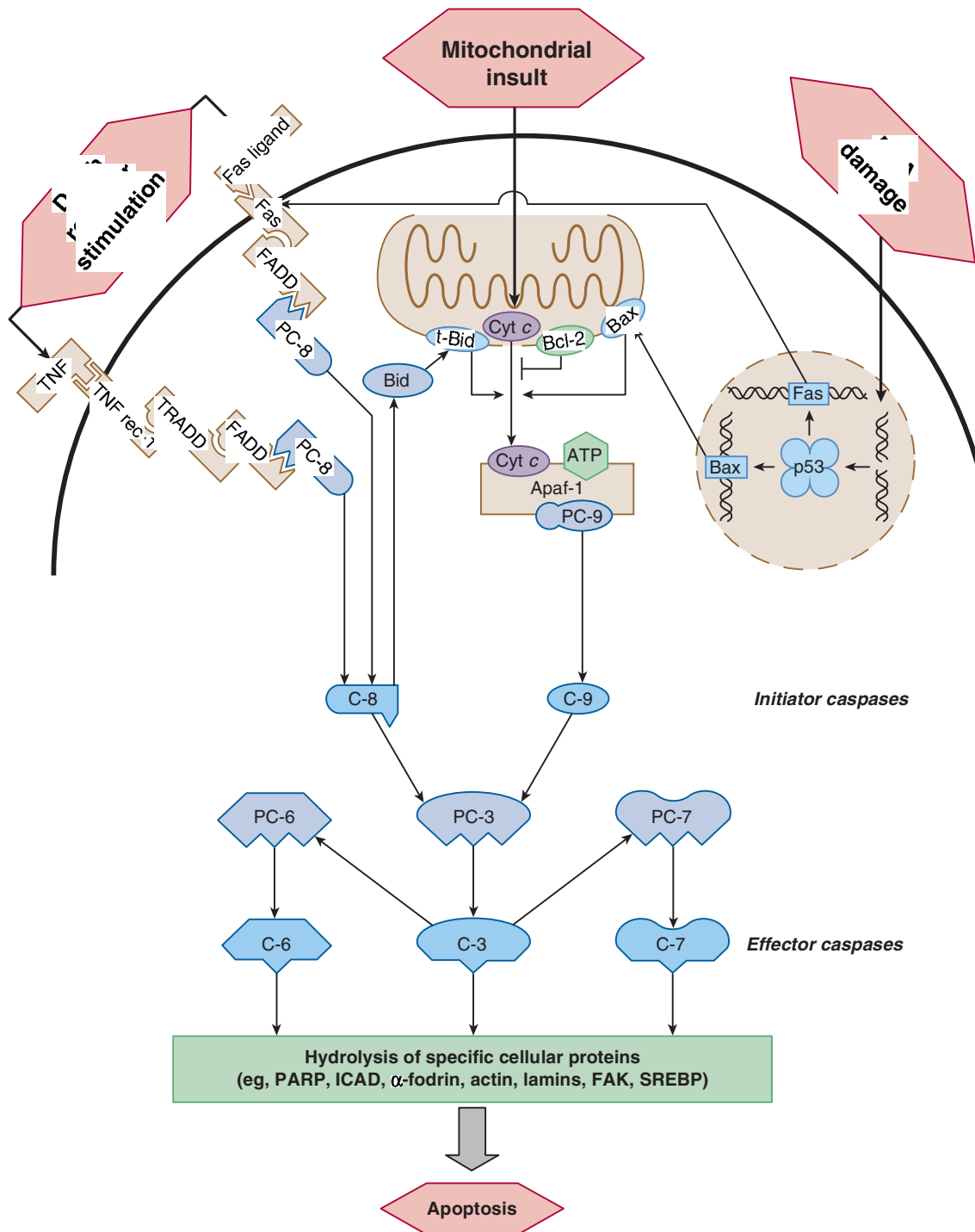
**An Alternative Outcome of MPT: Apoptosis** Chemicals that adversely affect cellular energy metabolism,  $\text{Ca}^{2+}$  homeostasis, and redox state and then ultimately cause necrosis may also induce apoptosis, another form of cell demise. Whereas the necrotic cell swells and lyses, the apoptotic cell shrinks; its nuclear and cytoplasmic materials condense, and then it breaks into membrane-bound fragments (apoptotic bodies) that are phagocytosed (Wyllie, 1997).

As discussed above, the multiple metabolic defects that a cell suffers in its way to necrosis are causal yet rather random in sequence. In contrast, the routes to apoptosis are ordered, involving cascade-like activation of catabolic processes that finally disassemble the cell. A scheme of the apoptotic pathways is presented in Fig. 3-19. Pathways initiated by EPR stress will be dealt with in the section “Mechanisms of Adaptation.”

It appears that most if not all chemical-induced cell deaths will involve the mitochondria, and the resulting mitochondrial dysfunction (such as  $\text{Ca}^{2+}$  accumulation, dissipation of  $\Delta\Psi_m$ , and overproduction of ROS/RNS) may ultimately trigger either necrosis or apoptosis, and that MPT can be a crucial event in both. A related event is release into the cytoplasm of cytochrome *c* (cyt *c*), a small positively charged heme protein that normally resides in the mitochondrial intermembrane space attached electrostatically to cardiolipin, a specific inner membrane phospholipid with excess negative charge. Peroxidation of cardiolipin by  $\text{HOOH}$ , a process catalyzed by cyt *c*, results in detachment of cyt *c* from the lipid, which may be a critical first step of cyt *c* release into the cytoplasm (Orrenius *et al.*, 2011).

The significance of cyt *c* release is two-fold (Cai *et al.*, 1998): (1) as cyt *c* is the penultimate link in the mitochondrial electron transport chain, its loss will block ATP synthesis, increase formation of  $\text{O}_2^{\cdot -}$  (instead of  $\text{O}_2^-$  as shown in Fig. 3-16), and potentially thrust the cell toward necrosis. (2) Simultaneously, the unleashed cyt *c* (and some other proteins set free from the mitochondria) represents a signal or an initial link in the chain of events directing the cell to the apoptotic path (Cain, 2003) (Fig. 3-19). Cyt *c* together with dATP/ATP induces the cytoplasmic adapter protein apoptotic protease activating factor (Apaf-1) to oligomerize and bind the latent procaspase-9 (forming a complex called apoptosome), facilitating its conversion to active caspase-9.

Caspases are cysteine proteases (ie, they possess a catalytically active cysteine) that cleave proteins after specific aspartate residues. They reside mostly in the cytoplasm in inactive forms, as procaspases, which are activated by either dimerization (initiators) or proteolytic cleavage (effectors) (Boatright and Salvesen, 2003). Perhaps in order to guarantee that inadvertent activation of caspases should not occur, IAPs, such as XIAP and cIAP, reside in the cytoplasm and physically capture caspases that might have become activated (Cain, 2003). Therefore, cyt *c* alone probably could not induce sufficiently strong caspase activation if it were not for proteins that physically remove the IAPs from the caspases just being activated. These proteins, named Smac and Omi (or Diablo and HtrA2, respectively), are not only the helpers of cyt *c* but also residents of the mitochondrial intermembrane space, from where they are jointly mobilized to promote the caspase cascade. Some caspases on the “top” of the cascade (eg, 8 and 9) cleave and activate procaspases. Thereby these initiator caspases carry the activation wave to the so-called effector caspases (eg, 3, 6, and 7), which cleave specific cellular proteins, activating or inactivating them. It is the hydrolysis of these specific proteins that accounts directly or indirectly for the morphological and biochemical alterations in apoptotic cells. For example, proteolytic inactivation of PARP prevents futile DNA repair and wasting of ATP; caspase-mediated cleavage of Beclin 1 aborts autophagy, hydrolysis of the inhibitor of



**Figure 3-19. Apoptotic pathways initiated by mitochondrial insult, nuclear DNA insult, and death receptor stimulation.** The figure is a simplified scheme of 3 pathways to apoptosis. (1) Mitochondrial insult (see text) ultimately opens the permeability transition pore spanning both mitochondrial membranes and/or causes release of cytochrome *c* (Cyt *c*) and other proapoptotic and antiapoptotic proteins (not shown) from the mitochondria. Cyt *c* release is facilitated by Bax or truncated Bid (tBid) proteins and opposed by Bcl-2 protein. (2) DNA insult, especially double-strand breaks, activates p53 protein that increases the expression of Bax (which mediates Cyt *c* release) and the membrane receptor protein Fas. (3) Fas ligand, tumor necrosis factor (TNF), or TNF-related apoptosis-inducing ligand (TRAIL, not shown) binds to and activates their respective receptor, Fas, TNF receptor-1 (TNFR1), and TRAIL receptors (not shown). The ligand-bound death receptors and the released Cyt *c* interact with specific adapter proteins (ie, FADD, TRADD, and Apaf-1) through which specific initiator procaspases (PC, eg, PC-8 and PC-9) become active caspases (C). The latter in turn cleave and activate other proteins, for example, Bid and the main effector procaspase PC-3. The active effector caspase-3 activates other effector procaspases (PC-6, PC-7). Finally, C-3, C-6, and C-7 cleave specific cellular proteins, leading to morphological and biochemical features of apoptosis. These pathways are not equally relevant in all types of cells and other apoptotic pathways, such as those triggered by TGF- $\beta$  as an extracellular signaling molecule (which upregulates the proapoptotic Bim protein) and ceramide as an intracellular signaling molecule, also exist. Note that through TNFR1 TNF may signal not only for apoptosis (via TNFR1–TRADD–FADD association, as shown above) but also for cell survival (via TNFR1–TRADD–TRAF2 association). The survival pathways thus triggered are the NF- $\kappa$ B and the MAPK (specifically Jun kinase) pathways (see Fig. 3-12). FAK, focal adhesion kinase; ICAD, inhibitor of caspase-activated DNase; PARP, poly(ADP-ribose) polymerase; SREBP, sterol regulatory element-binding protein.

caspase-activated DNase (ICAD) permits caspase-activated DNase (CAD) to translocate to the nucleus and cleave internucleosomal DNA; cleavage of structural proteins ( $\alpha$ -fodrin, actin, lamins) aids in disassembly of the cell; incapacitation of focal adhesion kinase (see Fig. 3-12) permits detachment of the cell from the extracellular matrix (ECM); and hydrolytic activation of sterol regulatory element-binding proteins may contribute to accumulation of sterols and externalization of phosphatidylserine in the plasma membrane that identify the apoptotic cell to phagocytes (and the latter also enables experimental detection of apoptotic cells with fluorescently labeled annexin-V that binds to phosphatidylserine residues on the cell surface). Besides caspases, DNA fragmentation can be induced by apoptosis-inducing factor (AIF) and endonuclease G that are also released from the mitochondrial intermembrane space and may contribute to DNA fragmentation during apoptosis and acetaminophen-induced caspase-independent hepatocellular necrosis (Jaeschke and Bajt, 2006). Prerequisite for the release of AIF is its hydrolysis by the  $\text{Ca}^{2+}$ -activated protease calpain localized to the intermembrane space. Oxidative modification of AIF by mitochondrial ROS increases its affinity to calpain (Orrenius *et al.*, 2011).

The decisive mitochondrial event of cell death, that is, release of cyt *c* and other proapoptotic proteins (eg, Smac, Omi, AIF), is controlled by the Bcl-2 family of proteins, which includes members that facilitate (eg, Bax, Bak, Bad, Bid, Bim, BNIP3, Puma, Noxa) and those that inhibit (eg, Bcl-2, Bcl-XL) these processes. The death-promoting members can oligomerize and form pores in the mitochondrial outer membrane (MOM). By doing so, they may facilitate release of cyt *c* and other intermembrane proapoptotic proteins via MPT induced by toxic insult of the mitochondria; however, mitochondrial outer membrane permeabilization (MOMP) alone by Bax and its congeners is sufficient to evoke egress of cyt *c* from the mitochondria. MOMP induced by Bax, Bak, and/or Bid is responsible for cyt *c* release in apoptosis initiated at extramitochondrial targets, including death receptors and the nuclear DNA (see below). The death suppressor Bcl-2 and Bcl-XL can dimerize with the death-inducing counterparts and neutralize them. Thus, the relative amount of these antagonistic proteins functions as a regulatory switch between cell survival and death (Reed *et al.*, 1998).

The proapoptotic Bax and Bid proteins, which can induce MOMP, represent links whereby death programs initiated extramitochondrially, for example, by DNA damage in the nucleus or by stimulation of the so-called death receptors (Fas receptor and TNF receptor-1 [TNFR1]) at the cell surface, can engage the mitochondria into the apoptotic process (Green, 1998) (Fig. 3-19). DNA damage evoked by ionizing and UV radiations, alkylating chemicals, and topoisomerase II inhibitors, such as doxorubicin (Adriamycin), induces stabilization and activation of p53 protein that can promote apoptosis at dual cellular locations and by dual mechanisms. In the nucleus, p53 acts as a TF, which increases expression of proapoptotic members of the Bcl-family of proteins, such as Bax, Puma, and Noxa (Liu and Chen, 2006) (see also Fig. 3-33). Cytoplasmic p53 participates in protein-protein interactions. In such a manner, p53 abrogates the function of antiapoptotic proteins (eg, Bcl-X<sub>L</sub>) and activates the MOMP-forming function of proapoptotic Bax and Bak (Green and Kroemer, 2009). As discussed further on, DNA damage is potentially mutagenic and carcinogenic; therefore, apoptosis of cells with damaged DNA is an important self-defense of the body against oncogenesis. Furthermore, the antitumor drugs targeting the nuclear DNA exert their desirable toxic effects against tumor cells (and also their undesirable cytotoxic effects against rapidly dividing normal cells such as hematopoietic cells and small intestinal mucosal cells) by inducing apoptosis primarily via a p53-dependent mechanism.

Stimulation of the Fas receptor (also known as CD95 or Apo-1), TNFR1, or TNF-related apoptosis-inducing ligand (TRAIL) receptors (DR4 and DR5, not shown) by their ligands induces receptor oligomerization and recruitment of adapter proteins. The thus formed death-inducing protein complex (DISC) activates caspase-8, an initiator caspase that sets the caspase cascade in motion (Fig. 3-19). In addition, death receptor activation can also engage the mitochondria into the apoptosis program via caspase-8-mediated cleavage of Bid to its active form. The Fas system is involved in cell-mediated cytotoxicity, as cytotoxic T lymphocytes express the Fas ligand that activates the Fas receptor in the membrane of potential target cells, such as those of the liver, heart, and lung. Cholestatic liver injury involves apoptosis induced by the retained hydrophobic bile acids mediated partly through their mitochondrial effect (ie, MPT) and through the death receptors (Higuchi and Gores, 2003). By activating PKC, toxic bile acids promote trafficking of Fas receptor and TRAIL receptor-2 (also known as DR5) from the Golgi complex into the plasma membrane of liver cells, where the increased receptor density induces spontaneous (ie, ligand-independent) receptor oligomerization and caspase activation. Increased expression of soluble FasL and Fas receptor is thought to play a causative role in the apoptosis of pulmonary alveolar epithelial cells underlying acute lung injury (or acute respiratory distress syndrome) (Martin *et al.*, 2005), a potentially lethal outcome of various pathologies and heroin intoxication. The Fas system also mediates germ cell apoptosis in the testes of rodents exposed to mono-(2-ethylhexyl)phthalate or 2,5-hexanedione, the ultimate toxicant formed from hexane. These chemicals damage the microtubules in the Sertoli cells that normally nurse the germ cells. Unable to support the germ cells, Sertoli cells overexpress the Fas ligand to limit the number of germ cells (which upregulate their Fas receptor) by deleting them via apoptosis (Cohen *et al.*, 1997; Lee *et al.*, 1997).

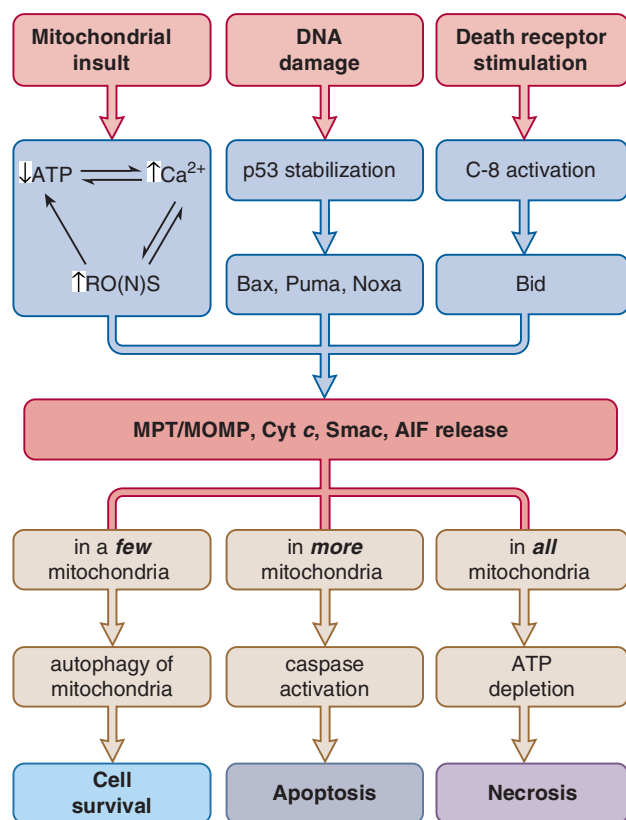
Thus, apoptosis can be executed via multiple pathways, most involving caspase activation. The route preferred will depend, among others, on the initial insult (Fig. 3-19) as well as on the type and state of the cell. For example, T lymphocytes lacking the Bax gene can still undergo p53-dependent death in response to ionizing radiation, probably by increasing Fas expression (Fig. 3-19), whereas Bax-null fibroblasts cannot. AIF appears to play a critical role in neuronal cell death (Orrenius *et al.*, 2011), whereas TRAIL is an important death inducer in various tumor cells. In contrast, keratinocytes and endothelial cells are resistant to death ligand-mediated apoptosis, as they strongly express cFLIP, an inhibitor of caspase-8.

**What Determines the Form of Cell Death?** For reasons discussed later, it is not inconsequential for the surrounding tissue whether a cell dies by apoptosis or necrosis. Therefore, considerable research has focused on what determines the form of cell death. Interestingly, there are some common features in the process of apoptosis and necrosis. Both forms of cell death caused by cytotoxic agents may involve similar metabolic disturbances and MPT (Kim *et al.*, 2003; Kroemer *et al.*, 1998; Qian *et al.*, 1999), and blockers of the latter (eg, cyclosporin A, Bcl-2 overexpression) prevent both apoptosis and necrosis in different settings. Furthermore, many xenobiotics—such as the hepatotoxicant 1,1-dichloroethylene, thioacetamide, and cadmium as well as the nephrotoxicant ochratoxin—can cause both apoptosis and necrosis (Corcoran *et al.*, 1994). However, toxicants tend to induce apoptosis at low exposure levels or early after exposure at high levels, whereas they cause necrosis later at high exposure levels. This indicates that the severity of the insult determines the mode of cell death. Based on

experimental evidence, it appears that a larger toxic insult causes necrotic cell death rather than apoptosis because it incapacitates the cell to undergo apoptosis. This incapacitation may result from three causatively related cellular events, that is, increasing number of mitochondria undergoing MPT, depletion of ATP, and failed activation of caspases or their inactivation.

Lemasters and coworkers (Kim *et al.*, 2003; Rodriguez-Enriquez *et al.*, 2004) used confocal microscopy to visualize mitochondria in cells exposed to an apoptogenic stimulus and found that MPT does not occur uniformly in all mitochondria. They proposed a model in which 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 more mitochondria suffer MPT, the autophagic mechanism becomes overwhelmed, and the released proapoptotic factors (eg, cyt *c*, Smac, and Omi) initiate caspase activation (Fig. 3-19). By hydrolyzing Beclin 1, a component of the autophagic mechanism, caspase-8 aborts mitophagy (Li *et al.*, 2011), and by hydrolyzing other cellular proteins (see above) and DNA, caspases and AIF, respectively, promote apoptosis. When eventually MPT involves virtually all mitochondria, cytolysis occurs. Fig. 3-20 illustrates an expanded version of this model.

For reasons discussed above, ATP is bound to become severely depleted in cells in which most mitochondria suffer MPT. The degree of ATP depletion may decide the fate of the cell. In experimental models as distinct as  $\text{Ca}^{2+}$ -exposed hepatocytes, Fas-stimulated T lymphocytes, and HOOH-exposed endothelial cells,



**Figure 3-20. "Decision plan" on the fate of injured cell.** See the text for details. MOMP, mitochondrial outer membrane permeabilization; MPT, mitochondrial permeability transition; Puma, p53-upregulated modulator of apoptosis; RO(N)S, reactive oxygen or nitrogen species.

necrosis occurs instead of apoptosis when cells are depleted of ATP, whereas apoptosis takes place rather than necrosis when ATP depletion is alleviated by providing substrates for ATP generation (Leist *et al.*, 1997; Kim *et al.*, 2003; Lelli *et al.*, 1998). 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 (Fig. 3-19).

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 (QACXG) with a reactive cysteine in it. At high concentrations, soft electrophiles, disulfides (eg, glutathione disulfide), and oxidants (eg, ROS), can react with this cysteine, causing caspase inactivation and ablation of the apoptotic program, with necrosis rather than apoptosis as the final outcome. Such a scenario has been demonstrated for cell death evoked by tributyltin, pyrrolidine dithiocarbamate, and arsenic trioxide (Orrenius, 2004) and might also underlie the acetaminophen-induced hepatocellular necrosis, which involves cyt *c* release with no caspase activation or apoptosis (Jaeschke and Bajt, 2006).

**Induction of Cell Death by Unknown Mechanisms** In addition to chemicals that ultimately injure mitochondria by disrupting oxidative phosphorylation and/or intracellular  $\text{Ca}^{2+}$  homeostasis, there are toxicants that cause cell death by affecting other functions or structures primarily. Included here are (1) chemicals that directly damage the plasma membrane, such as lipid solvents, detergents, and venom-derived hydrolytic enzymes; (2) xenobiotics that damage the lysosomal membrane, evoking release of lysosomal hydrolases and  $\text{Fe}^{2+}$ , such as aminoglycoside antibiotics and hydrocarbons binding to  $\alpha_1$ -globulin that injure renal tubular cells; (3) toxins that destroy the cytoskeleton, such as the microfilament toxins (eg, phalloidin and cytochalasins) and the microtubule toxins (eg, colchicine and 2,5-hexanedione); (4) the protein phosphatase inhibitor and hepatotoxic microcystin, which causes hyperphosphorylation of microfilaments and other cellular proteins (Toivola and Eriksson, 1999); (5) toxins that disrupt protein synthesis, such as  $\alpha$ -amanitin and ricin; and (6) cholesterol-lowering drugs (statins) that inhibit HMG-coenzyme A reductase, the rate-limiting enzyme in the mevalonate pathway, and rarely cause myotoxicity.

The events leading to cell death after exposure to these chemicals are generally unknown. It is likely that cell death caused by these chemicals is ultimately mediated by impairment of oxidative phosphorylation, sustained elevation of intracellular  $\text{Ca}^{2+}$ , and/or overproduction of ROS/RNS, and that it takes the form of necrosis if these processes are abrupt, but apoptosis if they are protracted. For example, direct injury of the plasma membrane would lead rapidly to increased intracellular  $\text{Ca}^{2+}$  levels. Neurofilamental toxins that block axonal transport cause energy depletion in the distal axonal segment. HMG-coenzyme A reductase inhibitors not only diminish the synthesis of cholesterol but may also compromise formation of other products of the mevalonate pathway. Such are the mitochondrial electron-transporting molecule ubiquinone (also called coenzyme Q) and isopentenyl pyrophosphate, which is utilized for isopentenylation of selenocysteinyl-tRNA, an obligatory step for synthesis of selenoproteins, including the antioxidant glutathione peroxidase (see Fig. 3-5) and thioredoxin reductase (see Fig. 3-22). These alterations may contribute to statin-induced myopathy and rhabdomyolysis.

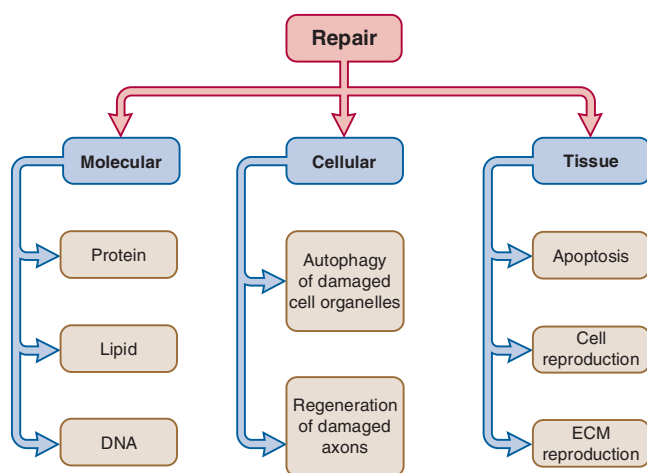
**Impairment of External Cellular Maintenance** Toxicants may also interfere with cells that are specialized to provide support to other cells, tissues, or the whole organism. Chemicals acting on the liver illustrate this type of toxicity. Hepatocytes produce and



release into the circulation a number of proteins and nutrients. They remove cholesterol and bilirubin from the circulation, converting them into bile acids and bilirubin glucuronides, respectively, for subsequent excretion into bile. Interruption of these processes may be harmful to the organism, the liver, or both. For example, inhibition of hepatic synthesis of coagulation factors by coumarins does not harm the liver, but may cause death by hemorrhage (Hardman *et al.*, 1995). This is the mechanism of the rodenticidal action of warfarin. In the fasting state, inhibitors of hepatic gluconeogenesis, such as hypoglycin (the ackee fruit-derived causative agent of Jamaican vomiting sickness; methylene cyclopropyl alanine), may be lethal by limiting the supply of glucose to the brain. Similarly, Reye syndrome, which is viewed as a hepatic mitochondrial injury caused by a combination of a viral disease (which may induce hepatic NOS) and intake of salicylate (which provokes MPT) (Fromenty and Pessayre, 1997; Kim *et al.*, 2003), causes not only hepatocellular injury but also severe metabolic disturbances (hypoglycemia, hyperammonemia) that affect other organs as well. Chemical interference with the  $\beta$ -oxidation of fatty acids or the synthesis, assembly, and secretion of lipoproteins overloads the hepatocytes with lipids, causing hepatic dysfunction (Fromenty and Pessayre, 1997).  $\alpha$ -Naphthylisothiocyanate (ANIT) causes separation of the intercellular tight junctions that seal bile canaliculi (Krell *et al.*, 1987), impairing biliary secretion and leading to the retention of bile acids and bilirubin; this adversely affects the liver as well as the entire organism.

## STEP 4—INAPPROPRIATE REPAIR AND ADAPTATION

The fourth step in the development of toxicity is inappropriate repair and adaptation (Fig. 3-1). As noted previously, many toxicants alter macromolecules, which eventually cause damage at higher levels of the biological hierarchy in the organism. Progression of toxic lesions can be intercepted by repair mechanisms operating at molecular, cellular, and tissue levels (Fig. 3-21). Another strategy whereby the organism can resist the noxious chemical is by increasing its own readiness to cope with it and with its harmful effects. This phenomenon is called adaptation. Because the capacity of the organism to repair itself and adapt to the toxic exposure and effects is so important in determining the outcome of chemical exposure, the mechanisms of repair and adaptation will be discussed below.



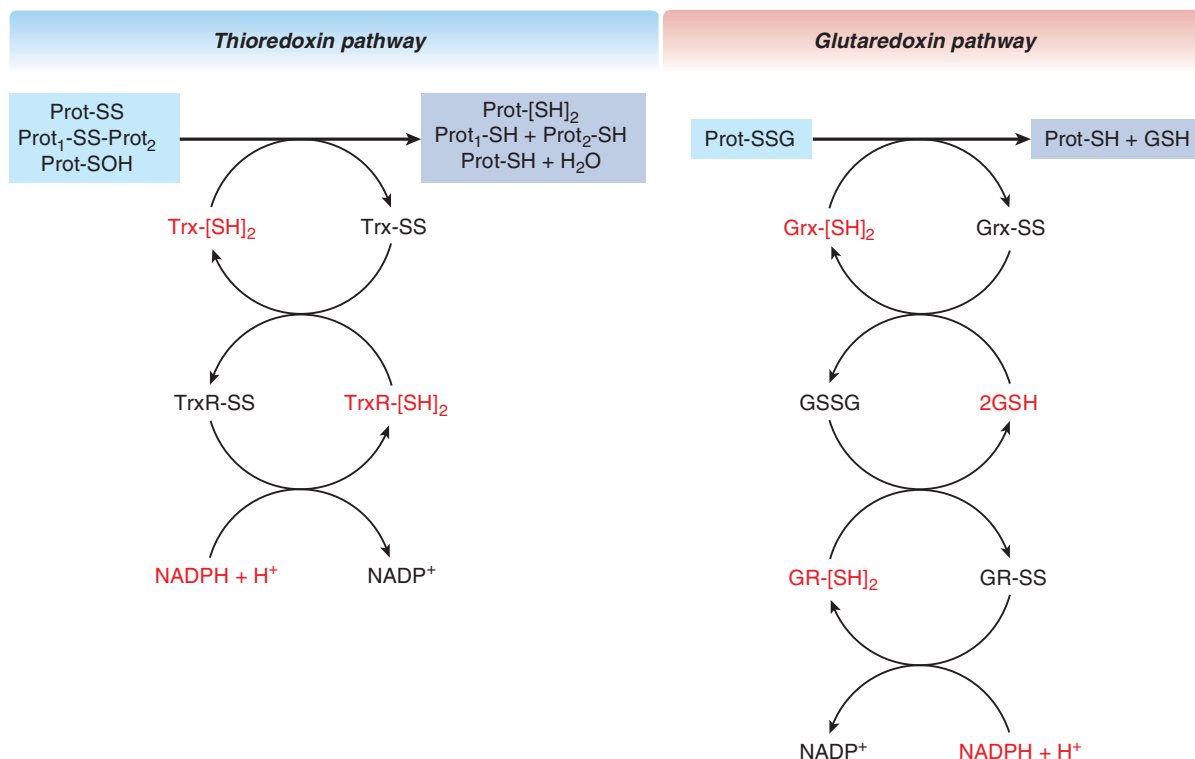
**Figure 3-21. Repair mechanisms.** Dysfunction of these mechanisms results in dysrepair, the fourth step in the development of numerous toxic injuries. ECM, extracellular matrix.

## Mechanisms of Repair

**Molecular Repair** Damaged molecules may be repaired in different ways. Some chemical alterations, such as oxidation of protein thiols and methylation of DNA, are simply reversed. Hydrolytic removal of the molecule's damaged unit or units and insertion of a newly synthesized unit or units often occur with chemically altered DNA 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 are essential for the function of numerous proteins, such as receptors, enzymes, cytoskeletal proteins, and TFs. Oxidation of protein thiols (Prot-SHs) to protein disulfides (Prot-SS, Prot<sub>1</sub>-SS-Prot<sub>2</sub>), protein-glutathione mixed disulfides (Prot-SSG), and protein sulfenic acids (Prot-SOH) can be reversed by reduction (Watson *et al.*, 2004; Gravina and Mieyal, 1993) (Fig. 3-22). The endogenous reductants are thioredoxins and glutaredoxins, small, ubiquitous proteins with two redox-active cysteines in their active centers (Holmgren *et al.*, 2005). These proteins as well as thioredoxin reductase have two isoenzymes; those labeled 1 are located in the cytosol, whereas those labeled 2 are mitochondrial. Because the catalytic thiol groups in these proteins become oxidized, they are reduced by NADPH, which is generated by NADP<sup>+</sup>-dependent isocitrate dehydrogenase localized in various cell compartments (cytosol, mitochondria, peroxisomes), as well as by the cytosolic glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in the pentose phosphate pathway. ROS may 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 (Moskovitz, 2005). Msr enzymes can reverse this modification at the expense of oxidation of their catalytic cysteine to sulfenic acid (Msr-Cys-S-OH). This then reacts with a neighboring thiol (in MsrA), forming an intramolecular disulfide, or with glutathione (in some other Msr proteins), forming a protein-glutathione disulfide. Finally the disulfide enzyme is reduced by thioredoxin or glutaredoxin, respectively, with subsequent steps depicted in Fig. 3-22. Reduction of methionine sulfoxides in lens proteins (eg,  $\alpha$ -crystallin) is especially critical for maintenance of the transparency of the eye lens. MsrA knockout mice develop cataract on repeated exposure to hyperbaric oxygen (Kantorow *et al.*, 2010). Repair of oxidized hemoglobin (methemoglobin) occurs by means of electron transfer from cytochrome  $b_5$ , which is then regenerated by a NADH-dependent cytochrome  $b_5$  reductase (also called methemoglobin reductase).

Soluble intracellular proteins such as cytosolic enzymes are typically folded into a globular form with their hydrophobic amino acid residues hidden inside, whereas the hydrophilic residues are located externally together with a hydrophobic cleft that constitutes the ligand (substrate) binding site. Physical or chemical insults may evoke an unduly large opening of this cleft that may lead to unfolding of the protein (denaturation) and its aggregation. Molecular chaperones such as the heat-shock proteins (Hsp, eg, Hsp90, Hsp70, and Hsp40) can prevent protein unfolding by "clamping down" onto the exposed hydrophobic region of their client protein, using the energy of ATP hydrolysis to execute their conformational change that entails this maneuver. According to a recent model (Pratt *et al.*, 2010), Hsp90 plays the leading role in stabilizing the cleft and impeding further unfolding, which would eventually lead to degradation of the protein by the ubiquitin-proteasome system (UPS) as described below. Indeed, the benzoquinone ansamycin Hsp90



**Figure 3-22. Repair of proteins oxidized at their thiol groups.** Protein disulfides (Prot-SS, Prot<sub>1</sub>-SS-Prot<sub>2</sub>) and protein sulfenic acids (Prot-SOH) are reduced by thioredoxin (Trx-[SH]<sub>2</sub>) or thioredoxin-like proteins. Protein-glutathione mixed disulfides (Prot-SSG) are reduced by glutaredoxin (Grx-[SH]<sub>2</sub>), which is also called thioltransferase. The figure also indicates how Trx-[SH]<sub>2</sub> and Grx-[SH]<sub>2</sub> are regenerated from their disulfides (Trx-SS and Grx-SS, respectively). In the mitochondria, Trx-SS also can be reduced by the dithiol dihydrofolipoic acid, a component of the pyruvate- and  $\alpha$ -ketoglutarate dehydrogenase complexes. GSH, glutathione; GSSG, glutathione disulfide; GR-[SH]<sub>2</sub> and GR-SS, glutathione reductase (dithiol and disulfide forms, respectively); TrxR-[SH]<sub>2</sub> and Trx-SS, thioredoxin reductase (dithiol and disulfide forms, respectively).

inhibitors (eg, geldanamycin and herbimycin) promote degradation of Hsp90 client proteins by the UPS.

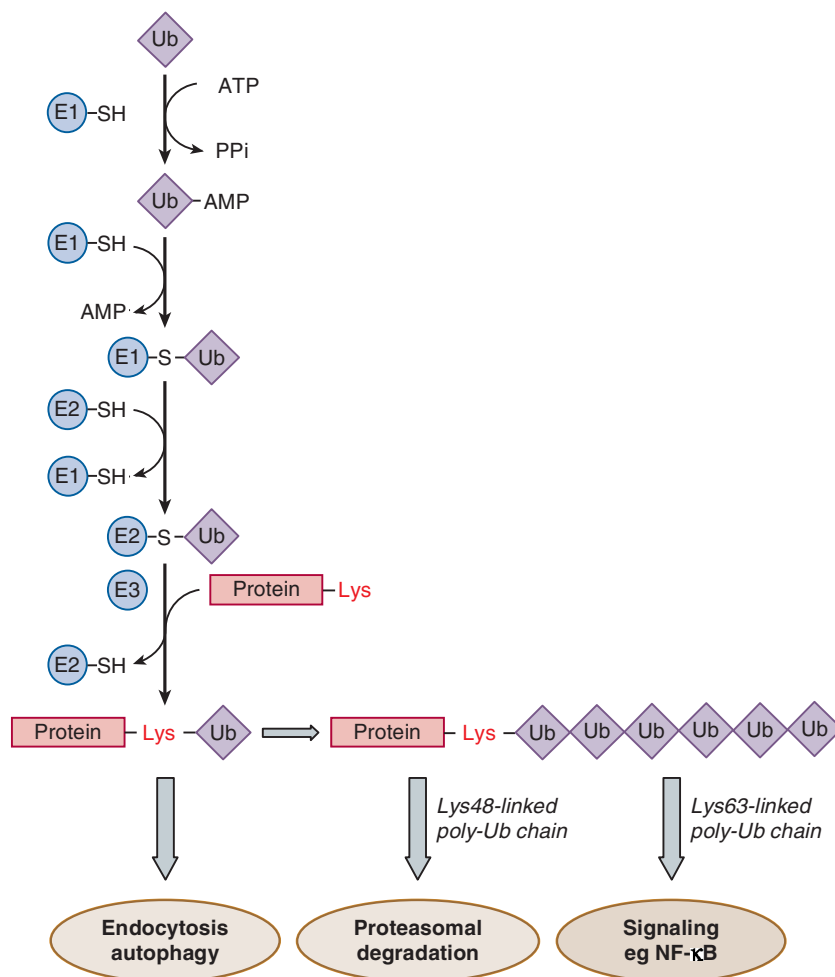
When unfolding progresses beyond a limit, Hsp90 dissociates. This may occur in response to chemical-induced protein damage, such as that inflicted by mechanism-based enzyme inactivators that are converted into reactive metabolites that bind covalently to the catalytic site of the enzyme. Dissociation of Hsp90 from the unfolding protein allows Hsp70 and its co-chaperone Hsp40 to recruit the Hsp70-dependent E3 ubiquitin ligases (eg, CHIP and Parkin) that in turn direct ubiquitin-charged E2 enzymes to the Hsp70-bound client protein to tag it with a polyubiquitin chain as the final step in a process presented in Fig. 3-23 (Bedford *et al.*, 2011). The protein tagged with a Lys48-linked polyubiquitin chain is then recognized and degraded in the proteasomes. The 26S proteasome is large barrel-shaped multiprotein complex that binds the ubiquitinated protein, deubiquitinates and unfolds it using ATP, and finally hydrolyzes the protein with its threonine-containing protease active sites into small peptides. The UPS controls the cellular level of numerous regulatory proteins (eg, p53, I $\kappa$ B, Nrf2,  $\beta$ -catenin, cyclins) and also has a prominent role in eliminating oxidized or otherwise damaged and unfolded intracellular proteins (Poppe and Grune, 2006). NOS exposed to its mechanism-based inactivator guanabenz that causes loss of tetrahydrobiopterin from the substrate-binding cleft will suffer this fate (Pratt *et al.*, 2010). CYP2E1, in which the heme becomes cross-linked to the protein as a consequence of reductive dechlorination of CCl<sub>4</sub> to the trichloromethyl free radical by this enzyme, is also eliminated by proteasomal degradation.

Oligomerization and aggregation of damaged and unfolded proteins preclude the proteasome from degrading them; such substrates can even trap proteasomes, rendering them nonfunctional.

After ubiquitination, protein aggregates can be eliminated by autophagy, a process described in more detail in the section “Cellular Repair.” Ubiquitin on the modified proteins is recognized and bound by autophagic adaptor proteins, such as p62 or Nbr1, which interact with LC3 to deliver them to autophagosomes, newly formed vesicles that encapsulate their protein cargo. After fusing of autophagosomes with lysosomes, the damaged protein is hydrolyzed by proteases. Lysosomal proteases degrade, for example, the immunogenic trifluoroacetylated proteins that are formed in the liver during halothane anesthesia (Cohen *et al.*, 1997). Erythrocytes have ATP-independent, nonlysosomal proteolytic enzymes that rapidly and selectively degrade proteins denatured by HO<sup>•</sup> (Davies, 1987). Red blood cells containing protein aggregates (Heinz body) after exposure to methemoglobin-forming chemicals are removed via phagocytosis by macrophages in the spleen.

**Repair of Lipids** Phospholipids containing fatty acid hydroperoxides are preferentially hydrolyzed by phospholipase A<sub>2</sub>, with the peroxidized fatty acids replaced by normal fatty acids (van Kuik *et al.*, 1987). Peroxidized lipids (eg, fatty acid hydroperoxides and phospholipid-associated hydroperoxides) may be reduced by the glutathione peroxidase–glutathione–glutathione reductase system (see Fig. 3-24) or by the peroxiredoxin–thioredoxin–thioredoxin reductase system (shown in part in Fig. 3-22). Again, NADPH is needed to “repair” the reductants that are oxidized in the process.

**Repair of DNA** Despite its high reactivity with electrophiles and free radicals, nuclear DNA is remarkably stable, in part because it is packaged in chromatin and because several repair mechanisms are available to correct alterations. The mitochondrial DNA, however,



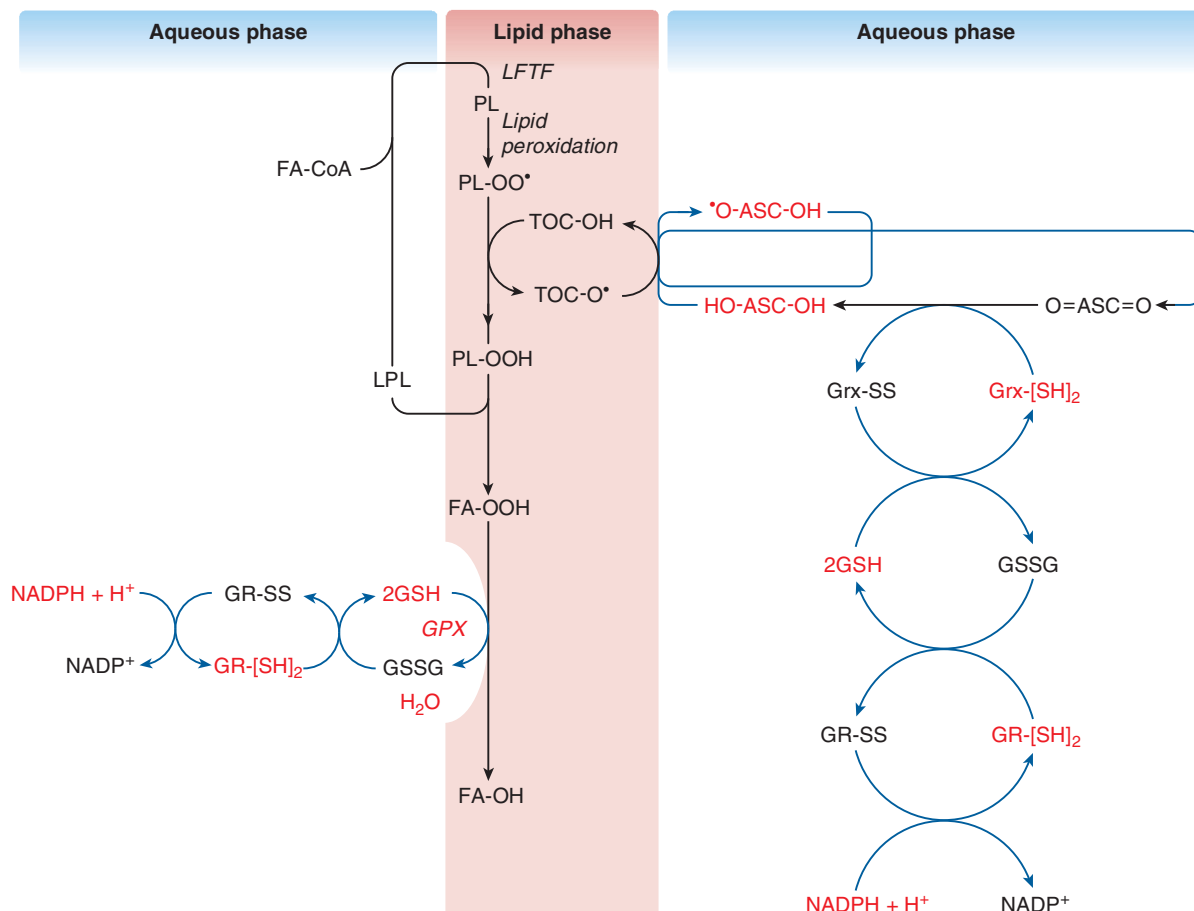
**Figure 3-23. The process of ubiquitination and its possible outcomes.** Ubiquitin (Ub) is a small (8.6 kDa) ubiquitous protein that when covalently linked to a target protein (as a monomer or as a polymer of Ub units) alters its fate and/or function. Ubiquitination is a posttranslational protein modification that takes place in an enzymatic cascade composed of 3 types of proteins—the E1 Ub-activating enzyme, the E2 Ub-conjugating enzyme, and the E3 Ub ligase. Humans possess 8 E1, over 30 E2, and hundreds of E3 enzymes. E1 first activates Ub in an ATP-consuming reaction by adenylating the terminal carboxyl group of Ub, thus forming Ub-AMP, and then it transfers Ub from Ub-AMP to its catalytic SH group, forming a high-energy thioester bond between E1 and Ub. In the subsequent transthioesterification reaction, Ub is transferred from E1 to the catalytic SH group of E2. Thereafter, E3 binds both the target protein and the Ub-charged E2 and catalyzes the transfer of Ub from E2 to the target protein, forming an isopeptide bond between the terminal carboxyl group of Ub and the  $\epsilon$ -amino group of a lysine in the substrate. Most E3 ligases, including the so-called RING finger domain-containing E3 ligases (which constitute 1 of the largest enzyme groups in the cell), ubiquitinate proteins this way. However, the HECT domain-containing E3 ligases, which possess an active site cysteine, take Ub from E2 in a transthioesterification reaction, and then they (rather than E2) transfer Ub to the target protein. As Ub contains 7 lysine residues, it can also be ubiquitinated. Multiple rounds of ubiquitination generate polyubiquitin chains. In general, monoubiquitination triggers endocytosis or autophagy of the protein. Substrates that are tagged with Lys48-linked Ub chains are recognized and degraded by the 26S proteasome into small peptides, with the Ubs released for reuse. Proteins polyubiquitinated with Lys63-linked Ub chains are generally not degraded but are essential components of signaling pathways, functioning as scaffolds to assemble signaling complexes. For example, the activation of transcription factor NF- $\kappa$ B on signaling (see Fig. 3-12) involves assembly of a signaling complex by means of Lys63-linked Ub chains. Ubiquitination can be reversed by deubiquitinating enzymes, which are Ub-specific proteases (cysteine proteases or zinc metalloproteases). These may rescue a substrate from degradation by removing a degradative Ub signal or may change or remove a nondegradative Ub signal. Instead of Ub, proteins may also be modified by covalent attachment of Ub-like proteins, such as NEDD8, SUMO, and ISG15, using the enzymatic machinery described above.

lacks histones and efficient repair mechanisms and therefore is more prone to damage. Different types of damages are corrected by specialized mechanisms, each employing a different set of repair proteins (Christmann *et al.*, 2003).

**Direct Repair** Certain covalent DNA modifications are directly reversed by enzymes such as DNA photolyase, which cleaves adjacent pyrimidines dimerized by UV light. Inasmuch as this chromophore-equipped enzyme uses the energy of visible light to correct damage, its function is restricted to light-exposed cells.

Minor adducts, such as methyl groups, attached to DNA bases by alkylating agents (eg, MMS) may be removed by special

enzymes (Christmann *et al.*, 2003). Such groups attached to the  $O^6$  position of guanine are cleaved off by  $O^6$ -methylguanine-DNA-methyltransferase (MGMT). While repairing the DNA, this alkyltransferase sacrifices itself, transferring the adduct onto one of its cysteine residues. This results in its inactivation, ubiquitination, and proteasomal degradation. Thus, like glutathione, which is depleted during detoxication of electrophiles, MGMT is consumed during the repair of DNA. Methyl groups attached to  $N^1$  of adenine and guanine, and  $N^3$  of thymine and cytosine are removed by oxidative demethylation catalyzed by DNA dioxygenases (ABH2 and ABH3). These peculiar  $O_2$ ,  $Fe^{2+}$ , ascorbate, and 2-oxoglutarate-dependent



**Figure 3-24. Repair of peroxidized lipids.** Phospholipid peroxyl radicals (PL-OO<sup>•</sup>) formed as a result of lipid peroxidation (Fig. 3-9) may abstract hydrogen from  $\alpha$ -tocopherol (TOC-OH) and yield phospholipid hydroperoxide (PL-OOH). From the latter, the fatty acid carrying the hydroperoxide group is eliminated via hydrolysis catalyzed by phospholipase (PLase), yielding a fatty acid hydroperoxide (FA-OOH) and a lysophospholipid (LPL). The former is reduced to a hydroxy fatty acid (FA-OH) by glutathione peroxidase (GPX), utilizing glutathione (GSH), or peroxiredoxins (not shown), whereas the latter is reacylated to phospholipid (PL) by lysophosphatide fatty acyl-coenzyme A transferase (LFTF), utilizing long-chain fatty acid-coenzyme A (FA-CoA). The figure also indicates regeneration of TOC-OH by ascorbic acid (HO-ASC-OH), regeneration of ascorbic acid from dehydroascorbic acid (O=ASC=O) by glutaredoxin (Grx-[SH]<sub>2</sub>), and reduction of the oxidized glutaredoxin (Grx-SS) by GSH. Oxidized glutathione (GSSG) is reduced by glutathione reductase (GR-[SH]<sub>2</sub>), which is regenerated from its oxidized form (GR-SS) by NADPH, the ultimate reductant. NADPH is produced by NADP<sup>+</sup>-dependent isocitrate dehydrogenases and during metabolism of glucose via the pentose phosphate shunt. TOC-O<sup>•</sup>, tocopheroxyl radical; <sup>•</sup>O-ASC-OH, ascorbyl radical.

enzymes oxygenate the methyl group adduct, which in turn leaves as formaldehyde, whereas 2-oxoglutarate is oxidatively decarboxylated to succinate.

**Excision Repair** Base excision and nucleotide excision are two mechanisms for removing damaged bases from DNA (see Chaps. 8 and 9). Lesions that do not cause major distortion of the helix typically are removed by base excision, in which the altered base is recognized by a relatively substrate-specific DNA glycosylase that hydrolyzes the *N*-glycosidic bond, releasing the modified base and creating an apurinic or apyrimidinic (AP) site in the DNA. For example, 8-OH-Gua, a major mutagenic product of oxidative stress, is removed from the DNA by a specific 8-OH-Gua DNA glycosylase. The AP site is recognized by the AP endonuclease, which hydrolyzes the phosphodiester bond adjacent to the abasic site. After its removal, the abasic sugar is replaced with the correct nucleotide by a DNA polymerase and is sealed in place by a DNA ligase. Interestingly, AP endonuclease is a bifunctional protein and is also called redox factor-1 (Ref-1). In concert with thioredoxin and thioredoxin reductase, it maintains TFs with sensitive thiol groups in their DNA-binding domain (Fos, Jun, NF- $\kappa$ B) in an active reduced state (Hansen *et al.*, 2006).

Bulky lesions such as adducts produced by aflatoxins or aminofluorene derivatives and dimers caused by UV radiation are removed by nucleotide excision repair system, which consists of approximately 30 proteins (Christmann *et al.*, 2003). Lesions in the nontranscribed strands or the nontranscribed regions of the genome are corrected by the global genomic repair system. This involves 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 together with the one containing the adduct. The excised section of the strand is restored by insertion of nucleotides into the gap by DNA polymerase and ligase, using the complementary strand as a template. Lesions in the transcribed DNA strand blocking the RNA polymerase in the actively transcribed genes are removed by another variation of nucleotide excision repair, the transcription-coupled repair system. This involves assembly of repair proteins to remove the stalled RNA polymerase before excision of the damage and filling the gap. Resynthesis of the removed section of the strand is designated "unscheduled DNA synthesis" and can be detected by the appearance of altered deoxynucleosides in urine. Excision repair has a remarkably low error rate of less than one mistake in 10<sup>9</sup> bases repaired.



PARP appears to be an important contributor to excision repair. On base damage or SSB, PARP binds to the injured DNA and becomes activated. The active PARP cleaves NAD<sup>+</sup> to use the ADP-ribose moiety of this cofactor for attaching long chains of polymeric ADP-ribose to nuclear proteins, such as histones. Because one ADP-ribose unit contains two negative charges, the poly(ADP-ribosyl)ated proteins accrue negativity and the resultant electrorepulsive force between the negatively charged proteins and DNA causes decondensation of the chromatin structure. It is hypothesized that PARP-mediated opening of the tightly packed chromatin allows the repair enzymes to access the broken DNA and fix it. Thereafter, poly(ADP-ribose) glycohydrolase gains access to the nucleus from its perinuclear localization and reverses the PARP-mediated modification of nuclear proteins (D'Amours *et al.*, 1999). Other features of PARP that are relevant in toxicity—such as destruction of PARP by caspases during apoptosis as well as the significance of NAD<sup>+</sup> (and consequently ATP) wasting by PARP in necrosis—have been discussed earlier in this chapter.

**Nonhomologous End Joining** This process repairs DSB that may be formed when two SSB occur in close proximity, or when DNA with SSB 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 end-processing enzymes, DNA polymerases, and DNA ligase. Although the nonhomologous end joining is error-prone, it can operate in any phase of the cell cycle and is the mechanism for DSB repair in nondividing terminally differentiated cells (eg, neurons). By contrast, the other and more faithful mechanism for DSB repair, homologous recombination, can function only after replication (in S and G2 phases), when sister chromatid sequences are available for use as templates (see below), and thus is not an option in nondividing cells.

**Recombinational (or Postreplication) Repair** Homologous recombination can be used to fix postreplication gaps and DSBs (Li and Heyer, 2008). The former defect may result when excision of a bulky adduct or an intrastrand pyrimidine dimer fails to occur before DNA replication begins. At replication, such a lesion prevents DNA polymerase from polymerizing a daughter strand along a sizable stretch of the parent strand that carries the damage. Then the replication results in two homologous (“sister”) yet dissimilar DNA duplexes; one has a large postreplication gap in its daughter strand and an intact duplex synthesized at the opposite leg of the replication fork. This intact sister duplex is utilized to complete the postreplication gap in the damaged sister duplex. This is accomplished by recombination (“crossover”) of the appropriate strands of the two homologous duplexes. After separation, the sister duplex that originally contained the gap carries in its daughter strand a section originating from the parent strand of the intact sister, which in turn carries in its parent strand a section originating from the daughter strand of the damaged sister. This strand recombination explains the phenomenon of “sister chromatid exchange,” which is indicative of DNA damage corrected by recombinational repair. After completing the postreplication gap by recombination, the damage still present in the parent strand may be removed by nucleotide excision.

In addition to eliminating postreplication gaps, homologous recombination can also repair DSB in a complex process employing numerous proteins. These include, for example, the MRN complex (the trimer of Mre11, Rad50, and Nbs1 proteins, which binds to the DSB and is involved in end processing by generation of single-stranded DNA), Rad51, Rad54, and the breast cancer susceptibility

proteins BRCA1 and BRCA2 (which assist the single-stranded DNA in invading the undamaged template in an ATP-dependent process), as well as polymerases, nucleases, helicases, and other components, which mediate DNA ligation and substrate resolution. A combination of excision and recombinational repairs occurs in restoration of DNA with interstrand cross-links caused by bifunctional electrophiles, such as nitrogen mustard-type drugs and cisplatin.

**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 is a mechanism specific for nerve cells.

**Autophagy of Damaged Cell Organelles** Cells suffering mild injury may repair themselves by removing and degrading damaged components, such as organelles and protein aggregates, in a process called autophagy, meaning eating of self (Rabinowitz and White, 2010). While autophagy serves the purpose of cell restitution in all cells, it is particularly important in terminally differentiated cells, such as neurons and myocytes, where cell renewal by cell replication is not possible. In addition, autophagy of normal cell constituents is an ultimate means of nutrient generation (see the section “Adaptation to Energy Depletion—The Energy Stress Response”).

In autophagy, a so-called isolation membrane (or phagophore) emerges possibly from the EPR, which engulfs the cytoplasmic material and then encapsulates it in a double-membrane vesicle (called autophagosome). This vesicle moves along microtubules, driven by dynein motors, to the lysosome and on fusing with the lysosome generates an autolysosome. The cargo as well as the inner membrane of the original autophagosome is then degraded by lysosomal hydrolytic enzymes, such as proteases, lipases, nucleases, and glycosidases (which work optimally at the acidic pH in the lysosomes) to amino acids, lipids, nucleosides, and carbohydrates. These breakdown products are then released by lysosomal permeases and transporters into the cytosol, where they may be further metabolized or used for synthesis of new macromolecules.

Autophagic removal of damaged cell organelles is a method of their quality control, disposal and recycling. This is well exemplified by deletion of depolarized mitochondria through selective autophagy or mitophagy in cells exposed to mitochondrial poisons, such as the protonophoric uncoupler CCCP, valinomycin, and paraquat (see Table 3-6). Several proteins mediate this process (Mehrpour *et al.*, 2010; Youle and Narendra, 2011), including (1) Pink1 (a protein kinase) localized in the MOM that accumulates there on dissipation of the mitochondrial membrane potential, (2) the cytosolic Parkin (an E3 ubiquitin ligase) that is recruited to the mitochondria by Pink1 and that then polyubiquitinates itself and other proteins, among them (3) the MOM protein mitofusins (Mfn1, Mfn2) and voltage-dependent anion channel (VDAC), (4) p62 (an autophagic adaptor protein) that binds to both ubiquitinated proteins and to (5) the cytosolic LC3 (microtubule-associated protein light chain 3), which, after cleavage to LC3-I, becomes conjugated with phosphatidylethanolamine by (6) a ubiquitin-like conjugation system that requires Atg7 (E1-like), Atg3 (E2-like), and the Atg12-Atg5:Atg16L complex (E3-like), (7) the phosphatidylinositol 3-kinase vesicular protein sorting 34 (Vps34), which is activated by interacting with (8) Beclin 1 (Bcl-2-interacting protein-1). The lipidated LC-3 (called LC3-II) can integrate into the membrane of autophagosome and can recruit the ubiquitin-tagged mitochondria into this structure. The latter protein (which later decomposes in the lysosome) is a marker for autophagosomes.

Removal of damaged mitochondria by autophagy is important not only for maintaining a functional mitochondrial pool but also for limiting oxidative cell damage and preventing apoptosis because injured mitochondria may be sources of ROS and apoptotic factors, such as cyt c, Smac, and AIF (see Fig. 3-20). Indeed, overexpression of Parkin suppresses cell death (Tanaka, 2010). In contrast, loss-of-function mutations in the genes encoding Pink1 and Parkin cause early onset monogenic forms of Parkinson disease probably through defective mitophagy of damaged mitochondria in the dopaminergic nigrostriatal neurons (Youle and Narendra, 2011). Autophagy also contributes to structural restitution of liver cells. For example, left-over peroxisomes after exposure to peroxisome proliferators (eg, fibrate esters) and lipid droplets in hepatic steatosis are also cleared by autophagy.

**Regeneration of Damaged Axons** Peripheral neurons with axonal damage can regenerate their axons with the assistance of macrophages and Schwann cells. Macrophages remove debris by phagocytosis and produce cytokines and growth factors, which activate Schwann cells to proliferate and transdifferentiate from myelinating operation mode into a growth-supporting mode. Distal to the injury, Schwann cells play an indispensable role in promoting axonal regeneration by increasing their synthesis of cell adhesion molecules (eg, N-CAM), by elaborating ECM proteins for base membrane construction, and by producing an array of neurotrophic factors (eg, brain-derived neurotrophic factor, glial cell line–derived neurotrophic factor, and nerve growth factor) and their receptors (Boyd and Gordon, 2003). While comigrating with the regrowing axon, Schwann cells physically guide as well as chemically lure the axon to reinnervate the target cell.

In the mammalian central nervous system, axonal regrowth is prevented partly by the nonpermissive external environment. At the site of injury, the oligodendrocytes produce growth inhibitory myelin-associated proteins (eg, Nogo, NI 35, myelin-associated glycoprotein, oligodendrocyte myelin glycoprotein, ephrin, and semaphorin), which act through specific receptors, whereas the astrocytes produce chondroitin sulfate proteoglycans and fibrotic scar (whose components include laminin, fibronectin, and collagen IV) (Johnson, 1993) that hinder axonal regrowth. Signaling by TGF- $\beta$  through Smad2 TF (see pathway #8 in Fig. 3-12) plays a leading role in overexpression of these extrinsic growth inhibitory factors. In addition, CNS neurons lose their intrinsic growth capacity postnatally, which occurs simultaneously with increased expression of some neuronal growth inhibitory Krüppel-like TFs, such as KLF4. For these reasons, damage to central neurons is irreversible but is compensated for in part by the large number of reserve nerve cells that can take over the functions of lost neurons. For example, in Parkinson disease, symptoms are not observed until there is at least an 80% loss of nigrostriatal neurons.

**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. The damaged cells are eliminated by apoptosis or necrosis.

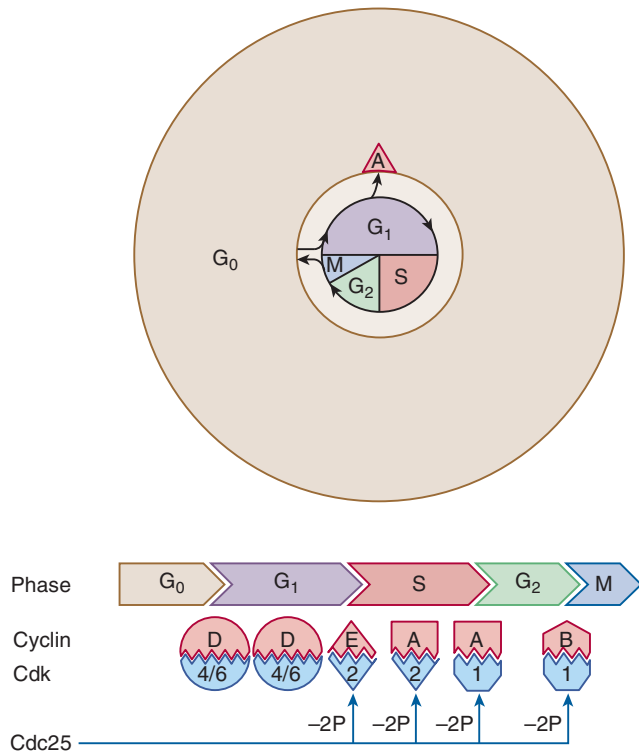
**Apoptosis: An Active Deletion of Damaged Cells** Apoptosis initiated by cell injury can serve as tissue repair for two reasons: first, because it may intercept the process leading to necrosis, as discussed earlier (see Fig. 3-20). Necrosis is a more harmful sequel than apoptosis for the tissue in which the injured cell resides. A cell destined for apoptosis shrinks; its nuclear and cytoplasmic materials condense, and then it breaks into membrane-bound fragments (apoptotic bodies) that are phagocytosed (Bursch *et al.*, 1992). During necrosis, cells and intracellular organelles swell and disintegrate with membrane lysis. Whereas apoptosis is orderly, necrosis

is a disorderly process that ends with cell debris in the extracellular environment. The constituents of the necrotic cells attract aggressive inflammatory cells, and the ensuing inflammation amplifies cell injury (see further on). With apoptosis, dead cells are removed without inflammation. Second, apoptosis may intercept the process leading to neoplasia by eliminating the cells with potentially mutagenic DNA damage. This function of apoptosis is discussed in more detail in the final section of this chapter.

It must be emphasized, however, that apoptosis of damaged cells may serve tissue restoration only for tissues that are made up of constantly renewing cells (eg, the bone marrow, the respiratory and GI epithelium, and the epidermis of the skin), or of conditionally dividing cells (eg, hepatic and renal parenchymal cells), because in these tissues the apoptotic cells are readily replaced. 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, because deletion of such cells, if extensive, can cause a deficit in the organ's function. Apoptosis may also be harmful when it occurs at a critical location. For example, in the pulmonary alveolar epithelium, an extremely tight barrier, apoptosis could cause flooding of the alveolar space with interstitial fluid, a potentially lethal outcome.

**Proliferation: Regeneration of Tissue** Tissues are composed of various cells and the ECM. Tissue elements are anchored to each other by transmembrane proteins. Cadherins allow adjacent cells to adhere to one another, whereas connexins associate into tubular structures and connect neighboring cells internally (gap junctions). Integrins link cells to the ECM. Therefore, repair of injured tissues involves not only regeneration of lost cells and the ECM but also reintegration of the newly formed elements. In parenchymal 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 blood monocytes, produce factors that stimulate parenchymal cells to divide and stimulate some specialized cells (eg, the stellate cells in the liver) to synthesize ECM molecules.

**Replacement of Lost Cells by Mitosis** Soon after injury, cells adjacent to the damaged area enter the cell division cycle (Fig. 3-25). Enhanced DNA synthesis is detected experimentally as an increase in the labeling index (which is the proportion of cells that incorporate administered  $^3\text{H}$ -thymidine or bromodeoxyuridine into their nuclear DNA during the S phase of the cycle), or by increased expression of proliferating cell nuclear antigen (PCNA, a trimeric protein that functions as a sliding clamp to hold DNA polymerase- $\delta$  to the template DNA strand during DNA replication). Also, mitotic cells can be observed microscopically. As early as 2 to 4 hours after administration of a low dose of carbon tetrachloride to rats, the mitotic index in the liver increases dramatically, indicating that cells already in the  $G_2$  phase progress rapidly to the M phase. The mitotic activity of the hepatocytes culminates at 36 to 48 hours, after a full transit through the cycle, indicating that quiescent cells residing in  $G_0$  enter and progress to mitosis (M). Peak mitosis of nonparenchymal cells occurs later, after activation and replication of parenchymal cells. In severe toxic liver injury, when hepatocyte replication is impaired (eg, in rats dosed with galactosamine or acetaminofluorene, and in humans intoxicated with acetaminophen), restoration of the liver may depend on stem cell–derived cells, called oval cells, which are located in the terminal bile ductules. These cells proliferate and differentiate into both hepatocytes and biliary epithelial cells (Fausto *et al.*, 2006; Vessey and Hall, 2001). As oval cells produce  $\alpha$ -fetoprotein, the increase in serum



**Figure 3-25.** The cell division cycle and the participating cyclins and cyclin-dependent protein kinases. Areas representing phases of the cycle are meant to be proportional to the number of cells in each phase. Normally, most cells are in G<sub>0</sub> phase, a differentiated and quiescent state. After receiving signals to divide, they progress into the G<sub>1</sub> phase of the cell division cycle. G<sub>0</sub>/G<sub>1</sub> transition involves activation of immediate early genes so that cells acquire replicative competence. Now increasingly responsive to growth factors, these cells progress to the phase of DNA synthesis (S). If this progression is blocked (eg, by the accumulated p53 protein), the cells may undergo apoptosis (A). After DNA replication, the cells prepare further for mitosis in the G<sub>2</sub> phase. Mitosis (M) is the shortest phase of the cell cycle (approximately 40 minutes out of the 40-hour-long cycle of hepatocytes) and most likely requires the largest energy expenditure per unit of time. The daughter cells produced may differentiate and enter into the pool of quiescent cells (G<sub>0</sub>), substituting for those which had been lost. During the cycle, the levels of various cyclins temporarily surge by synthesis and degradation (see figure). These proteins bind to and activate specific cyclin-dependent protein kinases (Cdk, see figure), which, in turn, phosphorylate and thus activate enzymes and other proteins required for DNA replication and cell division (Johnson and Walker, 1999) (see Fig. 3-32). In addition to cyclins, phosphorylation also regulates the activity of Cdks: phosphorylation by Wee1 protein kinase (not shown) inactivates Cdk1 and Cdk2, whereas dephosphorylation by cdc25 phosphatases activates them. After tissue necrosis, the number of cells entering the cell division cycle markedly increases at areas adjacent to the injury. The proportion of cells that are in S phase in a given period is reflected by the labeling index, whereas the percentage of cells undergoing mitosis is the mitotic index (see text).

$\alpha$ -fetoprotein levels indicates an improved outcome of acetaminophen-induced injury. Hepatic sinusoidal endothelial cells originate from bone marrow progenitors and when they are chemically injured (eg, by monocrotaline) bone marrow-derived precursor cells move into the sinusoids and contribute to their replacement, as demonstrated in a rat model of hepatic sinusoidal obstruction syndrome (Harb *et al.*, 2009). Accordingly, bone marrow infusion ameliorates, and myelosuppression aggravates, such liver injury. In an ozone-exposed lung, the nonciliated Clara cells and type II pneumocytes undergo mitosis and terminal differentiation to replace, respectively, the damaged ciliated bronchial epithelial cells and

type I pneumocytes (Mustafa, 1990). In some tissues, such as intestinal mucosa and bone marrow, stem cells first divide to provide self-renewal and then differentiate to replace more mature cells lost through injury.

Sequential changes in gene expression occur in cells that are destined to divide. In rats subjected to partial hepatectomy to study regeneration of the liver, oligonucleotide microarray analysis revealed that more than 150 genes are involved in the early gene response with upregulation or downregulation (Su *et al.*, 2002). The overexpressed genes include those that code for TFs important in proliferative signaling, such as *c-fos*, *c-jun*, *Egr1*, and *c-myc* (see Fig. 3-12), the genes of the antiapoptotic protein Bcl-X<sub>L</sub> (see Fig. 3-19), and that of *mdm2* that constrains p53, a proapoptotic and cell cycle inhibitory TF (see Fig. 3-32). Interestingly, some genes whose products decelerate the cell cycle also become temporarily overexpressed (eg, the cyclin-dependent kinase inhibitor p21 and *gadd45*; see Fig. 3-32), suggesting that this duality keeps tissue regeneration precisely regulated. Nevertheless, the genetic expression is apparently reprogrammed so that DNA synthesis and mitosis gain priority over specialized cellular activities. For example, as a result of dedifferentiation, regenerating hepatocytes underexpress cytochrome P450, *N*-acetyltransferase-2, as well as PPAR $\alpha$ , and hepatic stellate cells cease to accumulate fat and vitamin A.

It has been speculated that the regenerative process is initiated by the release of chemical mediators from damaged cells. The nonparenchymal cells, such as resident macrophages and endothelial cells, are receptive to these chemical signals and produce a host of secondary signaling molecules, cytokines, and growth factors that promote and propagate the regenerative process (Fig. 3-26). In rodents subjected to partial hepatectomy, the initial or priming phase of liver regeneration is controlled by the cytokines TNF and IL-6, whose hepatic mRNA and serum levels increase. TNF originates from the Kupffer cells. This cytokine acts on these macrophages in an autocrine manner, activating its receptor (item 2 in Fig. 3-12) and the coupled signal transducing network. This in turn causes activation of NF- $\kappa$ B, which increases IL-6 expression. The secreted IL-6 then acts on the hepatocytes and through its receptor (item 1 in Fig. 3-12) activates Janus kinase (JAK) and induces TFs (eg, Stat3, C/EBP $\beta$ ), which activate several target genes. This cytokine network promotes transition of the quiescent liver cells (G<sub>0</sub>) into cell cycle (G<sub>1</sub>) and makes them receptive to growth factors ("priming"). Growth factors, especially the hepatocyte growth factor (HGF), transforming growth factor- $\alpha$  (TGF- $\alpha$ ), and heparin-binding epidermal growth factor-like growth factor (HB-EGF), initiate the progression of the "primed" cells in the cycle toward mitosis (Costa *et al.*, 2003; Fausto *et al.*, 2006). 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 (Fig. 3-26). In rats intoxicated with carbon tetrachloride, the synthesis of HGF in hepatic and renal nonparenchymal cells increases markedly (Noji *et al.*, 1990) and HGF levels in blood rise rapidly (Lindroos *et al.*, 1991). The communication between parenchymal and nonparenchymal cells during tissue repair is mutual. For example, TGF- $\alpha$ , a potent mitogen produced by regenerating hepatocytes, acts both as an autocrine and a paracrine mediator on liver cells as well as on adjacent nonparenchymal cells (Fig. 3-26). By activating their receptors (item 4 in Fig. 3-12), these growth factors initiate signaling through the MAPK pathway and the PI3K-Akt pathway (Fig. 3-12), thereby mediating activation of TFs (*c-Jun*, *c-Fos*, *c-Myc*, FoxM1B, NF- $\kappa$ B, Stat3). These, among others, induce cyclins and the protein phosphatase cdc25, two groups of short-lived regulatory proteins. Then cyclins activate



Cdks by associating with them (Fig. 3-25), whereas cdc25 activates Cdk1 and Cdk2 by dephosphorylating their 2 amino acid residues (Thr14 and Tyr15). The activated Cdks accelerate the cell cycle mainly by phosphorylation of pRb. This in turn releases the TF E2F, which induces enzymes and regulatory proteins needed for cell cycle progression (Fig. 3-32). The growth factor signaling also activates mTOR that upregulates mRNA translation, thereby meeting the demand for increased protein synthesis (see Fig. 3-29). Similar signaling appears to mediate regeneration of the *S*-(1,2-dichlorovinyl)-L-cysteine-injured kidney that exhibits increased expression of the cytokine IL-6, the growth factors TGF- $\alpha$  and HB-EGF, the growth factor receptors EGFR (also receptor for TGF- $\alpha$ ) and IGF-1R, and the MAP kinase isoform Erk1 (Vaidya *et al.*, 2003).

Although the cytokine- and growth factor-controlled mitotic cell replacement is likely an essential part in the repair

of most tissues built up of cells with proliferative capacity, there are also tissue-specific features of tissue repair. For example in the liver, bile acids also stimulate hepatocyte proliferation through their nuclear receptor, FXR (Huang *et al.*, 2006). In mice subjected to partial hepatectomy, liver regrowth is hastened by bile acid feeding, but is markedly delayed when FXR is deleted or recirculation of bile acids from the intestine to the liver is prevented. Epithelia composed of a single cell layer form important barriers; therefore, replacement of mortally injured epithelial cells, which become detached from the basement membrane, is an urgent need. This can be achieved more rapidly by cell migration than by mitotic cell replacement. For example, in the damaged mucosa of the GI tract, cells of the residual epithelium rapidly migrate to the site of injury as well as elongate and become thin to reestablish the continuity of the surface even before this could be achieved by cell replication. Cell movement involves orderly dissociation of cadherin-mediated cell–cell contacts (involving  $\beta$ -catenin phosphorylation) and integrin-mediated cell–ECM contacts at focal adhesions (involving FAK phosphorylation), assembly of actin stress fibers, and formation of lamellopodia (cell projection filled with F-actin meshwork). Mucosal repair is dictated not only by growth factors and cytokines operative in tissue repair elsewhere but also by trefoil factors (TFFs). TFFs are small (7–12 kDa) protease-resistant proteins that are abundantly secreted from specific mucosal cells (eg, intestinal goblet cells) and are associated with the mucous layer of the GI tract (Taupin and Podolsky, 2003). TFF expression is rapidly upregulated at the margins of mucosal injury by cytokine and growth factor signaling. Whereas growth factors (eg, HGF and EGF) exert both motogenic (motility-increasing) and mitogenic effects on enterocytes, TFFs are potent mitogens, but are not mitogens. Although TFFs do not act alone, they are the only peptides shown to be essential for restitution of the injured intestinal mucosa. Normal mice exposed to dextran sodium sulfate in the drinking water develop diffuse colonic mucosal injury, some exhibiting bloody diarrhea. In contrast, the majority of TFF3-null mice develop frank bloody diarrhea and die in response to dextran sodium sulfate. The immediate target molecule (eg, receptor) for TFFs remains unknown. Migration of the surviving cells also precedes mitotic cell replacement in the tubular epithelium of the injured kidney. It appears that ECM components, such as collagen IV, beneath the tubular epithelial cells aid in the restitution of the injured epithelium and reestablishment of its polarity (Nony and Schnellmann, 2003).

**Replacement of the Extracellular Matrix** The ECM is composed of proteins, glycosaminoglycans, and the glycoprotein and proteoglycan glycoconjugates (Gressner, 1992). In liver, these molecules are synthesized by stellate or fat-storing cells located in the space of Disse, between the hepatic sinusoid and the hepatocytes (Fig. 3-26). The stellate cells become activated during liver regeneration, undergoing mitosis and major phenotypic changes. The latter changes include not only increased synthesis and secretion of ECM constituents but also expression of  $\alpha$ -smooth muscle actin as well as the myogenic TF MyoD and loss of fat, vitamin A, and PPAR $\gamma$  content. Thus, resting stellate cells become transdifferentiated into myofibroblast-like contractile and secretory cells. Activation of stellate cells is mediated chiefly by 2 growth factors—platelet-derived growth factor (PDGF) and TGF- $\beta$  (Fig. 3-26). Both may be released from platelets (which accumulate and degranulate at sites of injury) and later from the activated stellate cells themselves. The main sources of TGF- $\beta$ , however, are the neighboring tissue macrophages residing in the hepatic sinusoids (Gressner, 1992). A dramatic increase in TGF- $\beta$  mRNA levels in Kupffer cells is observed with in situ hybridization after carbon tetrachloride-induced hepatic



necrosis. Proliferation of stellate cells is induced by the potent mitogen PDGF. Through its receptor (like item 4 in Fig. 3-12), PDGF activates the PI3K–Akt pathway, which not only signals for proliferation but also inactivates the FoxO1 TF, which would halt the cell cycle in G1 phase by increasing the expression of p27, a cyclin-dependent kinase inhibitor. TGF- $\beta$  acts on the stellate cells to induce their transdifferentiation and to stimulate the synthesis of ECM components, including collagens, fibronectin, tenascin, and proteoglycans. TGF- $\beta$  acts through its Ser/Thr kinase receptor (item 8 in Fig. 3-12), which phosphorylates the TFs Smad2 and 3 (Flanders, 2004). TGF- $\beta$  also plays a central role in ECM formation in other tissues. In kidney and lung, for example, TGF- $\beta$  targets the mesangial cells and the septal fibroblasts, respectively. 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. The former group of these proteins originates from various types of nonparenchymal cells, including inflammatory cells; however, their inhibitors are mainly produced by stellate cells (Arthur *et al.*, 1999).

The way in which tissue regeneration is terminated after repair is unclear, but the gradual dominance of TGF- $\beta$ , which is a potent antimitogen and apoptogen, over mitogens is a contributing factor in the termination of cell proliferation. ECM production may be halted by an intracellular negative feedback mechanism in the ECM-producing cells, that is, by induction of Smad7 (an inhibitory Smad), which competitively inhibits phosphorylation of the receptor-activated Smads (Smad2 and 3). In addition, extracellular products of the proliferative response, such as the proteoglycan decorin and the positive acute-phase protein  $\alpha_2$ -macroglobulin, can bind and inactivate TGF- $\beta$ ; thereby they may contribute to its silencing (Gressner, 1992).

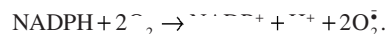
**Side Reactions to Tissue Injury** In addition to mediators that aid in the replacement of lost cells and the ECM, resident macrophages and endothelial cells activated by cell injury also produce other mediators that induce ancillary reactions with uncertain benefit or harm to tissues (Fig. 3-26). Such 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 the hallmarks of inflammation. These processes are largely initiated by resident macrophages secreting cytokines, such as TNF and interleukin-1 (IL-1), in response to tissue damage (Baumann and Gauldie, 1994) (Fig. 3-26). These cytokines, in turn, stimulate neighboring stromal cells, such as the endothelial cells and fibroblasts, to release mediators that induce dilation of the local microvasculature and cause permeabilization of capillaries. Activated endothelial cells also facilitate the egress of circulating leukocytes into the injured tissue by releasing chemoattractants and expressing cell adhesion molecules, which are cell surface glycoproteins (Jaeschke, 1997). One group of cell adhesion molecules, called selectins, located on the membrane of endothelial cells, interact with their ligands on the surface of leukocytes, thereby slowing down the flow of these cells and causing them to “roll” on the capillary surface. Subsequently a stronger interaction (adhesion) is established between the endothelial cells and leukocytes with participation of intercellular adhesion molecules (eg, ICAM-1) expressed on the endothelial cell membrane and integrins expressed on the membrane of leukocytes. This interaction is also essential for the subsequent transendothelial migration of leukocytes. This is facilitated by gradients of chemoattractants that induce expression of leukocyte integrins. Chemoattractants originate from various

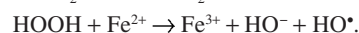
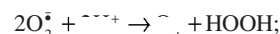
stromal cells and include chemotactic cytokines (or chemokines), such as the MCP-1 and IL-8 (whose rat homologue is the CINC-1), as well as lipid-derived compounds, such as platelet-activating factor (PAF) and leukotriene B<sub>4</sub> (LTB<sub>4</sub>). Ultimately all types of cells in the vicinity of injury express ICAM-1, thus promoting leukocyte invasion; the invading leukocytes also synthesize mediators, thus propagating the inflammatory response. Production of most inflammatory mediators is induced by signaling, turned on by TNF and IL-1, which results in activation of TFs, notably NF- $\kappa$ B and C/EBP (Poli, 1998) (see Fig. 3-12). Genes of many of the proteins mentioned above (eg, selectins, ICAM-1, MCP-1, IL-8) and below (eg, inducible NOS, acute-phase proteins) as well as the genes of TNF and IL-1 themselves contain binding sites for the NF- $\kappa$ B (Lee and Burckart, 1998).

**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 (Weiss and LoBuglio, 1982) (Fig. 3-26). Free radicals are produced in the inflamed tissue in three ways, each of which involves a specific enzyme: Nox, NOS, or myeloperoxidase.

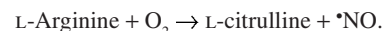
Nox is an electron-transporting protein complex composed of two transmembrane proteins (one of them the FAD- and heme-containing catalytic subunit) and 4 cytoplasmic proteins (including the G-protein Rac) (El-Benna *et al.*, 2005). In resting cells Nox is dormant; however, in activated cells the cytoplasmic subunits become extensively phosphorylated and move to the membrane to assemble the Nox complex. Constituents of microorganisms, such as the bacterial LPS (the active endotoxin component of gram-negative bacteria), acting through the cell surface Toll-like receptors (TLR), and PKC activators, such as phorbol myristate acetate (PMA), may evoke Nox activation. Unlike in nonphagocytic cells, in macrophages and granulocytes activation causes a sudden and rapid electron transfer from NADPH through the FAD and heme in Nox to the molecular oxygen, releasing the thus formed superoxide anion radical ( $O_2^{\cdot -}$ ) in a burst (“respiratory burst”) at the external membrane surface into the phagocytic vacuole:



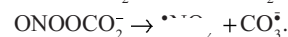
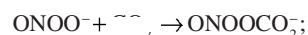
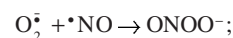
The  $O_2^{\cdot -}$  can give rise to the hydroxyl radical ( $HO^{\cdot}$ ) in two sequential steps: the first is spontaneous or is catalyzed by SOD, and the second, the Fenton reaction, is catalyzed by transition metal ions (see also Fig. 3-4):



Macrophages, but not granulocytes, generate another cytotoxic free radical, nitric oxide ( $^{\cdot}NO$ ). This radical is produced from arginine by NOS (Wang *et al.*, 1993), which is inducible in macrophages by bacterial endotoxin and the cytokines IL-1 and TNF:

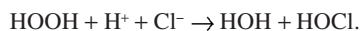


Subsequently,  $O_2^{\cdot -}$  and  $^{\cdot}NO$ , both of which are products of activated macrophages, can react with each other, yielding peroxynitrite anion; on reaction with carbon dioxide, this decays into two radicals, nitrogen dioxide and carbonate anion radical (Fig. 3-4):

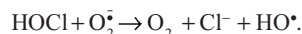


Granulocytes, but not macrophages, discharge the lysosomal enzyme myeloperoxidase into engulfed extracellular spaces, the phagocytic vacuoles (Wang *et al.*, 1993). Myeloperoxidase

catalyzes the formation of HOCl, a powerful oxidizing agent, from HOOH and chloride ion:



Like HOOH, HOCl can form  $\text{HO}^\bullet$  as a result of electron transfer from  $\text{Fe}^{2+}$  or from  $\text{O}_2^\bullet$  to HOCl:



All these reactive chemicals, as well as the discharged lysosomal proteases, are destructive products of inflammatory cells. Although these chemicals exert antimicrobial activity at the site of microbial invasion, at the site of toxic injury they can damage the adjacent healthy tissues and thus contribute to propagation of tissue injury (see the section "Tissue Necrosis"). Moreover, in some chemically induced injuries, inflammation plays the leading role. For example, ANIT, a cholestatic chemical, causes neutrophil-dependent hepatocellular damage. ANIT apparently acts on bile duct epithelial cells, causing them to release chemoattractants for neutrophil cells, which on invading the liver, injure hepatocytes (Hill *et al.*, 1999). Kupffer cell activation, TNF release, and subsequent inflammation are also prominent and causative events in galactosamine-induced liver injury in rats (Stachlewitz *et al.*, 1999).

Whereas it is well recognized that chemical-inflicted tissue injury can induce inflammation as a side reaction of tissue repair, it is becoming clear that inflammation (even if harmless alone) can precipitate an overt tissue injury on chemical exposure that is noninjurious alone. For example, a small harmless quantity of the macrophage activator LPS converts the nontoxic doses of monocrotaline, aflatoxin B<sub>1</sub>, and allyl alcohol into markedly hepatotoxic doses (Roth *et al.*, 2003). Moreover, in rats pretreated with LPS, unlike in untreated animals, chlorpromazine, ranitidine, and trovafloxacin caused liver injury. These drugs (and many others) when given to patients can induce rare, unexpected, and not obviously dose-related liver injury. Therefore, it is hypothesized that such idiosyncratic drug reactions develop when some endotoxin exposure decreases the threshold for drug toxicity by priming the Kupffer cells that produce ROS and inflammatory mediators discussed above. Manifest or subclinical infection, GI disturbance, or alcohol consumption (which greatly increases the intestinal permeability for endotoxin) may be the source of endotoxin. It is not surprising that most idiosyncratic drug reactions affect the liver, because this organ contains 80% to 90% of the body's fixed macrophages (ie, Kupffer cells), because the liver is the first organ to be exposed to LPS translocating from the intestinal lumen, and because the Kupffer cells not only are activated by LPS but also remove it from the circulation, thereby protecting other organs from its inflammatory effects (Roth *et al.*, 2003). However, the causative relationship between inflammation and idiosyncrasy needs further substantiation.

**Altered Protein Synthesis: Acute-Phase Proteins** Cytokines released from macrophages and endothelial cells of injured tissues also alter protein synthesis, predominantly in the liver (Baumann and Gauldie, 1994) (Fig. 3-21). Mainly IL-6 but also IL-1 and TNF act on cell surface receptors and increase or decrease the transcriptional activity of genes encoding certain proteins called positive and negative acute-phase proteins, respectively, utilizing primarily the TFs NF- $\kappa$ B, C/EBP, and STAT (Poli, 1998; see Fig. 3-15). Many of the hepatic acute-phase proteins, such as C-reactive protein and hepcidin, are secreted into the circulation, and their elevated levels in serum are diagnostic of tissue injury, inflammation, or neoplasm. Increased sedimentation of red blood cells, which is also indicative of these conditions, is due to enrichment of blood plasma with positive acute-phase proteins such as fibrinogen.

Apart from their diagnostic value, positive acute-phase proteins may play roles in minimizing tissue injury and facilitating repair. For example, many of them, such as  $\alpha_2$ -macroglobulin and  $\alpha_1$ -antiprotease, inhibit lysosomal proteases released from the injured cells and recruited leukocytes. Haptoglobin binds hemoglobin in blood, MT complexes metals in the cells, heme oxygenase oxidizes heme to biliverdin, and opsonins facilitate phagocytosis. Thus, these positive acute-phase proteins may be involved in the clearance of substances released on tissue injury. Induction of hepcidin, which triggers degradation of the iron exit channel ferroportin (see the section "Mechanisms of Adaptation") and thus decreases the duodenal  $\text{Fe}^{2+}$  absorption and  $\text{Fe}^{2+}$  export from macrophages, may be protective by limiting iron availability in the circulation for pathogens and by minimizing the  $\text{Fe}^{2+}$ -catalyzed Fenton reaction (Fig. 3-4), the source of reactive hydroxyl radicals that may cause tissue injury.

Negative acute-phase proteins include some plasma proteins, such as albumin, transthyretin, and transferrin, as well as hepatic enzymes (eg, several forms of cytochrome P450 and glutathione S-transferase), ligand-activated TFs (eg, PPAR $\alpha$  and the bile acid receptor FXR), and transporters, such as bile acid transporters at the sinusoidal and canalicular membrane of hepatocytes (NTCP and bile salt export pump [BSEP], respectively) and the bile canalicular export pump Mrp2. Because the latter enzymes and transporters play important roles in the toxication, detoxication, and excretion of endobiotics and xenobiotics, the disposition and toxicity of bile acids and toxicants may be altered markedly during the acute phase of tissue injury.

Although the acute-phase response is phylogenetically preserved, some of the acute-phase proteins are somewhat species-specific. For example, during the acute phase of tissue injury or inflammation, C-reactive protein and serum amyloid A levels dramatically increase in humans but not in rats, whereas the concentrations of  $\alpha_1$ -acid glycoprotein and  $\alpha_2$ -macroglobulin increase markedly in rats but only moderately in humans.

**Generalized Reactions** Cytokines released from activated macrophages and endothelial cells at the site of injury also may evoke neurohumoral responses. Thus, IL-1, TNF, and IL-6 alter the temperature set point of the hypothalamus, triggering fever. IL-1 possibly also mediates other generalized reactions to tissue injury, such as hypophagia, sleep, and "sickness behavior" (Rothwell, 1991). In addition, IL-1 and IL-6 act on the pituitary to induce the release of ACTH, which in turn stimulates the secretion of cortisol from the adrenals. This represents a negative feedback loop because corticosteroids inhibit cytokine gene expression.

## Mechanisms of Adaptation

Adaptation may be defined as a harm-induced capability of the organism for 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) strengthened mechanisms to compensate the toxicant-inflicted dysfunction. Mechanistically, adaptation involves sensing the noxious chemical and/or the initial damage or dysfunction, and a response that typically occurs through altered gene expression. Such mechanisms will be briefly overviewed below.

**Adaptation by Decreasing Delivery to the Target** The first step in the development of toxicity is delivery of the ultimate

toxicant (a xenobiotic, its metabolite or xenobiotic-generated ROS and RNS) to the target (Fig. 3-2). Certain chemicals induce adaptive changes that lessen their delivery by diminishing the absorption, increasing their sequestration by intracellular binding proteins, enhancing their detoxication, or promoting their cellular export.

**Repression of Iron Absorption** Adaptive mechanisms triggered by iron itself adjust the intestinal absorption of this essential yet potentially harmful metal ion, a catalyst of hydroxyl radical formation by the Fenton reaction (Fig. 3-4), to its demand.  $\text{Fe}^{2+}$  is taken up from the intestinal lumen into the enterocyte by the proton-coupled divalent metal transporter 1 (DMT1) localized in the luminal membrane and is exported across the basolateral membrane of these cells through the ferroportin iron channel. High iron intake diminishes the expression of both DMT1 and ferroportin in the enterocytes, whereas low intake has the opposite effect.

The expression of DMT1 is regulated at the translational level by the intracellular iron-regulatory proteins (IRP) that contain a [4Fe-4S] cluster. Iron supply influences the Fe content of this cluster and in turn affects the capacity of IRP to bind to mRNAs containing iron response element (IRE) in their untranslated regions and to alter translation. In iron deficiency, loss of Fe from the [4Fe-4S] cluster allows the thus formed IRP1 apoprotein to bind to and protect DMT1 mRNA from enzymatic degradation. On becoming more abundant, DMT1 mRNA translates into more DMT1 protein. Conversely, iron overload saturates IRP1 with Fe, making it incapable of binding to and stabilizing the DMT1 mRNA. The consequential loss of DMT1 mRNA and its translation into less DMT1 protein then limits  $\text{Fe}^{2+}$  absorption. This physiologically important adaptive mechanism may have toxicological corollary. Since DMT1 also mediates the transport of  $\text{Cd}^{2+}$ , DMT1 overexpression in iron deficiency increases intestinal absorption of this highly toxic metal ion (Park *et al.*, 2002).

Ferroportin allows exit of iron not only from enterocytes but also from macrophages (which obtain iron from phagocytosed senescent or damaged erythrocytes) and hepatocytes (which store iron). The abundance of ferroportin in the plasma membrane of these cells is regulated directly by hepcidin, a peptide hormone secreted by the hepatocytes of the liver (Ganz, 2011). By binding to ferroportin, hepcidin triggers the internalization and lysosomal hydrolysis of ferroportin. Hepatic production of hepcidin is sensitive to iron supply. Iron deficiency decreases hepcidin secretion, precluding ferroportin degradation and thus increasing the abundance of this iron exporter in the basolateral membrane of enterocytes. This (together with the upregulated DMT1 in the apical membrane of these cells) permits increased iron absorption from the duodenum. In contrast, iron overload increases the secretion of hepcidin, which in turn induces degradation of ferroportin, thereby preventing iron export from the enterocytes. Thus, decreased synthesis of DMT1 (see above) and degradation of ferroportin act in concert to shut down iron absorption from the intestine and protect against iron excess from the diet, from lysed red blood cells, or from ferritin. The toxicological significance of this mechanism is exemplified by findings with diquat, a redox cycling hepatotoxic herbicide that (like paraquat, see Fig. 3-3) can accept an electron, forming diquat monocation radical ( $\text{DQ}^{+}$ ), which in turn transfers the electron to  $\text{O}_2$ , producing superoxide anion radical ( $\text{O}_2^{\bullet}$ ). Soon after diquat injection to rats, hepatic free iron increased dramatically (purportedly because both  $\text{DQ}^{+}$  and  $\text{O}_2^{\bullet}$  had reductively released  $\text{Fe}^{2+}$  from ferritin) followed by a rise in hepatic hepcidin mRNA first, and later by a decrease in plasma iron (Higuchi *et al.*, 2011), a seemingly appropriate protective response.

Inappropriately elevated hepcidin, however, may cause iron deficiency. For example, one might speculate that elevated plasma levels of prohepcidin observed in chronic lead intoxication contribute to lead-induced anemia.

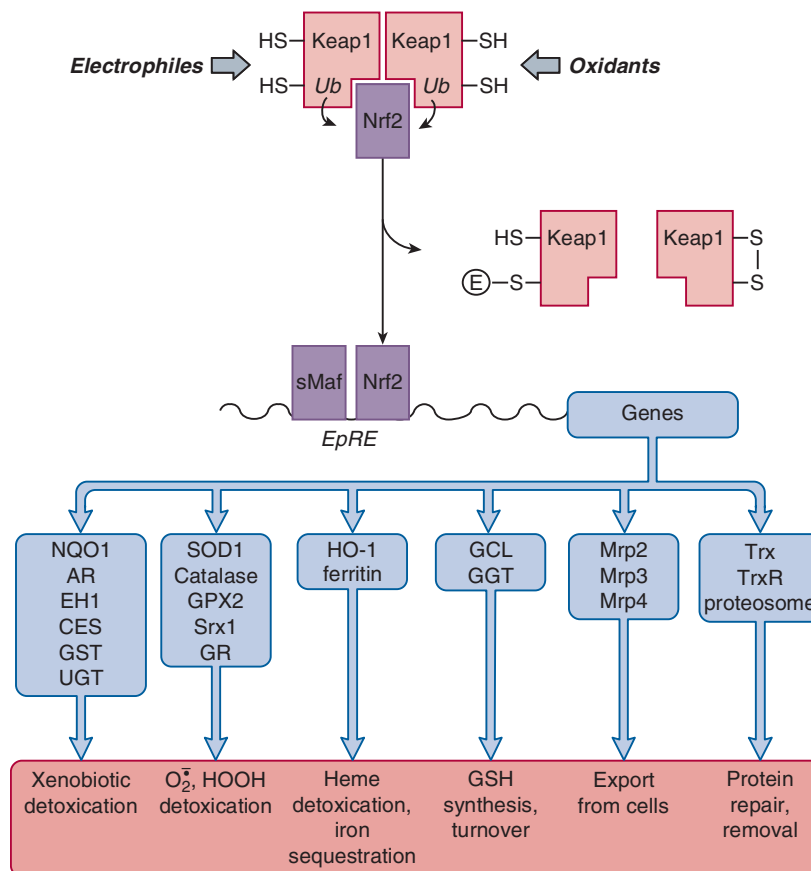
Hepcidin production by hepatocytes is transcriptionally regulated by Smad4 and STAT3 TFs through their cognate response elements in the hepcidin gene (Ganz, 2011). Two pathways converge on Smad4: one signals the amount of intracellular iron, whereas the other signals the quantity of plasma iron. Increase in intracellular iron in the liver cells is detected by unidentified sensors that induce secretion of bone morphogenetic protein (BMP6, a TGF- $\beta$ -like signaling molecule) acting in an autocrine or paracrine manner on its cell surface BMP receptor that activates Smad4 by phosphorylation (like TGF- $\beta$  does in pathway 8 shown in Fig. 3-12). Increase in plasma iron is detected by the transferrin receptor (TFR) of hepatocytes when activated by transferrin saturated with iron. Then TFR relays the signal through adapter proteins to the BMP receptor, which thus becomes increasingly sensitive to its ligand, BMP6. The pathway descending to STAT3 is initiated by IL-6 (see Fig. 3-12, item 1), a cytokine that triggers the acute-phase response, explaining why hepcidin is also a positive acute-phase protein (see above). In summary, the pathways initiated by transferrin and BMP receptors upregulate the hepatic production of hepcidin that reduces iron absorption in response to iron overload, whereas the pathway triggered by IL-6 has a similar effect in response to inflammation, tissue injury, or cancer, conditions with increased IL-6 production. The latter disorders may cause anemia by this mechanism.

**Induction of Ferritin and Metallothionein** Adaptive cellular accumulation of the binding proteins ferritin and MT is protective against their respective ligands, iron and cadmium ions. Interestingly, upregulation of ferritin by iron overload, like down-regulation of DMT1, is also translationally mediated by IRP1. However, the apoIRP1 (which is formed in iron deficiency) acts oppositely on ferritin mRNA and blocks its translation. Iron overload relieves this blockade, as it yields IPR1 with the [4Fe-4S] cluster, which does not bind to the IRE in ferritin mRNA, thus causing a surge in ferritin translation. Ferritin is protective as it removes  $\text{Fe}^{2+}$  from the Fenton reaction (Fig. 3-4) and incorporates it as  $\text{Fe}^{3+}$  through its ferroxidase activity.

MT is greatly induced by cadmium and elevated levels of MT protect the liver by restricting distribution of this toxic metal ion to sensitive intracellular targets (Klaassen *et al.*, 1999). Induction of MT by  $\text{Cd}^{2+}$  is likely indirect, mediated by Zn (displaced from intracellular binding sites), which activates the metal-responsive transcription factor 1 (MTF-1) that in turn augments transcription of the MT gene by binding to the metal-responsive elements in its promoter (Lichtlen and Schaffner, 2001).

**Induction of Detoxication—The Electrophile Stress Response, Part 1** Adaptive increases in detoxication (ie, elimination of xenobiotics, their reactive metabolites, harmful endobiotics, or ROS and RNS by biotransformation) and cellular export have a major role in limiting toxicity. Such adaptation is typically induced by compounds with thiol reactivity (ie, soft electrophiles, oxidants, and those generating oxidative stress), which are sensed by the cytosolic Keap1-Nrf2 protein complex. The response is initiated by the TF Nrf2, which activates genes with electrophile response element (EpRE) in their regulatory region (Fig. 3-27; Dinkova-Kostova *et al.*, 2005). Normally Nrf2 is retained in the cytoplasm by Keap1, a cysteine-rich homodimeric protein. Keap1 keeps Nrf2 inactive and at low intracellular levels by anchoring it to the cytoskeleton and also by linking it to cullin 3, a component in certain E3 ubiquitin ligase complexes, thereby initiating ubiquitination of





**Figure 3-27. 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 proteasomal degradation by ubiquitination. 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, the 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. Induction of such proteins represents an electrophile stress response that provides protection against a wide range of toxicants. Nrf1, a transcription factor structurally related to Nrf2, also interacts with Keap1 and Maf proteins as well as EpRE and its role is partially overlapping with that of Nrf2. *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.

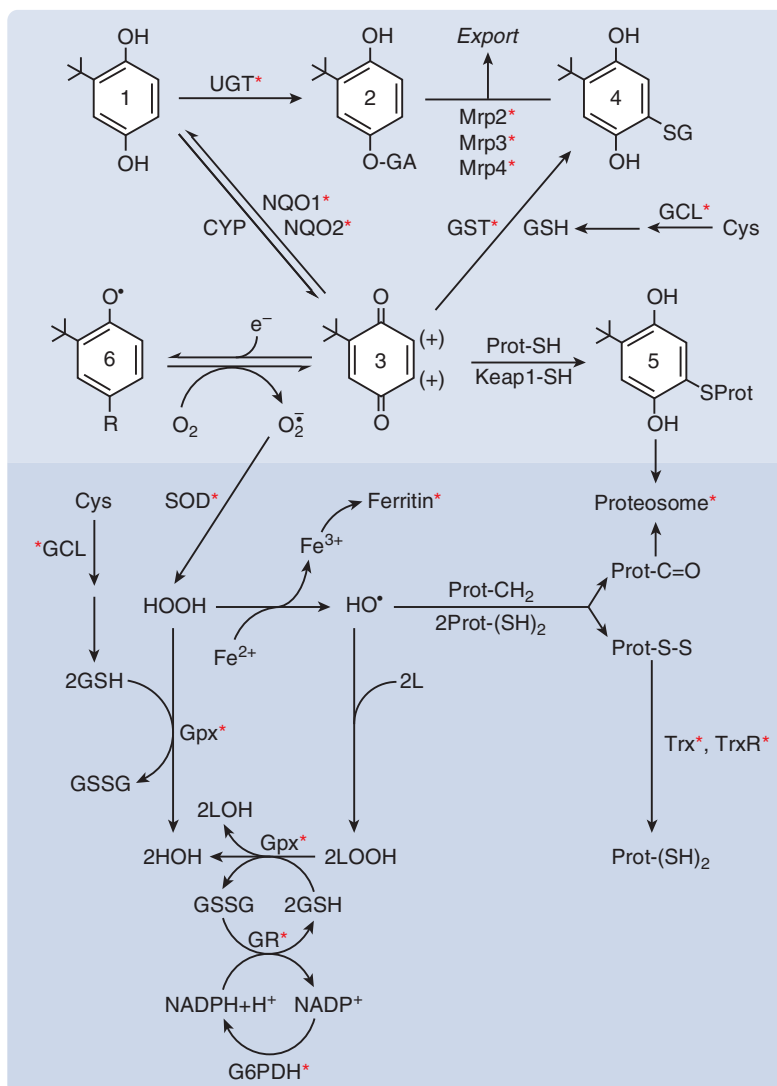
Nrf2 for subsequent proteasomal degradation. On disruption of the Keap1–Nrf2 complex, the active Nrf2 escapes rapid degradation and accumulates in the cell. Electrophiles, such as quinones (eg, *t*-butylquinone), quinoneimines (eg, NAPBQI derived from acetaminophen), quinone methides (eg, metabolite of butylated hydroxytoluene),  $\alpha,\beta$ -unsaturated aldehydes and ketones (eg, the lipid peroxidation products 4-oxonon-2-enal, 4-hydroxynon-2-enal, and 15- $A_2$ -isoprostane), isothiocyanates (eg, ANIT), thiol-reactive metal ions (eg,  $Cd^{2+}$ ), and trivalent arsenicals, as well as direct and indirect oxidants (eg, HOOH, diquat, quinones) may attack Keap1 at its reactive cysteine thiol groups by binding to them covalently or oxidizing them, thereby forcing Keap1 to release Nrf2. After being released from Keap1, Nrf2 translocates into the nucleus, forms a heterodimer with small Maf proteins, and activates genes through binding to EpREs.

There are many genes with EpRE motifs that encode proteins known to be important in detoxication and export (Fig. 3-27). These include genes that code for (1) enzymes that detoxify xenobiotics (eg, NQO1, NQO2, AR, EH-1, CES1e1, CES2a6, GST, and

UGT), (2) enzymes that eliminate  $O_2^{\cdot -}$  (ie, SOD1) and HOOH (eg, GPX2, catalase, and Srx1; the latter reduces the overoxidized peroxiredoxins that in turn can reduce not only HOOH [see Fig. 3-5] but also lipid hydroperoxides and peroxynitrite), (3) proteins that detoxify heme (HO-1) and  $Fe^{2+}$  (ferritin), (4) enzymes involved in the synthesis of GSH and its regeneration from GSSG (eg, GCL and the NADPH-forming G6PDH), and (5) transporters that pump xenobiotics and their metabolites out of cells (eg, Mrp2, 3, and 4). Other Nrf2-induced proteins of known toxicological relevance will be mentioned later.

As demonstrated in Fig. 3-28 through the example of *t*-butylhydroquinone (*t*BHQ), these Nrf2-mediated adaptive changes facilitate elimination and detoxication of electrophilic chemicals, as well as ROS that may be generated by them, and assist in repairing or removing damaged proteins (to be discussed later); therefore, Nrf2 conveys protection against a wide range of toxicants. Indeed, Nrf2 knockout mice are more sensitive to the hepatotoxicity of acetaminophen, pentachlorophenol, and carbon tetrachloride, the pulmonary toxicity of butylated hydroxytoluene, hyperoxia, or cigarette smoke,





**Figure 3-28.** Adaptive changes in response to the *t*-butylhydroquinone (tBHQ)-induced electrophile stress that influence the metabolic fate and some effects of tBHQ and of superoxide anion radical generated in the course of tBHQ biotransformation. This figure illustrates the numerous proteins (ie, enzymes, exporters, repair proteins) that, when induced in response to the tBHQ-induced electrophile stress, facilitate the detoxication and export of tBHQ and/or its metabolites, the detoxication of ROS formed during the biotransformation of tBHQ, and repair or removal of proteins damaged by the reactive metabolites. Proteins induced by Nrf2 as a result of adaptation to the electrophile stress are marked with an asterisk.

tBHQ (1) can be detoxified by UDP-glucuronosyltransferase to form tBHQ glucuronide (2) and toxified by cytochrome P450-catalyzed dehydrogenation to t-butylquinone (tBQ; 3), which contains electrophilic carbon atoms (+), and is believed to be the actual inducer of electrophile stress response. tBQ can undergo 3 biotransformations. First, it can be detoxified by conjugation with glutathione (GSH) to form tBHQ-SG (4), which, together with tBHQ-glucuronide, is exported from the cell by Mrp2, 3, and 4. Second, tBQ can covalently bind to SH groups in proteins (5), including Keap1. Third, by accepting an electron (e<sup>-</sup>), tBQ can form t-butylsemiquinone radical (6), which can pass the electron to molecular oxygen to form superoxide anion radical (O<sub>2</sub><sup>•-</sup>), completing a redox cycle. In a process catalyzed by superoxide dismutase (SOD), O<sub>2</sub><sup>•-</sup> can form HOOH, which is detoxified by GSH-peroxidase (GPX), using GSH (whose synthesis is rate-limited by GCL), or toxified by Fe<sup>2+</sup>-catalyzed Fenton reaction to form hydroxyl radical (HO\*). HO\* can react with proteins to form oxidized proteins (Prot-C=O, Prot-S-S), which can be degraded in the proteasome, or Prot-S-S can also be repaired through reduction by the thioredoxin (Trx)-thioredoxin reductase (TrxR) system. HO\* can also react with lipid (L) and form lipid hydroperoxide (LOOH), which can be reduced to lipid alcohol (LOH) by GPX at the expense of GSH. This results in formation of glutathione disulfide (GSSG), which can be reduced back to GSH by glutathione reductase (GR) at the expense of NADPH generated by glucose-6-phosphate dehydrogenase (G6PDH). Virtually all of these processes become more effective after the electrophile stress response.

the neurotoxicity of 3-nitropropionic acid, and the carcinogenicity of benzo[a]pyrene (Klaassen and Reisman, 2010). Conversely, liver-specific deletion of Keap1 constitutively activates Nrf2 in hepatocytes, causing them to overexpress many detoxifying enzymes and become resistant to acetaminophen-induced hepatotoxicity.

Nrf2 can be activated by treatment with several chemicals of low toxicity that are thiol-reactive as soft electrophiles or oxidants.

These include tBHQ, butylated hydroxyanisole (BHA), sulforaphane (a compound in broccoli with an electrophilic isothiocyanate group), curcumin (a compound in turmeric containing 2  $\alpha,\beta$ -unsaturated ketone groups), resveratrol (a hydroquinone-type compound in red grapes that may undergo oxidation to a quinone and redox cycling, like shown for tBHQ in Fig. 3-28), derivatives of oleoanolic acid, such as 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic

acid (CDDO, which contains 2 electrophilic enone groups), and oltipraz (a dithiolene compound whose metabolites can form mixed disulfides with thiols, probably also with those on Keap1). These chemicals induce Nrf2 target genes (Fig. 3-27) and protect from toxicant-induced tissue injury and cancer. For example, treatment with oleanolic acid or CDDO ameliorates acetaminophen-induced hepatotoxicity and aflatoxin-induced hepatocarcinogenesis (Klaassen and Reisman, 2010). The superpotent Nrf2 agonist CDDO is now tested clinically for use in chronic kidney diseases. Of these chemopreventive agents, some (eg, *t*BHQ, BHA, and resveratrol) are antioxidants; hence the inappropriate names “antioxidant response” and “antioxidant response element” (ARE) for the Nrf2-mediated adaptive alterations and the cognate DNA-binding site for Nrf2, respectively. It became apparent only later that the electrophilic quinone metabolites of these chemicals are the inducers and not the antioxidants.

**Adaptation by Decreasing the Target Density or Responsiveness** Decreasing the density and sensitivity of the xenobiotic target is an adaptation mechanism for several cell surface receptors. Such alterations underlie the tolerance induced by opioids, abused drugs of considerable clinical toxicological interests.

**Induction of Opioid Tolerance** The main target of opioids (eg, morphine, heroine, methadone) is the  $\mu$ -opioid receptor. Stimulation of this  $G_i$  protein-coupled inhibitory receptor by an agonist results in adenylyl cyclase inhibition (causing decline in cyclic AMP levels and PKA activity) and  $K^+$  channel opening (causing hyperpolarization) (Fig. 3-15) in neurons with opioid receptors, such as those in the midbrain periaqueductal gray. Even brief stimulation induces adaptive alterations: the receptor is desensitized by G-protein receptor kinase-mediated phosphorylation and  $\beta$ -arrestin binding, and then becomes uncoupled from the G protein and internalized via a clathrin-dependent pathway. Whereas some receptors are recycled to the cell membrane, others are degraded in the lysosomes, causing receptor downregulation (Bailey and Connor, 2005). On prolonged stimulation, adenylyl cyclase signaling undergoes a compensatory increase. Tolerance to opioids, though far from being clarified mechanistically, may result from downregulation of the receptors and upregulation of adenylyl cyclase signaling. These changes would require increasing doses of agonist to produce an effect (ie, inhibition of adenylyl cyclase signaling) as intensive as after its first application. These adaptive changes could also explain the withdrawal reaction, that is, appearance of clinical symptoms (dysphoria, excitement, pain sensation), contrasting with the pharmacologic effects of opioids (euphoria, sedation, analgesia), on abrupt termination of drug treatment, because withdrawal of the opioid would disinhibit the reinforced signaling it had inhibited. Nevertheless, mechanistic relationships between tolerance and the withdrawal reaction remain controversial (Bailey and Connor, 2005). An important clinical feature of opioid tolerance is that the tolerance to the respiratory depressive effect is short-lived and sensitivity returns after some abstinence. Therefore, abusers often kill themselves with a dose tolerated earlier.

**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 in regenerating the injured tissue.

**Induction of Enzymes Repairing Oxidized Proteins—The Electrophile Stress Response, Part 2** After sublethal exposure to chemicals, such as *t*BHQ, 4-hydroxynon-2-enal, and  $Cd^{2+}$ , not

only enzymes functioning in xenobiotic detoxication but also some of those mediating protein repair become overexpressed as part of the above-described electrophile response. The induced proteins include thioredoxin 1 (Trx1) and thioredoxin reductase 1 (TR1), which can reduce oxidized proteins (protein disulfides, -sulfenic acids, and -methionine sulfoxides) (Fig. 3-22), and several subunits of the proteasome complex, which hydrolyzes damaged proteins. These repair proteins are transcribed from genes containing EpRE, and their transcription is controlled by Nrf2 (Fig. 3-27). As Trx1 and TR1 are reduction partners for ribonucleotide reductase, they support this enzyme in forming deoxyribonucleotides for DNA synthesis. Thus, induction of Trx1 and TR1 also assists DNA repair.

**Induction of Chaperones Repairing Misfolded Proteins—The Heat-Shock Response** The cellular abundance of many molecular chaperones, which can disaggregate and refold denatured proteins, also increases after physical and chemical stresses (eg, heat, ionizing radiation, oxidants, electrophile reactants, and metal ions). Two adaptive reactions involving overexpression of chaperones are known; they are the heat-shock response and the EPR stress response.

Although first observed as a result of hyperthermia, the heat-shock response is an adaptive mechanism also triggered by various pathologic conditions (eg, trauma, tissue ischemia) and by virtually all reactive chemicals and/or their metabolites (eg, electrophiles, oxidants, lipid peroxidation products, metal ions, arsenite) that denature proteins. Thus, it takes place simultaneously with the electrophile response discussed above. This reaction, however, is governed by heat-shock transcription factors (HSF), mainly HSF1, which transactivate genes that encode Hsp through heat-shock response elements (HSE) within the promoter region. HSF1, like Nrf2, normally resides in the cytoplasm, where it associates with Hsp90, Hsp70, and Hsp40. On heat- or chemical-induced protein damage, these Hsps are purportedly sequestered by damaged proteins, allowing HSF1 to be released. HSF1 then migrates into the nucleus, trimerizes, undergoes phosphorylation, and stimulates the transcription of Hsp genes. As described in the section “Repair of Proteins,” the chaperones Hsp90 and Hsp70, together with co-chaperone proteins, are especially important in maintaining the integrity of hundreds of proteins (Pratt *et al.*, 2010). The client proteins include not only those carrying out housekeeping functions but also those involved in signaling and apoptosis. Therefore, induction of Hsps has pleiotropic effects besides increased protection from cytotoxicity. However, when the proteotoxic stress induces excessive protein unfolding, Hsp70 and Hsp40 recruit the ubiquitin system (Fig. 3-23) to tag the aberrant proteins for proteasomal degradation (Bedford *et al.*, 2011).

**Induction of Chaperones Repairing Misfolded Proteins—The Endoplasmic Reticulum Stress and the Unfolded Protein Response** All proteins that are destined for export or insertion into cellular membranes pass through the EPR. In this  $Ca^{2+}$ -rich oxidative environment, they may be subjected to *N*-glycosylation at asparagine residues by oligosaccharyltransferase, formation of disulfide bonds by protein disulfide isomerases, and folding assisted by EPR-resident chaperones, such as the glucose-regulated proteins Grp78 (also called binding immunoglobulin protein [BiP]) and Grp94 as well as the  $Ca^{2+}$ -binding proteins calreticulin and calnexin. Damage of proteins being processed in the EPR by reactive metabolites produced in situ by CYP enzymes (eg, the quinonimine metabolite of acetaminophen,  $Cl_3C^{\bullet}$  and  $Cl_3COO^{\bullet}$  radicals formed from  $CCl_4$ , and ROS generated by CYP2E1 during oxidation of ethanol) and/or depletion of  $Ca^{2+}$  in the EPR lumen (eg,

by inactivation of the EPR  $\text{Ca}^{2+}$ -ATPase by reactive metabolites) causes accumulation of unfolded or misfolded proteins, a condition known as EPR stress (Cribb *et al.*, 2005; Nagy *et al.*, 2007; Malhi and Kaufman, 2011). Protein folding disorder can also be experimentally induced by some natural compounds that disturb the homeostasis of EPR at specific steps, such as thapsigargin (which blocks SERCA-mediated  $\text{Ca}^{2+}$  uptake into EPR; see Table 3-7), tunicamycin (which inhibits *N*-glycosylation of proteins), castanospermine (which interferes with maturation of nascent glycoproteins by deglycosylation), and brefeldin A (which inhibits transport of proteins from EPR to Golgi).

When the load of unfolded or misfolded proteins in the EPR exceeds the capacity of EPR-resident chaperones, these proteins trigger a complex adaptive response, called unfolded protein response (UPR). This may constrain exacerbation of the disorder (1) by attenuation of mRNA translation to decrease the functional load on the EPR, (2) by increased transcription of EPR chaperones to boost the folding capacity, (3) by initiating the translocation of aberrant proteins via translocon peptide channels from the EPR into the cytosol for proteasomal degradation, and (4) by eliminating the affected cell via apoptosis, if the EPR stress is sustained or massive. The eukaryotic translation initiation factor  $2\alpha$  (eIF2 $\alpha$ ) and TFs X-box binding protein 1 (XBP1), ATF6, ATF4, and C/EBP homologous protein (CHOP) (or Gadd153) are the major executioners of this adaptive response.

Three transmembrane EPR proteins sense the overload of the EPR lumen with misfolded proteins and transduce this signal to initiate the UPR. These are (1) PERK, (2) inositol-requiring enzyme 1 $\alpha$  (IRE1 $\alpha$ ), a protein with kinase and endoribonuclease (RNase) activities, and (3) the precursor form ATF6 (activating TF 6). Normally, these sensor proteins are turned off by being associated at their luminal domain with the EPR-resident soluble chaperone BiP (or Grp78). However, when EPR stress occurs, the unfolded proteins that accumulate in the lumen of the EPR sequester BiP away from these sensors, thereby turning them on to signal for the UPR. PERK stripped off BiP is activated by dimerization and trans-autophosphorylation. With its kinase activity thus raised, PERK catalyzes the phosphorylation of the eIF2 $\alpha$ , with the phosphorylated eIF2 $\alpha$  causing a global translation attenuation and decreased synthesis of most (but not all) proteins. The second EPR stress sensor IRE1 $\alpha$  also undergoes dimerization and trans-autophosphorylation on dissociation from BiP. These changes boost the RNase activity of IRE1 $\alpha$ , enabling it to cleave and process a mRNA into one that translates into XBP1, an active TF, another executioner of UPR. The third sensor ATF6 resides as a 90 kDa precursor protein in the EPR with its Golgi localization sequences masked by BiP. On dissociation from BiP, ATF6 translocates to the Golgi, undergoes a so-called regulated intramembrane proteolysis (by site-1 and site-2 proteases), yielding a 50 kDa fragment, the active TF ATF6. XBP1 and ATF6 bind alone or as heterodimers to cognate DNA sequences, such as EPR response element (ERSE) and unfolded protein response element (UPRE), and activate the transcription of genes coding for chaperone proteins that promote protein folding (eg, Grp78, Grp94, Mdj1/Erdj4, Herp) and for proteins that assist in degradation of misfolded proteins (eg, EPR degradation-enhancing  $\alpha$ -mannosidase-like protein [EDEM]).

On excessive EPR stress, the three activated sensors, that is, PERK, ATF6, and IRE1 $\alpha$ , may act in concert to initiate apoptosis (Malhi and Kaufman, 2011). While PERK-catalyzed phosphorylation of eIF2 $\alpha$  decreases the synthesis of most proteins (as stated above), it increases the synthesis of some, including that of ATF4. This TF (as well as ATF6) promotes the expression of CHOP, yet another TF. CHOP in turn can transcriptionally induce

the expression of the proapoptotic Bim protein and the cell surface death receptor TRAIL-2, while inhibiting the expression of the antiapoptotic Bcl-2 protein (see Fig. 3-19). The activated IRE1 $\alpha$  may initiate cell death via recruiting the adaptor protein TNF receptor-associated factor-2 (TRAF2), with subsequent activation of apoptosis signal-regulating kinase 1 (ASK1) and JNK. By phosphorylation, JNK can activate the proapoptotic Bim and inactivate the antiapoptotic Bcl-2 proteins (Malhi and Kaufman, 2011). The IRE1 $\alpha$ -TRAF2 complex may also recruit and activate procaspase 12. Alternatively,  $\text{Ca}^{2+}$  released from the EPR can activate calpains, which cleave the EPR-associated procaspase 12 into active caspase 12. The latter then engages the caspase cascade (see Fig. 3-19) by activating effector caspases therein to sacrifice the cell by apoptosis. Several toxicants have been shown to induce EPR stress in experimental animals and in isolated cells by demonstrating expression of some elements of the UPR on toxicant exposure (Cribb *et al.*, 2005; Nagy *et al.*, 2007; Malhi and Kaufman, 2011).

### Induction of Enzymes Repairing DNA—The DNA Damage Response (DDR)

DDR is initiated by detection of the DNA damage. Double-stranded DNA breaks are recognized by the MRN complex (the trimer of Mre11, Rad50, and Nbs1 proteins) or the Ku protein (the dimer of Ku70 and Ku80; see the section “Repair of DNA”) that bind and activate protein kinases ataxia telangiectasia mutated (ATM) and DNA-PKcs, respectively. A third kinase, ataxia telangiectasia and Rad3-related (ATR), is activated in response to persistent single-stranded DNA coated with replication protein A (RPA), an intermediate during nucleotide excision or recombination DNA repair processes. Directly or through checkpoint kinases (Chk1, Chk2), these kinases phosphorylate p53 (Christmann *et al.*, 2003; McGowan and Russell, 2004), a protein that can play the role of a TF regulating gene expression and the role of an associate protein affecting the function of its interacting protein partner. Normally, p53 is kept inactive and at low levels by its binding protein mdm2 (see Fig. 3-33), which ubiquitinates p53, facilitating its proteasomal degradation. On phosphorylation, p53 escapes from mdm2, allowing its activation and stabilization. Indeed, the levels of p53 protein in cells increase dramatically in response to DNA damage caused by UV or gamma irradiation or genotoxic chemicals. p53 then facilitates DNA repair by a number of mechanisms. For example, mainly by transcriptionally upregulating the cyclin-dependent kinase inhibitor protein p21, p53 arrests cells in G1 phase of the cell cycle (see Fig. 3-33), allowing more time for DNA repair. As a TF, p53 also increases expression of proteins directly involved in DNA repair (Harms *et al.*, 2004). Such proteins include (a) growth arrest and DNA damage inducible (gadd45), which interacts with histones and facilitates access of proteins (eg, topoisomerase) to DNA, (b) XPE and XPC, members of the xeroderma pigmentosum group of proteins important in UV-induced DNA damage recognition before nucleotide excision repair, (c) MSH2 operating in mismatch repair, (d) PCNA that holds DNA polymerase- $\delta$  to DNA during DNA replication as well as reparative DNA synthesis in the excision and the recombination repair processes, and (e) a form of ribonucleotide reductase that provides deoxyribonucleotides for sealing DNA gaps. As a partner protein, p53 supports the function of several proteins of the nucleotide excision machinery (eg, TFIIH, XPB, XPD). (Other roles p53 plays in apoptosis and in carcinogenesis as a tumor suppressor protein are illustrated in Figs. 3-19 and 3-33 and discussed elsewhere in this chapter.) The DNA-damage-activated protein kinases (ATM, ATR, and DNA-PK) also phosphorylate histone H2AX adjacent to the damage, which then becomes ubiquitinated. These markings in turn recruit repair proteins (including BRCA1) and induce chromatin



relaxation for better access of repair enzymes to the lesion. The DDR-initiating kinases have numerous other protein substrates, including other kinases; therefore, DDR may extend to diverse cellular functions. It is important to realize that while both the complex DDR and distinct DNA repair mechanisms (eg, direct repair by MGMT) protect normal cells that suffered DNA damage from transformation into cancer cells (a process to be discussed below), these mechanisms, if remain operative in cancer cells, can protect them from mortal DNA damage inflicted by radiotherapy or chemotherapy, and thereby DDR and DNA repair can contribute to resistance of tumor cells to anticancer treatments.

#### Adaptive Increase in Tissue Repair—A Proliferative Response

Many toxicants potentially injurious to cells, for example, electrophiles, oxidants, and those inducing oxidative stress, can initiate mitogenic signaling as a prelude to tissue repair via cell replacement. It appears that the need for mitogenesis is sensed by PTP (eg, PTP1B) and the lipid phosphatase PTEN, which contains reactive cysteine thiols at their active site (Rhee *et al.*, 2005). These phosphatases serve as brakes on the growth factor receptor-initiated mitogenic signaling, as PTPs dephosphorylate (and inactivate) the receptors themselves (eg, EGFR, PDGFR, IGFR) as well as some protein kinases (eg, Src and JAK), whereas PTEN dephosphorylates  $PIP_3$ , an important second messenger in the PI3K–Akt–IKK–NF- $\kappa$ B pathway (Fig. 3-12). Electrophiles covalently bind to essential cysteine-SH groups in these phosphatases. HOOH can oxidize the critical -SH group in PTEN to an intramolecular disulfide, whereas it oxidizes the -SH group of PTP1B, through sulfenic acid (-S-OH), to a 5-membered cyclic sulfenyl amide species in which the sulfur atom is covalently linked to the nitrogen of the neighboring serine (Rhee *et al.*, 2005). Inactivation of PTPs and PTEN, which decrease the proliferative signal transduction, amplifies intracellular signaling for mitosis and survival.

It has been known for some time that oxidative stress, if not severe, activates the TF NF- $\kappa$ B (Dalton *et al.*, 1999). For example, silica, which can produce ROS on its surface, activates NF- $\kappa$ B as well as PI3K when added to various cells (Castranova, 2004). In light of new information discussed above, NF- $\kappa$ B activation is now attributed to the fact that this TF is situated downstream of growth factor receptors (which are negatively controlled by the ROS-sensitive PTP) and  $PIP_3$  (which is eliminated by ROS-sensitive PTEN) (Fig. 3-12). Furthermore, NF- $\kappa$ B is at the focal point of proliferative and prolife signaling, as it transactivates genes producing cell cycle accelerators (eg, cyclin D1 and c-Myc) and apoptosis inhibitors (eg, antiapoptotic Bcl proteins and the caspase IAPs) (Karin, 2006). In addition, NF- $\kappa$ B also transactivates the genes of ferritin, GST, SOD1, HO-1, a proteasome subunit, and gadd45, facilitating detoxication and molecular repair. All these roles of NF- $\kappa$ B explain its involvement in tolerance to chemically induced tissue injury, resistance against cholestatic liver injury caused by bile acids, adaptation to ionizing radiation, as well as the phenomenon termed preconditioning. This is a tolerance to ischemic tissue injury (eg, myocardial infarction), a tolerance induced by temporarily enhanced ROS formation evoked by hyperoxia or brief periods of ischemia–reperfusion.

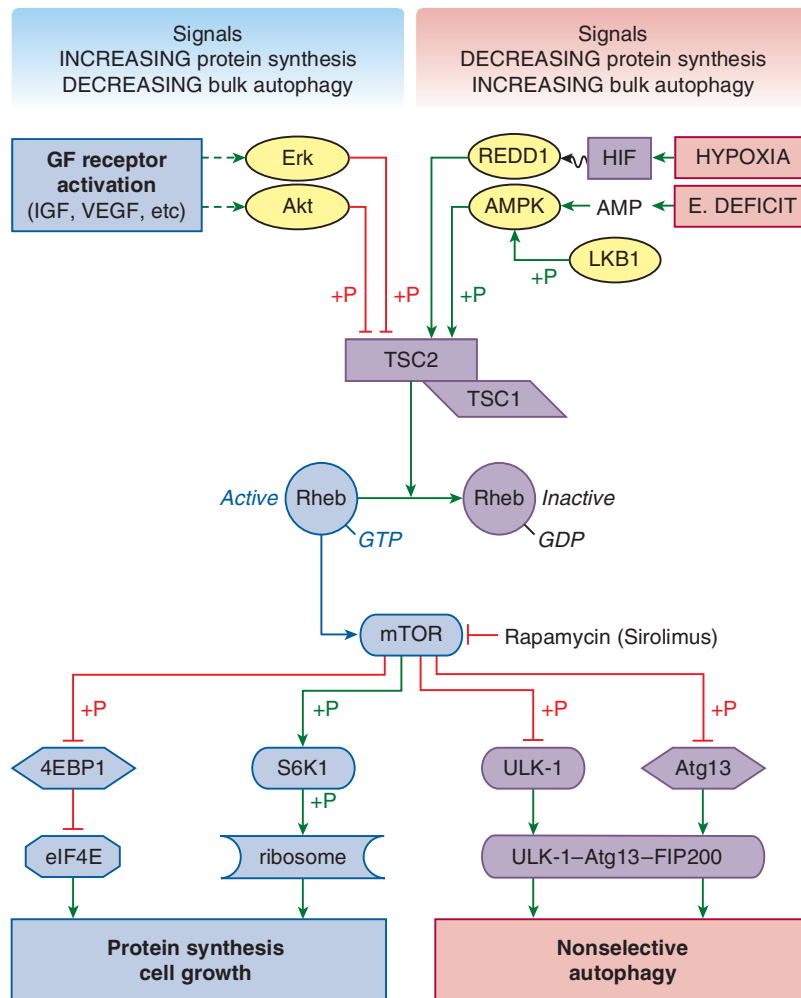
In addition to signaling for cell replacement in damaged tissue—in which NF- $\kappa$ B plays a leading role—the growing cells need to boost protein synthesis. This is done under the control of the protein kinase mTOR. As shown in Fig. 3-29, mTOR activation results from signaling through both pathways coupled to growth factor receptors, that is, the MAPK pathway leading to phosphorylation of the MAPK isoform Erk and the PI3K pathway leading to phosphorylation of Akt (see Fig. 3-12). Importantly,

these pathways are subject to activation in response to oxidant or electrophile exposure as they are controlled by PTPs and PTEN (Fig. 3-12). Erk and Akt protein kinases activate mTOR through a complex mechanism (Fig. 3-29), and mTOR in turn 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 (Shaw and Cantley, 2006). As described in the sections “Adaptation to Hypoxia—The Hypoxia Response” and “Adaptation to Energy Depletion—The Energy Stress Response,” mTOR signaling is switched off in the cell to save energy as a measure to adapt to the energy shortage caused by hypoxia or toxic impairment of ATP synthesis.

**Adaptation by Compensating Dysfunction** Dysfunctions caused by toxicants or drug overdose manifested at the level of organism (eg, hypoxia), organ system (eg, hypotension and hypertension), or organ (eg, renal tubular dysfunction) may evoke compensatory mechanisms.

**Adaptation to Hypoxia—The Hypoxia Response** When  $O_2$  delivery is impaired and hypoxia persists for more than a few minutes, a response involving gene expression alteration is initiated. This reaction is mainly orchestrated by hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ), a ubiquitous TF whose activity and cellular abundance is greatly increased in response to hypoxia (Maxwell and Salnikow, 2004; Pouyssegur *et al.*, 2006). HIF-1 $\alpha$  is maintained at very low intracellular levels because of continuous hydroxylation of its 2 proline residues by HIF-prolyl hydroxylases. This permits a ubiquitin ligase subunit (called von Hippel Lindau protein [VHL]) to capture HIF-1 $\alpha$  and initiate its destruction by proteasomal degradation. Indeed, HIF-1 $\alpha$  is one of the shortest lived proteins with a half-life of less than 5 minutes. In addition, HIF-1 $\alpha$  is kept transcriptionally inactive by hydroxylation at one of its asparagine residues by HIF-asparagine hydroxylases, which prevents interaction of HIF-1 $\alpha$  with transcriptional coactivators, such as p300 and CREB-binding protein (CBP). These two types of HIF hydroxylases are  $O_2$  sensors: they use  $O_2$  as a substrate to carry out proline/asparagine hydroxylations with concomitant oxidative decarboxylation of 2-oxoglutarate to succinate, with the  $K_M$  of  $O_2$  being close to the ambient  $O_2$  concentration. As the  $O_2$  concentration falls, decreases in the hydroxylation rate of HIF-1 $\alpha$  as well as its VHL-mediated ubiquitination and proteasomal degradation occur, and this increases its abundance and transcriptional activity. HIF hydroxylases belong to the  $Fe^{2+}$  and ascorbate-dependent dioxygenases (the largest group of non-heme oxidases); therefore, not only hypoxia but also  $Fe^{2+}$  deficiency impairs their activity. The latter feature explains why iron chelators (eg, deferoxamine) or  $Fe^{2+}$ -mimicking metal ions (eg,  $Co^{2+}$  and  $Ni^{2+}$ ) also induce and activate HIF-1 $\alpha$  (Maxwell and Salnikow, 2004). When induced after hypoxic conditions, HIF-1 $\alpha$  dimerizes with HIF-1 $\beta$  (also called Arnt, which, coincidentally, is the dimerization partner for the Ah receptor as well). The HIF complex transactivates a vast array of genes with hypoxia response element (HRE) in their promoter. Many of the gene products assist in acclimatization to hypoxia (Pouyssegur *et al.*, 2006). These include (1) erythropoietin (EPO) that is produced largely in kidney and activates erythropoiesis in bone marrow, (2) proteins involved in iron homeostasis (eg, transferrin, TFR, ceruloplasmin, and heme oxygenase) that may increase availability of iron for erythropoiesis, (3) vascular endothelial growth factor (VEGF) and angiopoietin-2 that stimulate blood vessel growth (ie, angiogenesis), (4) proteins facilitating anaerobic ATP synthesis from glucose (ie, glycolysis), such as the glucose transporter GLUT1 and





**Figure 3-29. Modulation of protein synthesis and autophagy by signaling through the mTOR kinase pathway as a means for cellular adaptation—**increased signaling via mTOR promotes protein synthesis at translational level and suppresses autophagy, thereby permitting cell growth and proliferation, whereas attenuated signaling via mTOR permits energy saving (by halting protein synthesis) and a desperate attempt for energy generation (by starting autophagy) when hypoxia, nutrient shortage, or toxic injury causes energy deficit. Growth factor receptors signaling via either the MAPK pathway (leading to phosphorylation of Erk) or the PI3K pathway (leading to phosphorylation of Akt) (see Fig. 3-12) activate the serine/threonine kinase, mammalian target of rapamycin (mTOR; rapamycin is also called sirolimus) by an indirect mechanism. The protein kinases Erk and Akt catalyze inactivating phosphorylation of TSC2, a member of TSC1/2 complex. TSC2 is a GTPase-activating protein whose substrate is Rheb, a small G protein (Ras homologue), which is active in the GTP-bound form and inactive in the GDP-bound form. With its GTPase activating capacity blocked by Erk or Akt, TSC2 cannot convert Rheb-GTP into inactive Rheb-GDP, and thus Rheb-GTP activates mTOR. In turn, mTOR phosphorylates 2 substrates that are necessary to initiate translation of mRNA into proteins, that is: (1) eukaryotic initiation factor 4E-binding protein-1 (4EBP1), which thus releases the translational initiation factor eIF4E, and (2) ribosomal protein S6 kinase-1 (S6K1), which phosphorylates ribosomal protein S6, thereby increasing translational efficiency of mRNAs that encode ribosomal proteins (“ribosomal biogenesis”). Simultaneously, mTOR phosphorylates and inactivates unc-51-like kinase-1 (ULK-1) and Atg13, thereby preventing nonselective or bulk autophagy that is dependent on formation of the stable complex of ULK-1, Atg13, and focal adhesion kinase family-interacting protein of 200 kDa (FIP200).

Protein synthesis for cell growth and proliferation is halted, whereas bulk autophagy is facilitated in times of energy deficit resulting from hypoxia, nutrient shortage, or toxic impairment of ATP production. Then AMP levels increase and AMP binds to the AMP-activated protein kinase (AMPK), facilitating its phosphorylation by protein kinase LKB1. Activated AMPK phosphorylates TSC2 (at a site different from that targeted by Akt and Erk), thereby increasing the GTPase-activating capacity of TSC2. This in turn switches Rheb off, making mTOR inactive. This suspends mRNA translation and starts autophagy. Hypoxia can initiate this process in a more specific way as well, that is, via stabilization of the hypoxia-inducible factor (HIF). This transcription factor induces the synthesis of REDD1 (by a mechanism that is not completely understood), which activates TSC2. See text for further details on cellular responses to hypoxia and energy deficit.

some glycolytic enzymes, (5) proteins that correct acidosis caused by glycolytic overproduction of lactate (eg, a monocarboxylate transporter and a  $\text{Na}^+/\text{H}^+$  exchanger for export of lactate and  $\text{H}^+$ , respectively), (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 the protein kinase mTOR (Fig. 3-29), and (7) many other proteins, such as

those that promote ECM remodeling (eg, matrix metalloproteinase-2) and cell migration (perhaps to facilitate access of the cells to the blood vessel), as well as BNIP3, a proapoptotic MOM protein that is also involved in autophagy of mitochondria in reticulocytes (perhaps to induce apoptosis of cells subjected to extreme hypoxia and to facilitate the terminal differentiation of red blood cells). Experiments on mice kept in low  $\text{O}_2$  environment (hypoxic

preconditioning) demonstrated that HIF-1 $\alpha$  became stabilized in the retina of these animals, hypoxia-responsive genes (EPO, VEGF) were induced, and the retina became resistant to light toxicity (Grimm *et al.*, 2005). Adaptation to hypoxia also occurs, for example, in response to high-altitude hypoxia, chronic cardiorespiratory dysfunction, and ischemic preconditioning, along with other adaptive responses discussed above. The hypoxia response is also expected to develop as a result of toxicities causing hypoxia acutely or subacutely (eg, respiratory muscle weakness after organophosphate intoxication, diquat-induced pulmonary injury) or as a delayed sequel (eg, respiratory surface restriction in hard metal disease).

### Adaptation to Energy Depletion—The Energy Stress Response

Cells try to maintain their adenosine nucleotide pool in triphosphorylated, energized state, which is in the form of ATP. When the rephosphorylation rate of AMP and ADP to ATP does not keep up with the rate of ATP use, because, for example, oxidative phosphorylation is impaired or ATP use for muscle contraction or ion pumping is excessive, the ratio of AMP to ATP increases. A cellular mechanism has evolved to sense this menacing energy deficit and, in order to compensate, boosts ATP production and curtails ATP consumption (Hardie *et al.*, 2006). The sensor is a ubiquitous heterotrimeric intracellular protein complex called AMP-activated protein kinase (AMPK). AMP strongly activates AMPK allosterically, and also by making it susceptible for phosphorylation by protein kinase LKB1 (or by the calmodulin-dependent protein kinase kinase [CaMKK] in neurons). The phosphorylated, and thus activated AMPK, targets 2 sets of proteins. One set includes those whose activation facilitates ATP production from catabolism of glucose and fatty acids as well as by promoting the biogenesis of mitochondria. For example, AMPK activation increases (a) glucose uptake (via recruiting to the cell membrane or activating glucose transporters GLUT4 and GLUT1), (b) glycolysis (via phosphorylation and activation of 6-phosphofructo-2-kinase [PFK-2] whose product, fructose-2,6-bisphosphate, is a glycolytic activator), and (c) fatty acid oxidation in mitochondria (via phosphorylation and inactivation of acetyl-CoA-carboxylase, whose product, malonyl-CoA, is an allosteric inhibitor of carnitine palmitoyltransferase-1 [CPT-1], which mediates uptake of long-chain fatty acid CoA esters into mitochondria). Another set of proteins, which are inactivated by AMPK (directly or indirectly), includes those that are involved in biosynthetic ATP-consuming reactions. Thus, AMPK inhibits (a) glycogen synthesis via phosphorylation and inactivation of glycogen synthase, (b) lipid synthesis by phosphorylating and inactivating acetyl-CoA-carboxylase, whose product, malonyl-CoA, is an essential substrate for fatty acid synthesis, (c) cholesterol synthesis by phosphorylating and inactivating HMG-CoA reductase, (d) glucose synthesis via inactivating phosphorylation of a transcriptional coactivator, TORC2, which then decreases expression of key gluconeogenic enzymes, such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase, and (e) protein synthesis, and thus cell growth, by inhibiting the protein kinase mTOR (Fig. 3-29). AMPK-mediated modulation of cellular energy supply and consumption involves mainly kinase reactions rather than new protein synthesis. Therefore, this adaptation is a rapid process. It can be a response to any harmful condition that compromises oxidative phosphorylation, such as hypoxia, hypoglycemia (especially in neurons), and chemically induced mitochondrial toxicity. For example, cells exposed to arsenite exhibit rapid increases in the AMP/ATP ratio and AMPK activity, with concomitant declines in HMG-CoA reductase activity as well as fatty acid and cholesterol synthesis (Corton *et al.*, 1994).

Apart from the above-described rapid AMPK-dependent reprogramming of the cell's intermediary metabolism from energy-consuming operation to the energy-producing mode, there is an ultimate means for the cell to generate fuel for ATP production in case of nutrient shortage. This slower process involves consumption of the cell's own constituents (lipid droplets, glycogen particles, proteins, and organelles) by nonselective or bulk autophagy (Rabinowitz and White, 2010) involving their lysosomal hydrolysis in order to gain fuel (amino acids, fatty acids, nucleosides, and carbohydrates). An essential actor in this form of autophagy is a protein complex of unc-51-like kinase-1 (ULK-1), autophagy-related protein 13 (Atg13), and focal adhesion kinase family-interacting protein of 200 kDa (FIP200), which is an initiator of autophagosome formation (Fig. 3-29). When nutrients are abundant, the ULK-1–Atg13–FIP200 complex associates with mTOR complex 1 (mTORC1), with both ULK-1 and mTOR kinase being bound by Raptor, a scaffolding protein in mTORC1. Then mTOR phosphorylates and inactivates ULK-1 and Atg13, thus inhibiting autophagy. In nutrient shortage, signaling pathways, such as those activated by AMP and hypoxia (see Fig. 3-29), inactivate mTOR kinase, thereby forcing mTORC1 to release the complex of ULK-1–Atg13–FIP200, which in turn initiates autophagosome nucleation (Hosokawa *et al.*, 2009).

In summary, mTOR kinase is a key regulator of the cell's energy homeostasis, permitting or limiting cell growth according to availability of resources, the use of which can also be controlled by mTOR through switching autophagy on and off. In the presence of abundant nutrients and growth factors, mTOR is activated, thereby promoting cell growth and metabolic activity while suppressing nonselective autophagy. In nutrient deprivation or stress (eg, energetic failure, hypoxia), signaling pathways inactivate mTOR kinase activity. This both suppresses cell growth to reduce energy demand and induces autophagy to enable stress adaptation and survival (Fig. 3-29).

After surveying the major cellular adaptation mechanisms to toxicants, it is easy to recognize that one noxious effect may initiate several adaptive responses. For example, cells exposed to a hypoxic environment can rapidly respond with both AMPK-mediated program of energy stabilization and HIF-1 $\alpha$ -directed adaptation to oxygen shortage. Theoretically, an electrophile toxicant that can bind covalently to cellular macromolecules and can also generate oxidative stress, such as a redox cycling quinone, would be expected to induce a number of adaptive processes, including the electrophile response, the heat-shock response, the EPR stress response, the DDR, and the proliferative response, and if it compromises ATP synthesis as well, it even induces the energy stress response.

**Adaptation by Neurohumoral Mechanisms** There are numerous adaptive responses to dysfunctions of organs or organ systems that are mediated by humoral or neuronal signals between cells located in the same or different organs. For example, the rapid hyperventilation evoked by acute hypoxia or HCN inhalation is mediated by a neural reflex initiated by glomus cells in the carotid body. These chemosensitive cells generate a Ca<sup>2+</sup> signal via the above-described AMP sensor, AMPK, which becomes activated by hypoxia or CN<sup>-</sup> through impairment of oxidative phosphorylation in these cells, causing rise in the AMP/ATP ratio (Evans *et al.*, 2005). Besides CN<sup>-</sup>, mitochondrial electron transport inhibitors (eg, rotenone, antimycin A, myxothiazole), uncouplers (eg, 2,4-dinitrophenol), or ATP-synthase inhibitors (eg, oligomycin) (see Table 3-6 and Fig. 3-16) as well as AMPK activators mimic the response to hypoxia in these cells. There are numerous other neurohumoral adaptive mechanisms

in the body, such as the sympathetic reflex as well as activation of the renin–angiotensin–aldosterone system in response to hypotension, and the feedback systems between endocrine glands and the hypothalamus–hypophysis, which correct abnormal hormone levels. For information on these and other mechanisms, the reader is referred to textbooks of physiology.

## When Repair and Adaptation Fail

**When Repair Fails** Although repair mechanisms operate at molecular, cellular, and tissue levels, for various reasons they often fail to provide protection against injury. First, the fidelity of the repair mechanisms is not absolute (eg, the nonhomologous end joining that restores DNA DSB), making it possible for some lesions to be overlooked or erroneously fixed. However, repair fails most typically when the damage overwhelms the repair mechanisms, as when protein thiols are oxidized faster than they can be reduced. In other instances, the capacity of repair may become exhausted when necessary enzymes or cofactors are consumed. For example, alkylation of DNA may lead to consumption of MGMT (a self-sacrificing enzyme), lipid peroxidation can deplete  $\alpha$ -tocopherol, and overproduction of oxidized or otherwise damaged proteins can exhaust the pool of ubiquitin. Sometimes the toxicant-induced injury adversely affects the repair process itself. For example, ethanol generates ROS via CYP2E1 that impairs the proteasomal removal of damaged proteins. Autophagic removal of cell constituents may be compromised by lysosomotropic drugs (lipophilic amines) that increase the pH in these organelles, thus decreasing the activity of lysosomal hydrolases. This may underlie the mechanism of chloroquine-induced myopathy in rats. Thiol-reactive chemicals may inactivate ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and the HECT domain-containing ubiquitin ligases (E3), each of which contains a catalytically active cysteine, as well as the lysosomal cysteine proteases (eg, cathepsins B, H, and L), thereby compromising the clearance of damaged proteins by both the UPS and autophagy. Indeed, diminished proteolysis occurs in hepatocytes exposed to toxic concentrations of acetaminophen. These repair mechanisms are especially important in neurons, as genetic deletion of proteins mediating UPS and autophagy causes accumulation of intraneuronal inclusions, neuronal loss, or neurodegeneration. In fact, human neurodegenerative diseases can be directly attributed to some dysfunction of the UPS and/or autophagy (Bedford *et al.*, 2011). The finding that individual overexpression of some E2 and E3 enzymes confers resistance to methylmercury toxicity in yeast (Hwang *et al.*, 2006) lends some support to the speculation that impairment of the UPS and/or autophagy might contribute to the neurotoxicity of methylmercury. After exposure to necrogenic chemicals, mitosis of surviving cells may be blocked and restoration of the tissue becomes impossible (Mehendale, 2005). Finally, some types of toxic injuries cannot be repaired effectively, as occurs when xenobiotics are covalently bound to proteins or when protein carbonyls are formed. Thus, toxicity is manifested when repair of the initial injury fails because the repair mechanisms become overwhelmed, exhausted, or impaired or are genuinely inefficient.

It is also possible that repair contributes to toxicity. This may occur in a passive manner, for example, if excessive amounts of  $\text{NAD}^+$  are cleaved by PARP when this enzyme assists in repairing broken DNA strands, or when too much  $\text{NAD(P)H}$  is consumed for the repair of oxidized proteins and endogenous reductants. Either event can compromise oxidative phosphorylation, which is also dependent on the supply of reduced cofactors (see Fig. 3-16), thus causing or aggravating ATP depletion that contributes to cell injury.

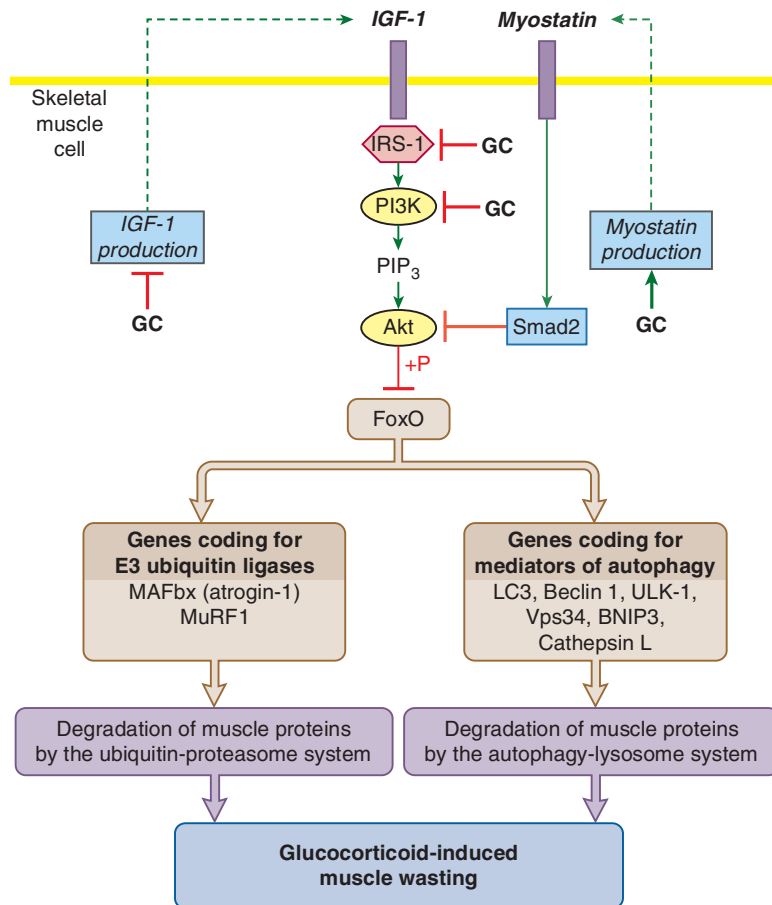
Excision repair of DNA and reacylation of lipids also contribute to cellular deenergization and injury by consuming significant amounts of ATP. However, repair also may play an active role in toxicity. This is observed after chronic tissue injury, when the repair process goes astray and leads to uncontrolled proliferation instead of tissue remodeling. Such proliferation of cells may yield neoplasia, whereas overproduction of ECM results in fibrosis. A specific case is when the cellular repair processes that normally degrade damaged proteins, such as the UPS and the autophagy pathway, become inappropriately stimulated by adverse signaling, causing degradation of ordinary intracellular proteins. This occurs in muscle wasting induced by glucocorticoids, such as cortisol and dexamethasone. This condition appears to be secondary to suppression of the growth-promoting IGF-1–PI3K–Akt signaling that leaves FoxO TFs unchecked, which in turn increase the expression of muscle-specific ubiquitin ligases (MAFbx, also called atrogen-1, and MuRF1) as well as several components of the autophagy–lysosome pathway (Fig. 3-30).

**When Adaptation Fails** Although adaptation mechanisms, such as the Nrf2-mediated electrophile response and the NF- $\kappa$ B-induced proliferative reaction, boost the capacity of the organism to withstand toxicant exposure and damage, excessive exposure can overwhelm these protective responses. Moreover, toxicants may impair the adaptive process. For example, moderate oxidative stress activates NF- $\kappa$ B, AP-1, and Nrf2 to initiate adaptive protection. However, extensive oxidant exposure aborts this program because it leads to oxidation of thiol groups in the DNA-binding domain of these TFs (Hansen *et al.*, 2006). Similarly,  $\text{Hg}^{2+}$  can incapacitate NF- $\kappa$ B, thus inhibiting the prolife program activated by this TF. This promotes  $\text{Hg}^{2+}$ -induced renal tubular cell injury (Dieguez-Acuna *et al.*, 2004).

Some adaptive mechanisms may be harmful under extreme conditions. For example, acute tubular injury, which impairs tubular reabsorption and causes polyuria, triggers a tubuloglomerular feedback mechanism that reduces glomerular blood flow and filtration. Ultimately, this may precipitate anuric renal failure. It is possible that an adaptive mechanism that is beneficial in the short term may become harmful when forced to operate for a prolonged period of time. Bulk autophagy likely contributes to lethal wasting syndrome induced by TCDD in experimental animals. Chronic inflammation, tissue injury, or cancer may lead to iron deficiency and anemia because IL-6 (the acute-phase-triggering cytokine overproduced in these conditions) upregulates hepcidin secretion from the liver, which in turn reduces intestinal iron absorption. As discussed earlier, NF- $\kappa$ B activation is indispensable for repair via proliferation of the acutely injured tissue. However, NF- $\kappa$ B also targets cytokine genes, and the cytokines (eg, TNF, IL-1 $\beta$ ) in turn activate NF- $\kappa$ B through their receptors (see Fig. 3-12). This vicious cycle may lead to chronic inflammation and cancer when repetitive tissue injury maintains NF- $\kappa$ B signaling (Karin, 2006). This occurs after occupational exposure to silica (Castranova, 2004). Sustained activation of HIF-1 $\alpha$  in tumors facilitates invasiveness, in part by increasing VEGF expression and angiogenesis. In the kidney, HIF-1 $\alpha$  may be involved in fibrogenesis, as it targets critical genes, such as tissue inhibitor of metalloproteinase-1 (TIMP-1).

## Toxicity Resulting from Inappropriate Repair and Adaptation

Like repair, dysrepair occurs at the molecular, cellular, and tissue levels. Some toxicities involve dysrepair at an isolated level. For example, hypoxemia develops after exposure to methemoglobin-forming chemicals if the amount of methemoglobin produced overwhelms



**Figure 3-30. A model of glucocorticoid-induced muscle wasting:** by attenuating the IGF-1–PI3K–Akt growth signaling pathway, glucocorticoids disinhibit FoxO transcription factors that upregulate the degradation of muscle proteins by both the ubiquitin–proteasome system and the autophagy–lysosome pathway. The trophic condition of the skeletal muscle is positively regulated by IGF-1, a muscle anabolic growth factor. IGF-1 acts in autocrine and paracrine manner on its tyrosine kinase receptor to trigger signaling through insulin receptor substrate 1 (IRS-1), phosphatidylinositol 3-kinase (PI3K), and the lipid mediator phosphatidylinositol (3,4,5)-trisphosphate (PIP<sub>3</sub>), causing activation of the serine/threonine protein kinase Akt (see also Fig. 3-12). As shown in Fig. 3-29, Akt activation leads to activation of mTOR, which boosts protein translation and halts bulk autophagy. In addition, the active Akt also phosphorylates and inactivates Forkhead box O transcription factors (eg, FoxO1 and FoxO3), thereby preventing them from translocating into the nucleus and activating the expression of genes whose products would mediate degradation of muscle proteins. These mechanisms collectively contribute to muscle hypertrophy.

Glucocorticoids (GC) induce muscle atrophy mainly by suppressing the IGF-1–PI3K–Akt signaling pathway in the skeletal muscle. Under this condition the phosphorylation of FoxO by Akt ceases, and then the nonphosphorylated FoxO translocates into the nucleus and activates the transcription of its target genes. Among these are the genes coding for muscle-specific E3 ubiquitin ligases, that is, muscle atrophy F-box (MAFbx, also called atrogin-1) and muscle ring finger 1 (MuRF1). By ubiquitinating their substrates, which include structural proteins (eg, myosin heavy chain) and regulatory proteins (eg, the transcription factor MyoD and the translation initiation factor eIF3f), these ubiquitin ligases promote their proteasomal degradation. FoxO also turns on the other degradative machinery, the autophagy–lysosome system (Mehrpour *et al.*, 2010), by increasing the expression of a number of its components, such as LC3, Beclin 1, ULK-1, Vps34, BNIP3, and cathepsin L (see more details in the sections “Cellular Repair” and “Adaptation to Energy Depletion—The Energy Stress Response”).

GC suppress the IGF-1–PI3K–Akt signaling by multiple mechanisms. They decrease production of IGF-1 by the muscle, downregulate the expression of IRS-1, and inhibit PI3K activity (apparently by direct interaction of the activated GC receptor with the regulatory subunit of PI3K). In addition, GC upregulate the expression of myostatin in the muscle probably by downregulating the expression of miRNA-27a and b, which target myostatin mRNA (see elsewhere). Myostatin is a TGF- $\beta$  family member protein, which is a negative regulator of muscle mass. It acts by both endocrine and paracrine fashion on its receptor (similar to item 8 in Fig. 3-12) and activates Smad2, which inhibits Akt (Glass, 2010). Other mechanisms may also contribute to GC-induced muscle wasting (Hasselgren *et al.*, 2010).

the capacity of methemoglobin reductase. Because this repair enzyme is deficient at early ages, neonates are especially sensitive to chemicals that cause methemoglobinemia. Formation of cataracts purportedly involves inefficiency or impairment of lenticular repair enzymes, such as the endopeptidases and exopeptidases, which normally reduce oxidized crystalline and hydrolyze damaged proteins to their constituent amino acids. Dysrepair also is thought to contribute to the formation of Heinz bodies, which are protein aggregates formed in oxidatively stressed and aged red blood cells. Defective proteolytic degradation of the immunogenic

trifluoroacetylated proteins may make halothane-anesthetized patients victims of halothane hepatitis.

Several types of toxicity involve failed and/or derailed repairs at different levels before they become apparent. This is true for the most severe toxic injuries, such as tissue necrosis, fibrosis, and chemical carcinogenesis.

**Tissue Necrosis** As discussed above, several mechanisms may lead to cell death. Most or all involve molecular damage that is potentially reversible by repair mechanisms. If repair mechanisms



operate effectively, they may prevent cell injury or at least retard its progression. For example, prooxidant toxicants cause no lipid fragmentation in microsomal membranes until  $\alpha$ -tocopherol is depleted in those membranes. Membrane damage ensues when this endogenous antioxidant, which can repair lipids containing peroxyl radical groups (Fig. 3-24), becomes unavailable (Scheschonka *et al.*, 1990). This suggests that cell injury progresses toward cell necrosis if molecular repair mechanisms are inefficient or the molecular damage is not readily reversible.

Progression of cell injury to tissue necrosis can be intercepted by 2 repair mechanisms working in concert: apoptosis and cell proliferation. As discussed above, 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 injury by releasing cytotoxic mediators. Indeed, the activation of Kupffer cells, the source of such mediators in the liver, by the administration of bacterial LPS (endotoxin) greatly aggravates the hepatotoxicity of galactosamine. In contrast, when the Kupffer cells are selectively eliminated by pretreatment of rats with gadolinium chloride, the necrotic effect of carbon tetrachloride is markedly alleviated (Edwards *et al.*, 1993). Blockade of Kupffer cell function with glycine (via the inhibitory glycine receptor; see item 4 in Fig. 3-15) also protects the liver from alcohol-induced injury (Yin *et al.*, 1998).

Another important repair process that can halt the propagation of toxic injury is proliferation of cells adjacent to the injured cells. This response is initiated soon after cellular injury. A surge in mitosis in the liver of rats administered a low (non-necrogenic) dose of carbon tetrachloride is detectable within a few hours. This early cell division is thought to be instrumental in the rapid and complete restoration of the injured tissue and the prevention of necrosis. This hypothesis is corroborated by the finding that in rats pretreated with chlordecone, which blocks the early cell proliferation in response to carbon tetrachloride, a normally non-necrogenic dose of carbon tetrachloride causes hepatic necrosis (Mehendale, 2005). The sensitivity of a tissue to injury and the capacity of the tissue for repair are apparently two independent variables, both influencing the final outcome of the effect of injurious chemical—that is, whether tissue restitution ensues with survival or tissue necrosis occurs with death. For example, variations in tissue repair capacity among species and strains of animals appear to be responsible for certain variations in the lethality of hepatotoxicants (Soni and Mehendale, 1998).

It appears that the efficiency of repair is an important determinant of the dose-response relationship for toxicants that cause tissue necrosis. Following chemically induced liver or kidney injury, the intensity of tissue repair increases up to a threshold dose, restraining injury, whereupon it is inhibited, allowing unrestrained progression of injury (Mehendale, 2005). Impaired signaling to mitosis (see Fig. 3-12), caused by high tissue concentrations of toxicants (eg, acetaminophen in the liver or S-(1,2-dichlorovinyl)-L-cysteine in the kidney) and their reactive metabolites may account for lagging tissue repair (Boulares *et al.*, 1999; Vaidya *et al.*, 2003), but maintenance of DNA and protein synthesis, mitotic machinery, and energy supply may also be impaired at high-dose chemical exposures. That is, tissue necrosis is caused by a certain dose of a toxicant not only because that dose ensures sufficient concentration of the ultimate toxicant at the target site to initiate injury but also because that quantity of toxicant causes a degree of damage sufficient to compromise repair, allowing for progression of the injury. Experimental observations with hepatotoxicants indicate that apoptosis and cell proliferation are operative with latent tissue injury caused by low (non-necrogenic) doses of toxicants, but

are inhibited with severe injury induced by high (necrogenic) doses. For example, 1,1-dichloroethylene, carbon tetrachloride, and thioacetamide all induce apoptosis in the liver at low doses, but cause hepatic necrosis after high-dose exposure (Corcoran *et al.*, 1994). Similarly, there is an early mitotic response in the liver to low-dose carbon tetrachloride, but this response is absent after administration of the solvent at necrogenic doses (Mehendale, 2005). This suggests that tissue necrosis occurs because the injury overwhelms and disables the repair mechanisms, including (1) repair of damaged molecules, (2) elimination of damaged cells by apoptosis, and (3) replacement of lost cells by cell division.

As in tissues and organs several types of cells are integrated and support the function of each other, toxic injury to different cell types may exacerbate the tissue damage and promote its progression to tissue necrosis. This principle is exemplified by the acetaminophen-induced hemorrhagic hepatic necrosis. Even before causing manifest injury to the parenchymal liver cells, acetaminophen overdose in mice has a deleterious effect on the sinusoidal endothelial cells (McCuskey, 2008). These cells swell and lose normal function (eg, endocytosis); their fenestrae coalesce into gaps that permit red blood cells to penetrate into the space of Disse. The subsequent collapse of sinusoids reduces blood flow, thus impairing oxygen and nutrient supply of hepatocytes that also endure direct damage by the reactive metabolite of acetaminophen.

**Fibrosis** Fibrosis is a pathologic condition characterized by excessive deposition of an ECM of abnormal composition. Hepatic fibrosis, or cirrhosis, results from chronic consumption of ethanol or high-dose retinol (vitamin A), treatment with methotrexate, and intoxication with hepatic necrogens such as carbon tetrachloride and iron. Pulmonary fibrosis is induced by drugs such as bleomycin and amiodarone and prolonged inhalation of oxygen or mineral particles. Doxorubicin may cause cardiac fibrosis, whereas drugs acting as agonists on 5-HT<sub>2B</sub> receptors of human valvular interstitial cells, such as bromocriptine, ergotamine, methysergide, and fenfluramine after long-term use as well as 5-HT itself when overproduced in carcinoid syndrome, induce proliferative valve disease with fibrosis. Exposure to high doses of ionizing radiation induces fibrosis in many organs. Most of these agents generate free radicals and cause chronic cell injury.

Fibrosis is a specific manifestation of dysrepair of the chronically injured tissue. As discussed above, cellular injury initiates a surge in cellular proliferation and ECM production, which normally ceases when the injured tissue is remodeled. If increased production of ECM is not halted, fibrosis develops.

The cells that manufacture the ECM during tissue repair (eg, stellate cells and myofibroblasts in liver, mesangial cells in the kidney, fibroblast-like cells in lungs and skin) are the ones that overproduce the matrix in fibrosis. These cells are controlled and phenotypically altered (“activated”) by cytokines and growth factors secreted by nonparenchymal cells, including themselves (see Fig. 3-26). TGF- $\beta$  appears to be the major mediator of fibrogenesis, although other factors are also involved. These include growth factors, such as connective tissue growth factor (CTGF, a TGF- $\beta$ -induced growth factor) and PDGF, vasoactive peptides, such as endothelin-1 and angiotensin-II, and the adipocyte-derived hormone leptin (Lotersztajn *et al.*, 2005). The evidence is compelling to indicate that TGF- $\beta$ , acting through its receptor (item 8 in Fig. 3-12), and receptor-activated TFs (Smad2 and 3), is a highly relevant causative factor of fibrosis. For example, subcutaneous injection of TGF- $\beta$  induces local fibrosis, whereas overexpression of TGF- $\beta$  in transgenic mice produces hepatic fibrosis. Smad3-null mice are relatively resistant to radiation-induced cutaneous fibrosis,

bleomycin-induced pulmonary fibrosis, and  $\text{CCl}_4$ -induced hepatic fibrosis. TGF- $\beta$  antagonists, such as anti-TGF- $\beta$  immunoglobulin and decorin, as well as Smad3 antagonists, such as halofuginone and overexpressed Smad7 protein (which is antagonistic to Smad2 and 3), ameliorate chemically induced fibrogenesis (Flanders, 2004). In several types of experimental fibrosis and in patients with active liver cirrhosis, overexpression of TGF- $\beta$  in affected tissues has been demonstrated. Specific factors may also be involved in the pathomechanism of chemically induced fibrosis. For example, in alcoholic liver cirrhosis stellate cells may be activated directly by acetaldehyde (formed by alcohol dehydrogenase), by ROS (generated by the ethanol-induced CYP2E1), and by bacterial endotoxin (LPS), which is increasingly absorbed from the gut, the permeability of which is enhanced by chronic alcohol exposure. LPS can stimulate stellate cells both directly and indirectly through Kupffer cells (see Fig. 3-26) because both cells express TLR through which LPS acts.

The increased expression of TGF- $\beta$  is a common response mediating regeneration of the ECM after an acute injury. However, whereas TGF- $\beta$  production ceases when repair is complete, this does not occur when tissue injury leads to fibrosis. Failure to halt TGF- $\beta$  overproduction could be caused by continuous injury or a defect in the regulation of TGF- $\beta$ . Indeed, after acute  $\text{CCl}_4$ -induced liver injury, hepatic stellate cells exhibit a TGF- $\beta$ -mediated induction of Smad7 (which purportedly terminates the fibrotic signal by inhibiting activation of Smad2 and Smad3 by TGF- $\beta$  receptor); however, after chronic injury, Smad7 induction fails to occur (Flanders, 2004).

The fibrotic action of TGF- $\beta$  is due to increased production and decreased degradation of ECM components. TGF- $\beta$  stimulates the synthesis of individual ECM components (eg, collagens) by specific target cells via the Smad pathway (see Fig. 3-12) and also by downregulation of the transcription of miR-29 miRNA family members, which inhibit the translation of collagen. Downregulation of miR-29 family members occurs in murine hepatic stellate cells exposed to TGF- $\beta$ , in the stellate cells of mice with hepatic fibrosis induced by  $\text{CCl}_4$  or bile duct ligation, and in the liver of patients with advanced liver cirrhosis (Roderburg *et al.*, 2011). TGF- $\beta$  inhibits ECM degradation by disproportionately increasing the expression of inhibitor proteins that antagonize ECM-degrading enzymes, such as TIMP-1 and plasminogen activator inhibitor-1 (PAI-1), compared with the expression of ECM-degrading metalloproteinases (Arthur *et al.*, 1999; Flanders, 2004). Interestingly, TGF- $\beta$  induces transcription of its own gene in target cells (Flanders, 2004), suggesting that the TGF- $\beta$  produced by these cells can amplify in an autocrine manner the production of the ECM. This positive feedback (autoinduction) may facilitate fibrogenesis.

Fibrosis involves not only excessive accumulation of the ECM but also changes in its composition. The basement membrane components, such as collagen IV and laminin, as well as the fibrillar-type collagens (collagen I and III), which confer rigidity to tissues, increase disproportionately during fibrogenesis (Gressner, 1992).

Fibrosis is detrimental in a number of ways:

1. The scar compresses and may ultimately obliterate the 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 and its rigidity unfavorably affect the elasticity and flexibility of the whole tissue,

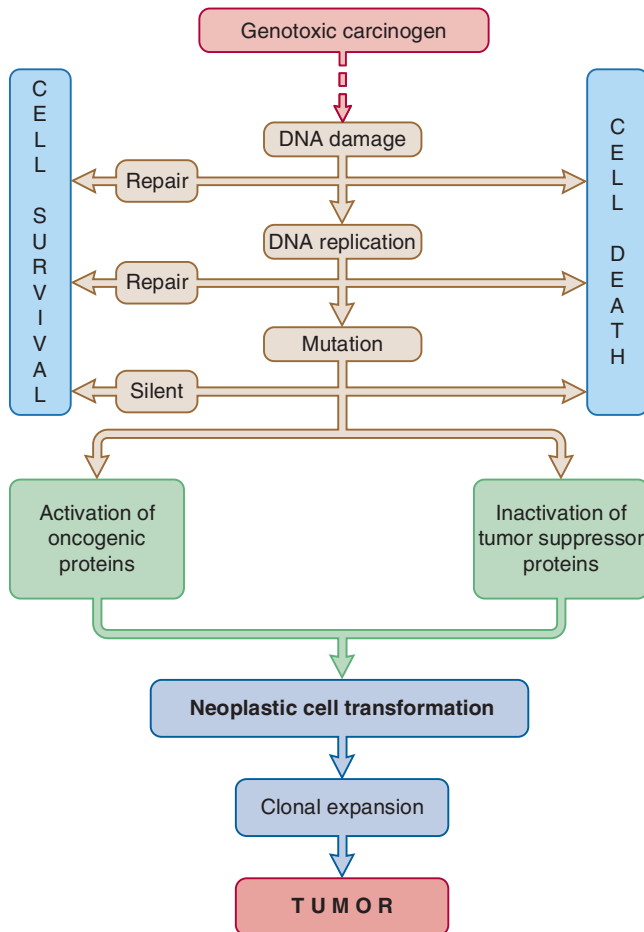
compromising the mechanical function of organs such as the heart and lungs.

4. Furthermore, the altered extracellular environment is sensed by integrins. Through these transmembrane proteins and the coupled intracellular signal transducing networks (see Fig. 3-12), fibrosis may modulate several aspects of cell behavior, including polarity, motility, and gene expression (Raghow, 1994).

**Carcinogenesis** Chemical carcinogenesis involves malfunctions in various repair and adaptive mechanisms. At the molecular level, a crucial feature of carcinogenesis is altered expression of critical proteins, that is, proto-oncogenic proteins and tumor suppressor proteins. This may result (1) from mutation of critical genes due to insufficient adaptive response to DNA damage and missed DNA repair or (2) from inappropriate transcriptional control at the regulatory regions of critical genes that gives rise to overexpression or underexpression of their products. (Note that the genes that we designate here as “critical” include those that are transcribed into mRNAs that in turn translate into proto-oncogenic or tumor suppressor proteins, as well as those that are transcribed into miRNAs that in turn regulate the translation of proto-oncogenic or tumor suppressor proteins. These proteins will be defined below.) At the cellular level, the fundamental feature of tumorigenesis is proliferation of cells, which may result (1) from failure to execute apoptosis and/or (2) from failure to restrain cell division.

As to be described in more detail later, carcinogenesis entails gene expression alterations initiated by two fundamentally distinct types of mechanisms that often work simultaneously and in concert, that is, genetic and epigenetic mechanisms. Genetic mechanisms bring about a *qualitative* change in gene expression, that is, expression of an altered gene product, a mutant protein, or miRNA, with gain or loss in activity. In contrast, epigenetic mechanisms cause a *quantitative* change in gene expression resulting in more or less gene product. Whereas genetic mechanisms alter the coding sequences of critical genes, epigenetic mechanisms eventually influence the regulatory (promoter) region of genes. Thus, chemical and physical insults may induce neoplastic transformation of cells by affecting critical genes through genotoxic and nongenotoxic (ie, epigenetic) mechanisms. However, either mechanism ultimately induces cancer by causing cellular failures in executing apoptosis and/or restraining cell division, thereby giving rise to an uncontrollably proliferating cell population.

**Genotoxic Mechanisms of Carcinogenesis: Chemical Damage and Disrepair in the Coding Region of Critical Genes Leading to Mutation** Chemicals that react with DNA may cause damage such as adduct formation, oxidative alteration, and strand breakage. In most cases, these lesions are repaired or the injured cells are eliminated. If neither event occurs, a lesion in the parental DNA strand may induce a heritable alteration, or 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 most unfortunate scenario for the organism occurs when the altered genes express mutant proteins that reprogram cells for multiplication and escaping apoptosis (ie, immortalization). When such cells undergo mitosis, their descendants also have a similar propensity for proliferation. Moreover, because enhanced DNA replication and cell division increases the likelihood of mutations (for reasons discussed below), these cells eventually acquire additional mutations that may further augment their growth advantage over their normal



**Figure 3-31.** The process of carcinogenesis initiated by genotoxic carcinogens. The figure indicates that activating mutation of proto-oncogenes that encode permanently active oncoproteins and inactivating mutation of tumor suppressor genes that encode permanently inactive tumor suppressor proteins can cooperate in neoplastic transformation of cells. It is important to realize that overexpression of normal proto-oncogenes (eg, by hypomethylation of their promoter) and underexpression (silencing) of normal tumor suppressor genes (eg, by hypermethylation of their promoter) may also contribute to such transformation (see text for explanation).

counterparts. The final outcome of this process is a nodule, followed by a tumor consisting of rapidly proliferating transformed cells (Fig. 3-31).

The critical role of DNA repair in preventing carcinogenesis is attested by the human heritable disease xeroderma pigmentosum. Affected individuals lack excision repair proteins of the XP series and exhibit a greatly increased incidence of sunlight-induced skin cancers. Cells from these patients are also hypersensitive to DNA-reactive chemicals, including aflatoxin B<sub>1</sub>, aromatic amines, polycyclic hydrocarbons, and 4-nitroquinoline-1-oxide (Lehmann and Dean, 1990). Also, mice with ablated PARP gene are extremely sensitive to  $\gamma$ -rays and *N*-methyl-*N*-nitrosourea and show genomic instability, as indicated by sister chromatid exchanges and chromatid breaks following genotoxic insult (D'Amours *et al.*, 1999).

A small set of cellular genes is the target for genetic alterations that initiate neoplastic transformations. Included are proto-oncogenes and tumor suppressor genes (Barrett, 1992).

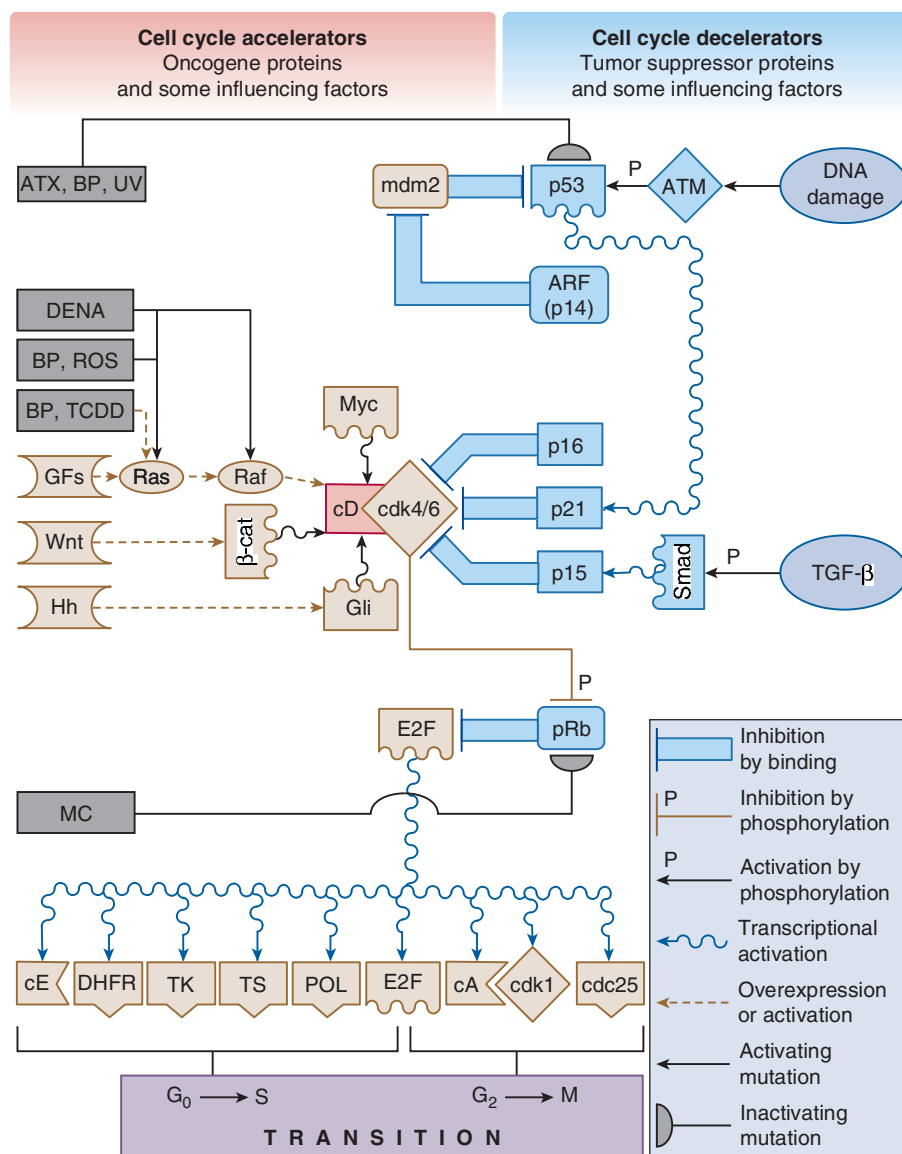
**Mutation of Proto-Oncogenes** Proto-oncogenes are highly conserved genes encoding proteins that stimulate the progression of cells through the cell cycle, or oppose apoptosis (Smith *et al.*, 1993). The products of proto-oncogenes that accelerate the

cell division cycle include (1) growth factors; (2) growth factor receptors; (3) intracellular signal transducers such as G proteins, protein kinases, cyclins, and cyclin-dependent protein kinases; and (4) nuclear TFs (see Figs. 3-12 and 3-32). A notable proto-oncogene product that inhibits apoptosis is Bcl-2 (see Fig. 3-19).

Fig. 3-32 depicts several proto-oncogene products that are closely involved in initiating the cell division cycle. The legend of that figure outlines some important details on the function of these proteins and their interaction with tumor suppressor proteins (to be discussed below). Transient increases in the production or activity of proto-oncogene proteins are required for regulated growth, as during embryogenesis, tissue regeneration, and stimulation of cells by growth factors or hormones. In contrast, permanent activation and/or overexpression of these proteins favor neoplastic transformation. One mechanism whereby genotoxic carcinogens induce neoplastic cell transformation is by producing an activating mutation of a proto-oncogene. Such a mutation is so named because the altered gene (then called an oncogene) encodes a permanently active protein that forces the cell into the division cycle.

An example of mutational activation of an oncogene protein is that of the Ras proteins. Ras proteins are G-proteins with GTP/GDP-binding capacity as well as GTPase activity (Anderson *et al.*, 1992). They are localized on the inner surface of the plasma membrane and function as crucial mediators in the signaling pathways initiated by growth factors (see Figs. 3-12 and 3-32). Ras is located downstream from growth factor receptors and nonreceptor protein tyrosine kinases and upstream from mitogen-activated protein kinase (MAPK) cascade whose activation finally upregulates the expression of cyclin D and initiates the mitotic cycle (Fig. 3-32). In this pathway, Ras serves as a molecular switch, being active in the GTP-bound form and inactive in the GDP-bound form. Some mutations of the Ras gene (eg, a point mutation in codon 12) dramatically lower the GTPase activity of the protein. This in turn locks Ras in the permanently active GTP-bound form. Continual rather than signal-dependent activation of Ras can lead eventually to uncontrolled cell division and transformation. Indeed, microinjection of Ras-neutralizing monoclonal antibodies into cells blocks the mitogenic action of growth factors as well as cell transformation by several oncogenes. Ionizing radiation and carcinogenic chemicals (eg, *N*-methyl-*N*-nitrosourea, polycyclic aromatic hydrocarbons, benzidine, aflatoxin B<sub>1</sub>) induce mutations of Ras proto-oncogenes that lead to constitutive activation of Ras proteins (Anderson *et al.*, 1992). Most of these chemicals induce point mutations by transversion of G<sub>35</sub> to T in codon 12.

Another example for activating mutation of a proto-oncogene is B-Raf mutation, although Ras and Raf mutations are mutually exclusive (Shaw and Cantley, 2006). Raf proteins are protein kinases, lying just downstream from Ras and being the first signal transducers in the MAP kinase pathway (see Fig. 3-12). After recruitment by Ras to the cell membrane, Raf is activated by the growth factor receptor (see item 4 in Fig. 3-12) through phosphorylation in its activating segment. B-Raf mutations occur in mouse liver tumors induced by diethylnitrosamine (Jaworski *et al.*, 2005) in 66% of malignant melanomas and a wide range of human cancers. All mutations are within the activation segment of B-Raf, with a single amino acid substitution (V599E) accounting for the majority. The mutant B-Raf protein has elevated kinase activity probably because substitution of the nonpolar valine with the negatively charged glutamate mimics an activating phosphorylation. Thus, the constitutively active B-Raf continually sends Ras-independent proliferative signal down the MAPK pathway. Indeed, transfection of the mutant B-Raf gene into cells induced neoplastic transformation even in the absence of Ras proteins (Davies



**Figure 3-32. Key regulatory proteins controlling the cell division cycle with some signaling pathways and xenobiotics affecting them.** Proteins on the left, represented by brown symbols, 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 and thus suppress oncogenesis, unless they are inactivated (eg, by mutation).

Accumulation of cyclin D (cD) is a crucial event in initiating the cell division cycle. cD activates cyclin-dependent protein kinases 4 and 6 (cdk4/6), which in turn phosphorylate the retinoblastoma protein (pRb) causing dissociation of pRb from transcription factor E2F (Johnson and Walker, 1999). Then the unleashed E2F is able to bind to and transactivate genes whose products are essential for DNA synthesis, such as dihydrofolate reductase (DHFR), thymidine kinase (TK), thymidylate synthetase (TS), and DNA polymerase (POL), or are regulatory proteins, such as cyclin E (cE), cyclin A (cA), and cyclin-dependent protein kinase 1 (cdk1), which promote further progression of the cell cycle. Expression of cD is increased, for example, by growth factors signaling through Ras proteins and the MAPK pathway (see Fig. 3-12) as well as by Wnt and Hedgehog (Hh) ligands that ultimately signal through B-cat and Gli transcription factors, respectively (see Figs. 3-13 and 3-14). Some carcinogens, for example, benzpyrene (BP) and reactive oxygen species (ROS), and diethylnitrosamine (DENA) may cause mutation of the *Ras* or *Raf* gene that results in permanently active mutant Ras or Rab protein, but BP as well as TCDD may also induce simple overexpression of normal Ras protein.

Cell cycle progression is counteracted, for example, by pRb (which inhibits the function of E2F), by cyclin-dependent protein kinase inhibitors (such as p15, p16, and p21), by p53 (which transactivates the *p21* gene), and by ARF (also called p14 that binds to mdm2, thereby neutralizing the antagonistic effect of mdm2 on p53). Signals evoked by DNA damage and TGF- $\beta$  will ultimately result in accumulation of p53 and p15 proteins, respectively, and deceleration of the cell cycle. In contrast, mutations that disable the tumor suppressor proteins facilitate cell cycle progression and neoplastic conversion and are common in human tumors. Aflatoxin B<sub>1</sub> (ATX), BP, and UV light cause such mutations of the *p53* gene (Bennett *et al.*, 1999), whereas *pRb* mutations occur invariably in methylcholanthrene (MC)-induced transplacental lung tumors in mice (Miller, 1999).



*et al.*, 2002). Another proto-oncogene product that often undergoes activating mutation in breast and colon tumors is p110- $\alpha$ , the catalytic subunit of PI3K (Shaw and Cantley, 2006). This can cause permanent proliferative signaling via the GF receptor–PI3K–Akt pathway (see Fig. 3-12).

Whereas constitutive activation of oncogene proteins, as a result of point mutation, is a common initiator of chemical carcinogenesis, permanent overexpression of such proteins also can contribute to neoplastic cell transformation. Overexpression of proto-oncogene proteins may result from amplification of the proto-oncogene, that is, the formation of more than one copy (Anderson *et al.*, 1992). Such an event may be initiated by DNA strand breaks, and therefore often observed after exposure to ionizing radiation; however, proto-oncogene amplification also occurs in spontaneous human cancer. An example for a proto-oncogene protein that is overexpressed in response to gene damage is the antiapoptotic Bcl-2 protein (see Fig. 3-19). The aberrantly increased expression of Bcl-2 is caused by a chromosomal translocation and is responsible for B-cell lymphoma, a spontaneous human malignancy (see later). Overexpression of proto-oncogene proteins as a result of nongenotoxic, epigenetic mechanisms will be discussed later.

**Mutation of Tumor Suppressor Genes** Tumor suppressor genes encode proteins that inhibit the progression of cells in the division cycle, or promote DNA repair or apoptosis on DNA damage. Fig. 3-32 depicts such proteins, which include, for example, cyclin-dependent protein kinase inhibitors (eg, p15, p16, and p21), TFs (eg, p53 and Smad4) that activate genes encoding cyclin-dependent protein kinase inhibitors, proteins (eg, pRb) that block TFs involved in DNA synthesis and cell division, and proteins (eg, ARF) that block inhibitors of tumor suppressor proteins. Other notable tumor suppressor gene products include, for example, the protein kinases (eg, ATM, ATR) that sense the DNA damage and signal for the p53-controlled response shown in Fig. 3-33, proteins involved in DNA repair, such as *O*<sup>6</sup>-methylguanine-DNA methyltransferase (which removes alkyl groups adducted to guanine) as well as BRCA1 and BRCA2 proteins (which contribute to recombinational DNA repair), proapoptotic proteins (eg, Bax, Puma, Noxa, and Bim) induced after DNA damage (Fig. 3-33), the suppressor of cytokine signaling (Socs) protein (Fig. 3-12), the phosphatase PTEN (which dephosphorylates the membrane lipid PIP<sub>3</sub>, an essential intermediate in the PI3K–Akt pathway) that turns off the PI3K–Akt pathway-mediated proliferative signaling (Fig. 3-12), and the tuberous sclerosis complex-2 (TSC2) that prevents activation of mTOR (Fig. 3-29). Uncontrolled proliferation can occur when the mutant tumor suppressor gene encodes a protein that cannot suppress cell division. Inactivating mutations of specific tumor suppressor genes in germ cells are responsible for the inherited predisposition to cancer, as in familial retinoblastoma (pRb; see Fig. 3-32), Wilms tumor (WT1, WTX, and  $\beta$ -catenin; see Fig. 3-13), familial polyposis (Smad4; see Figs. 3-12 and 3-32), and Li–Fraumeni syndrome (p53; see Figs. 3-19, 3-32, and 3-33). Mutations of tumor suppressor genes in somatic cells contribute to nonhereditary cancers. The genes of p16, PTEN, and pRb are frequently mutated in human cancer. The best known tumor suppressor gene involved in both spontaneous and chemically induced carcinogenesis is p53.

The p53 tumor suppressor gene encodes a 53 kDa protein with multiple functions (Fig. 3-33). Acting as a transcriptional modulator, the p53 protein (1) activates protein-coding genes whose products arrest the cell cycle (eg, p21 and gadd45), repair damaged DNA (eg, XPE, MSH2), or promote apoptosis (eg, Bax, Puma, and Fas receptor); (2) activates miRNA-coding genes whose products (eg,

miR-34a) repress the translation of mitogenic TFs and cell cycle accelerator proteins (eg, Myc, E2F3, cyclin D, and CDK4/6); and (3) represses protein-coding genes that encode cell cycle accelerators (eg, cyclin B1, Cdk1), or antiapoptotic proteins (eg, Bcl-2 and IGF-1 receptor) (Bennett *et al.*, 1999; Liu and Chen, 2006). DNA damage activates protein kinases (ATM, ATR, Chk1, Chk2) to phosphorylate and stabilize the p53 protein, causing its accumulation (Fig. 3-33). The accumulated p53 may induce cell cycle arrest and apoptosis of the affected cells. Thus, p53 eliminates cancer-prone cells from the replicative pool, counteracting neoplastic transformation; therefore, it is commonly designated as guardian of the genome.

Indeed, cells that have no p53 are a million times more likely to permit DNA amplification than are cells with a normal level of this suppressor gene. Furthermore, mice with the p53 gene deleted develop cancer by 6 to 9 months of age, attesting to the crucial role of the p53 tumor suppressor gene in preventing carcinogenesis.

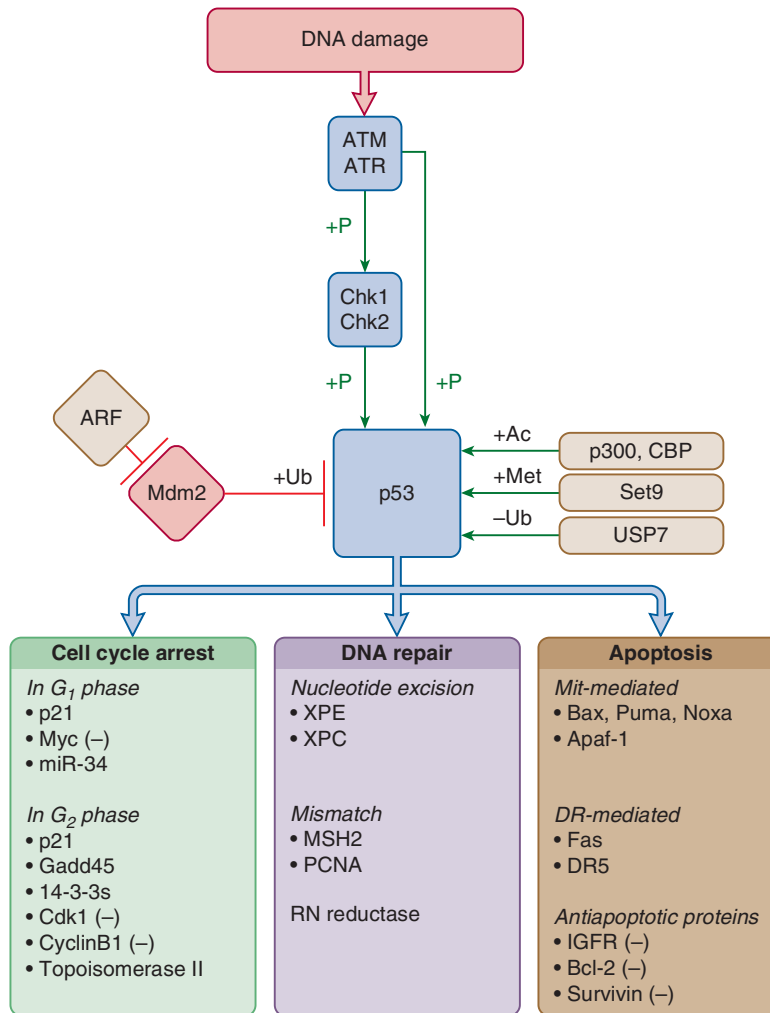
Mutations in the p53 gene are found in 50% of human tumors and in a variety of induced cancers. The majority are “missense mutations” that change an amino acid and result in a faulty or altered protein (Bennett *et al.*, 1999). The faulty p53 protein forms a complex with endogenous wild-type p53 protein and inactivates it. Thus, the mutant p53 not only is unable to function as a tumor suppressor protein but also prevents tumor suppression by the wild-type p53.

Carcinogens may cause characteristic mutations in the p53 tumor suppressor gene. An example is the point mutation in codon 249 from AGG to AGT, which changes amino acid 249 in the p53 protein from arginine to serine. This mutation predominates in hepatocellular carcinomas in individuals living where food is contaminated with aflatoxin B<sub>1</sub> (Bennett *et al.*, 1999). Because in human hepatocytes the CYP-activated metabolites of aflatoxin B<sub>1</sub> induce the transversion of G to T in codon 249 of the p53 tumor suppressor gene (Aguilar *et al.*, 1993), it appears likely that this mutation in primary human liver cancer is indeed caused by this mycotoxin. Although the incriminated mutation probably contributes to the hepatocarcinogenicity of aflatoxin B<sub>1</sub> in humans, it is not involved in aflatoxin B<sub>1</sub>-induced hepatocarcinogenesis in rats, as the transformed liver cells from the toxin-exposed rats do not show this mutation.

Another example for inactivating mutation of a tumor suppressor gene of environmental origin is that of Patched, which often occurs in basal cell carcinoma of the skin that may be induced by UV and ionizing radiation, as well as arsenic exposure (Tang *et al.*, 2007). Normal Patched, the membrane receptor of the Hh ligand, suppresses the mitogenic Hh pathway in absence of Hh ligand, which would inhibit Patched (Fig. 3-14). Mutant Patched loses its power to restrain this signaling network, which thus promotes cell division even in absence of its inhibitor ligand.

In addition to aberrations in critical protein-coding genes, damage in genes coding for miRNA (which repress the translation of critical proteins; see Fig. 3-11) may also contribute to carcinogenesis. For example, amplification of miR-21 (which is oncogenic by repressing the translation of PTEN; see Fig. 3-12) and deletion of miR-15 and miR-16 (which is tumor suppressive by repressing the translation of cdc25A; see Fig. 3-25) occur in spontaneous human malignancies. Therefore, miRNA gene damage may also underlie the mechanism of chemical carcinogenesis. The role of altered miRNA expression as an epigenetic mechanism of chemical carcinogenesis will be discussed below.

**Epigenetic Mechanisms in Carcinogenesis: Inappropriate Activation or Responsiveness of the Regulatory Region of Critical Genes** Whereas some chemicals cause cancer by reacting with DNA and inducing a mutation, others do not damage DNA,



**Figure 3-33. The guardian of the genome: p53 tumor suppressor protein—its role and regulation.** When activated on DNA damage, the p53 protein may mediate cell cycle arrest, DNA repair, and apoptosis. When inducing these effects, p53 acts chiefly as a transcription factor that can activate the transcription of most target genes, while repressing some of others, such as those marked with (–) in the figure (Liu and Chen, 2006). For example, p53 transactivates *p21* and *gadd45* genes (whose products are inhibitors of cyclin–cyclin-dependent protein kinase complexes) and arrest the cell cycle in  $G_1$  and  $G_2$  phases, respectively, but p53 represses the *Cdk1* and *cyclin B1* genes (whose products are indispensable for the cells to transit from  $G_2$  phase to M) (see Fig. 3-25). p53 also induces the expression of miR-34 (a microRNA), which in turn represses the translation of cyclin D, CDK4/6, and E2F, important cell cycle accelerator proteins (see Fig. 3-32) (Chen *et al.*, 2010). p53 also transactivates the genes of some DNA repair proteins and proapoptotic proteins (eg, bax and fas; see Fig. 3-19) and represses the genes of antiapoptotic proteins (eg, Bcl-2 and IGF-1 receptor), whereby it promotes apoptosis. These (and other) p53-induced proapoptotic mechanisms may be cell-specific, that is, all are not necessarily occurring in the same cell at the same time.

The intracellular level and activity of p53 depends primarily on the presence of mdm2 protein, which inactivates p53 by ubiquitinating it; monoubiquitination causes export of p53 from the nucleus, whereas polyubiquitination promotes its proteasomal degradation. The influence of mdm2 on p53 may be disrupted by overexpression of the ARF (or p14) protein (which binds to mdm2 and removes it from p53) or by post-translational modification of p53 through phosphorylation by protein kinases (see below), acetylation by acetyltransferases (eg, p300 and CBP), methylation by methyltransferases (eg, Set9) and deubiquitination by ubiquitin-specific-processing protease 7 (USP7, also called HAUSP) (Liu and Chen, 2006). These mechanisms release p53 from mdm2 and stabilize the p53 protein, thereby greatly increasing its abundance and activity. Phosphorylation of p53 is induced by DNA damage. This is sensed by kinases, such as ataxia telangiectasia mutated (ATM) and ataxia telangiectasia related (ATR), which directly or through checkpoint kinases (Chk1, Chk2) phosphorylate p53 to induce cell cycle arrest, DNA repair, or apoptosis (McGowan and Russell, 2004).

It is important to emphasize that there is also a p53-independent mechanism to arrest the cells suffering DNA damage before mitosis. Like induction of p53, this is also initiated by the activated Chk1, which phosphorylates and inactivates cdc25A, a protein phosphatase, which normally would dephosphorylate Cdk1 and activate the Cdk1–cyclin B complex (see Fig. 3-25). Thus, when cdc25A is inactivated, Cdk1 stalls and mitosis is delayed. Interestingly, p53 assists in keeping Cdk1, this mitosis-driving molecular motor, off track as it induces 14-3-3 $\sigma$ , a cytoplasmic binding protein, which associates with both cdc25A and the Cdk1–cyclin B complex and sequesters them in the cytoplasm.

By arresting division of cells with potentially mutagenic DNA damage, facilitating the DNA repair or eliminating such cells, p53 protein counteracts neoplastic development. *p53*-null mice, like *ARF*-null mice, develop tumors with high incidence. Mutational inactivation of the p53 protein is thought to contribute to the carcinogenic effect of aflatoxin B<sub>1</sub>, sunlight, and cigarette smoke in humans. Overexpression of mdm2 can lead to constitutive inhibition of p53 and thereby promotes oncogenesis even if the *p53* gene is unaltered. See the text for more details.

yet induce cancer after prolonged exposure (Barrett, 1992). These chemicals are designated *nongenotoxic* (or epigenetic) *carcinogens* and include (1) xenobiotic mitogens, that is, chemicals that promote proliferative signaling, such as the PKC activator phorbol esters and fumonisin B<sub>1</sub>, as well as the protein phosphatase inhibitor okadaic acid (see Fig. 3-12); (2) endogenous mitogens, such as growth factors (eg, TGF- $\alpha$ ) and hormones with mitogenic action on specific cells, for example, estrogens on mammary gland or liver cells, TSH on the follicular cells of the thyroid gland, and luteinizing hormone on Leydig cells in testes; (3) toxicants that, when given chronically, cause sustained cell injury (such as chloroform and D-limonene); (4) xenobiotics that are nongenotoxic carcinogens in rodents but not in humans, such as phenobarbital, DDT, TCDD, and peroxisome proliferators (eg, fibrates, WI-14643, dihalogenated and trihalogenated acetic acids); and (5) ethionine and diethanolamine, which interfere with formation of the endogenous methyl donor S-adenosyl methionine (Poirier, 1994). Because several of the listed chemicals promote the development of tumors after neoplastic transformation has been initiated by a genotoxic carcinogen, they are referred to as *tumor promoters*. Despite the initial belief that promoters are unable to induce tumors by themselves, studies suggest that they can do so after prolonged exposure.

It appears that nongenotoxic chemicals, like the genotoxic ones, eventually also influence the expression of proto-oncogenes and/or tumor suppressor genes, but in a different manner. When continuously present, nongenotoxic carcinogens can permanently induce the synthesis of normal proto-oncogene proteins and/or repress the synthesis of normal tumor suppressor proteins, rather than inducing the synthesis of permanently active mutant proto-oncogene proteins or permanently inactive mutant tumor suppressor proteins, as the mutagenic genotoxic carcinogens do.

Carcinogens may alter the synthesis of proto-oncogene proteins and tumor suppressor proteins at transcriptional and/or translational levels. Chemicals may modify transcription of the genes into mRNAs for proto-oncogene and tumor suppressor proteins by perturbing the signal transduction to the promoter region of these genes and/or by altering the signal receptivity of this gene region by DNA methylation and histone modifications. Translation of proto-oncogene proteins and tumor suppressor proteins from their mRNA may be controlled by specific miRNAs, which repress this process (Fig. 3-11). Carcinogen-induced perturbations in signaling and promoter alterations may not only influence transcription of protein-coding genes but also affect the transcription of miRNA-coding genes. The resultant changes in the abundance in miRNAs in turn alter, in a reciprocal (inverse) manner, the synthesis of proto-oncogene proteins and tumor suppressor proteins, provided the miRNA in question targets the mRNA of such a protein. The forthcoming discussion deals with the importance of signaling, DNA as well as histone modifications, and miRNA expression in carcinogenesis.

**Role of Signaling in Carcinogenesis** An important target of nongenotoxic carcinogens is the promoter region of critical genes where they can act via two distinct modes. First, they may alter the abundance and/or activity of TFs, typically by influencing upstream signaling elements ranging from extracellular signaling molecules (eg, TSH) to intracellular transducer proteins (eg, PKC). Obviously both xenobiotic and endogenous mitogens mentioned above can thus activate proliferative pathways (see Fig. 3-12) that descend to TFs (eg, Myc) that act on the promoter of proto-oncogenes coding for mRNAs of proto-oncogene proteins (eg, cyclins), and, as to be discussed below, on the promoter of genes coding for oncogenic miRNAs (eg, miR-17-92). It is easy to recognize that

even nongenotoxic carcinogens of the cytotoxic type act in this manner. As described under tissue repair, cell injury evokes the release of mitogenic growth factors such as HGF and TGF- $\alpha$  from tissue macrophages and endothelial cells. Thus, cells in chronically injured tissues are exposed continuously to endogenous mitogens. Although these growth factors are instrumental in tissue repair after acute cell injury, their continuous presence is potentially harmful because they may ultimately transform the affected cells into neoplastic cells. Indeed, transgenic mice that overexpress TGF- $\alpha$  develop hepatomegaly at a young age and tumors by 12 months (Fausto *et al.*, 2006). Mitogenic cytokines secreted by Kupffer cells are apparently involved in hepatocyte proliferation and, possibly, tumor formation induced by peroxisome proliferators in rats (Rose *et al.*, 1999) and in the formation of the endothelial cell-derived hepatic hemangiosarcoma in mice exposed to 2-butoxyethanol (Corthals *et al.*, 2006).

**Role of DNA Methylation and Histone Modification in Carcinogenesis** As a second mode of action, nongenotoxic carcinogens may alter expression of critical genes by modifying the responsiveness of the promoter region of these genes to TFs. Promoter responsiveness is typically controlled by DNA methylation. This takes place at C<sub>5</sub> of specific cytosine residues located in CpG islands (ie, clusters of CpG dinucleotides) in the promoter and is catalyzed by DNA cytosine methyltransferases (eg, DNMT1, DNMT3a, and DNMT3b) using S-adenosyl methionine as the methyl donor. It is well known that promoter methylation decreases the transcriptional activity of genes. For example, tissue-specific genes (eg, the genes of GI TFFs) are hypermethylated in tissues where they are not expressed, but are typically hypomethylated in tissues where they are expressed.

Promoter methylation can silence genes because it weakens binding of TFs to the promoter and because it triggers secondary alterations in histone proteins that reduce the accessibility of the promoter for TFs (Esteller, 2005). The latter mechanism involves modification of the protruding amino-terminal tails of core histone proteins by deacetylation and methylation of lysine residues. This makes the histone more compact, thereby diminishing access of TFs and other proteins involved in transcription initiation to the gene promoter. Histone deacetylases that remove the acetyl group from histone tails may be recruited by DNMTs directly, or through proteins that recognize and bind to the methylated CpG dinucleotides (eg, MeCP1, MeCP2, MBD2, MBD3). Some of these methyl-CpG-binding domain-containing proteins (eg, MBD2, MBD3) also have histone deacetylase activity. Finally, both DNMTs and MBDs can recruit histone methyltransferases to methylate histone tail lysines.

While methylation of CpG dinucleotides codes for gene silencing via histone deacetylation and methylation ("histone code"), hypomethylated genes are alert. In fact, CpG islands in the 5'-end region (promoter, untranslated region, exon 1) of genes are relatively hypomethylated in normal tissues, except for tissue- or germline-specific genes, and genomically imprinted genes (see earlier). Thus, if the appropriate TFs are available for a particular gene, and if the CpG island remains hypomethylated, the histones acetylated and hypomethylated, then the gene will be transcribed (Esteller, 2005). Histones are acetylated by histone acetyltransferases, such as p300 and CBP, which also acetylate other proteins, including p53 (see Fig. 3-33), and are also transcriptional coactivators.

It is well documented that the normal methylation pattern of DNA is disrupted in cancer cells. Such cells are characterized by global (average) hypomethylation, that is, decreased content of genomic 5'-methylcytosine. Paradoxically, DNA hypomethylation occurs in the face of hypermethylation of the CpG islands in



the promoter region tumor suppressor genes, which are normally demethylated (Esteller, 2005). The frequently hypermethylated tumor suppressor genes in human cancers are, for example, the Cdk inhibitor p15 and p16, the mdm2-binding protein ARF, pRb that tethers E2F (see Fig. 3-30), the DNA repair enzyme MGMT, and the lipid phosphatase PTEN (see Fig. 3-12). Importantly, both global hypomethylation and tumor suppressor gene hypermethylation intensify with increased malignancy of the tumor. Relevance of hypermethylation-induced silencing of tumor suppressor genes in carcinogenesis is supported by the finding that inhibitors of DNMTs (such as 5-aza-2'-deoxycytidine) can stop the growth of cancer cells and induce their differentiation by demethylating the dormant tumor suppressor genes, thereby restoring their expression (Esteller, 2005). The consequence of global hypomethylation of DNA is less clear. Nevertheless, hypomethylation of proto-oncogenes and increased expression of their products is a plausible mechanism (Goodman and Watson, 2002), although others have also been proposed (eg, chromosomal instability, reactivation of transposable DNA elements, and loss of genomic imprinting). On comparing mouse strains, it has been suggested that their sensitivity to chemical carcinogens may be related inversely to their capacity to maintain normal patterns of DNA methylation. It is worth noting that DNA methylation is more stable in human cells than in rodent cells (Goodman and Watson, 2002).

It appears that some of the nongenotoxic carcinogens alter DNA methylation. Inhibition of DNA methylation is a plausible mechanism that underlies tumorigenesis induced by ethionine, which depletes S-adenosyl-methionine (the methyl donor for DNMTs), and diethanolamine, which inhibits cellular uptake of choline, a dietary methyl group source for methylation of homocysteine to methionine. On prolonged administration, virtually all nongenotoxic rodent liver carcinogens listed above decrease DNA methylation. In addition to global DNA hypomethylation, promoter hypomethylation of the following proto-oncogenes have been observed in mouse or rat liver: Ras or Raf, following phenobarbital treatment, c-Jun, c-Myc, and IGF-2, after treatment with dichloroacetic acid, dibromoacetic acid, or trichloroacetic acid, and c-Myc after WY-14643 dosing. Long-term arsenic exposure of mice also induces hypomethylation of hepatic DNA globally and in the promoter of ER $\alpha$ , a hormone-activated TF. This is associated with an increase of cyclin D, a potentially ER $\alpha$ -linked gene (Chen *et al.*, 2004). In contrast, in human keratinocytes TCDD induces promoter hypermethylation in the tumor suppressor genes p16 and p53 as well as immortalization of these cells (Ray and Swanson, 2004). Although the mechanisms that initiate altered promoter methylation are currently unknown, it is possible that that altered promoter methylation plays a role in tumor promotion by these nongenotoxic carcinogens.

**Role of MicroRNAs in Carcinogenesis** As miRNAs almost always repress the translation of proteins from their mRNAs (Fig. 3-11), a miRNA plays an oncogenic role if it represses the translation of tumor suppressor proteins, that is, proteins that counter cell division, repair DNA, or promote apoptosis. For example, the miR-17-92 cluster is considered oncogenic, because its members promote mitosis by repressing the translation of PTEN (an inhibitor of the mitogenic PI3K–Akt signaling; see Fig. 3-12) and p21 (a cyclin-dependent kinase inhibitor; see Fig. 3-32) and because they inhibit apoptosis by repressing the translation of the proapoptotic protein Bim. Other examples for oncogenic miRNAs include miR-29b (which also targets the mRNAs of p21 and Bim), as well as miR-221/222, the targets of which include mRNAs translating into tumor suppressor proteins, such as PTEN, p27, p57, FOXO3 (see Fig. 3-30), TIMP3 (metalloproteinase inhibitor 3), and Bim

(Chen *et al.*, 2010; Garofalo and Croce, 2011). Overexpression of such oncogenic miRNAs promotes neoplastic cell transformation, whereas their underexpression opposes it.

Conversely, a miRNA has tumor suppressive role if it represses the translation of proto-oncogene proteins, that is, proteins that facilitate cell division or oppose apoptosis. For example, the let-7 family of miRNAs is regarded tumor suppressive as they silence the translation of Ras and Myc proteins, components of proliferative signaling (see Fig. 3-12). Furthermore, miR-15a and miR-16-1 repress the translation of protein phosphatase cdc25, which dephosphorylates and thus activates CDK1 and 2 that drives the cell cycle (see Fig. 3-25), and of Bcl-2, which inhibits apoptosis (see Fig. 3-19). Another tumor suppressor miRNA is miR-34, whose targets include the mRNAs that translates into the mitogenic TFs Myc and E2F3, as well as the cell cycle accelerator proteins cyclin D and CDK4/6 (see Figs. 3-25 and 3-32) (Chen, 2010; Garofalo and Croce, 2011). Upregulation of such tumor suppressive miRNAs counters neoplastic cell transformation, whereas their downregulation stimulates it.

Expression of miRNAs, like that of mRNAs coding for proteins, can be regulated at the promoter region of their genes by TFs as well as DNA methylation and histone modification. For example, Myc activates the transcription of the oncogenic miR-17-92, whereas p53 activates the transcription of the tumor suppressive miR-34. Therefore, the latter event is part of the adaptive DNA damage stress response discussed earlier and depicted in Fig. 3-33. Indeed, genotoxic carcinogens such as *N*-ethyl-*N*-nitrosourea and tamoxifen (which forms a DNA-reactive metabolite in rats, but not in humans) as well as ionizing radiation typically induce overexpression of mi-34.

The role of miRNAs in tumorigenesis induced by nongenotoxic carcinogens is well exemplified by the rodent hepatocarcinogenesis induced by the PPAR $\alpha$ -activator WY-14643. This process involves transcriptional downregulation of the gene coding for let-7c and consequential derepression of the translation of c-Myc mRNA into c-Myc protein. Due to its increased abundance, this TF (see Figs. 3-12 and 3-32) increasingly activates the gene coding for cyclin D, a mitogenic protein, as well as the gene coding for miR-17-92, an oncogenic miRNA. miR-17-92 in turn represses the translation of proteins that counter mitosis (eg, PTEN and p21) and promote apoptosis (eg, Bim), thus forcing hepatocytes to proliferate (Gonzalez and Shah, 2008).

**Cooperation of Genotoxic and Epigenetic Mechanisms in Carcinogenesis** Genotoxic and epigenetic mechanisms most likely complement and amplify each other in chemical carcinogenesis. One chemical may exert both genotoxic and epigenetic effects. For example, estrogens produce mutagenic free radicals via redox cycling of their quinone and hydroquinone metabolites and induce receptor-mediated proliferative effect (Newbold, 2004). Well-known genotoxic carcinogens can also cause epigenetic alterations. For example, 2-acetylaminofluorene does not only form DNA adducts but also induces DNA methylation in the promoter of Rassf1a and p16 genes as well as histone methylation around the promoter of Rassf1a, p16, Socs1, Cdh1, and Cx26 genes, thereby silencing all these genes that encode tumor suppressor proteins (Pogribny *et al.*, 2011). The p16, ARF, and MGMT tumor suppressor genes are also hypermethylated in aflatoxin B<sub>1</sub>-induced mouse lung tumor and in 7,12-dimethylbenzanthracene-induced skin tumor. The antiestrogen tamoxifen, a hepatocarcinogen in rats (but not in humans), exerts genotoxic effect and induces global hypomethylation of DNA and histones in rat liver (Tryndyak *et al.*, 2006). Thus, epigenetic alterations evoked by genotoxic carcinogens may drive the process of carcinogenesis after the initiating



genotoxic event (Pogribny *et al.*, 2011). Conversely, global DNA hypomethylation and tumor suppressor gene hypermethylation may increase the occurrence of mutations in cells exposed to non-genotoxic carcinogens. In this sense, epigenetic mechanisms may also initiate carcinogenesis (Goodman and Watson, 2002).

**Failure to Execute Apoptosis Promotes Mutation and Clonal Growth** As discussed earlier, in cells suffering DNA damage, the stabilized p53 protein may induce cell death by apoptosis (Fig. 3-33). Apoptosis thus eliminates cells with DNA damage, preventing mutations to initiate carcinogenesis. Initiated preneoplastic cells have much higher apoptotic activity than do normal cells (Bursch *et al.*, 1992) and this can counteract their clonal expansion. In fact, facilitation of apoptosis can induce tumor regression. This occurs when hormone-dependent tumors are deprived of the hormone that promotes growth and suppresses apoptosis. This is the rationale for the use of tamoxifen, an antiestrogen, and gonadotropin-releasing hormone analogues to combat hormone-dependent tumors of the mammary gland and the prostate gland, respectively (Bursch *et al.*, 1992).

Thus, inhibition of apoptosis is detrimental because it facilitates both mutations and clonal expansion of preneoplastic cells. Indeed, apoptosis inhibition plays a role in the pathogenesis of human B-cell lymphomas, in which a chromosomal translocation results in aberrantly increased expression of Bcl-2 protein, which overrides programmed cell death after binding to and inactivating the proapoptotic Bax protein (see Fig. 3-19). Increased levels of Bcl-2 are also detected in other types of cancer, and a high Bcl-2/Bax ratio in a tumor is a marker for poor prognosis (Jäättelä, 1999). Other antiapoptotic proteins, such as Hsp27, Hsp70, and Hsp90 (Sreedhar and Csermely, 2004) and IAP family members, may also contribute to progression of neoplasia. Survivin, a member of the IAP family, is expressed in all cancer cells but not in adult differentiated cells (Jäättelä, 1999).

Inhibition of apoptosis is one mechanism by which the rodent tumor promoter phenobarbital promotes clonal expansion of preneoplastic cells. This has been demonstrated in rats given a single dose of *N*-nitrosomorpholine followed by daily treatments with phenobarbital for 12 months to initiate and promote, respectively, neoplastic transformation in liver (Schulte-Hermann *et al.*, 1990). From 6 months onward, phenobarbital did not increase DNA synthesis and cell division in the preneoplastic foci, yet it accelerated foci enlargement. The foci grow because phenobarbital lowers apoptotic activity, allowing the high cell replicative activity to manifest itself. The peroxisome proliferator nafenopin, a nongenotoxic hepatocarcinogen, also suppresses apoptosis in primary rat hepatocyte cultures (Bayly *et al.*, 1994). Based on a recent study with WY-14643, another peroxisome proliferator (Gonzalez and Shah, 2008), it may be suggested that miR-17-92 upregulation and consequential suppression of Bim translation accounts for apoptosis inhibition.

**Failure to Restrain Cell Division Promotes Mutation, Proto-Oncogene Expression, and Clonal Growth** Enhanced mitotic activity, whether it is induced by oncogenes inside the cell or by external factors such as xenobiotic or endogenous mitogens, promotes carcinogenesis for a number of reasons:

1. **First, the enhanced mitotic activity increases the probability of mutations.** This is due in part to activation of the cell division cycle, which invokes a substantial shortening of the G<sub>1</sub> phase, allowing less time for the repair of injured DNA before replication, thus increasing the chance that the damage will yield a mutation. Although repair still may be feasible after

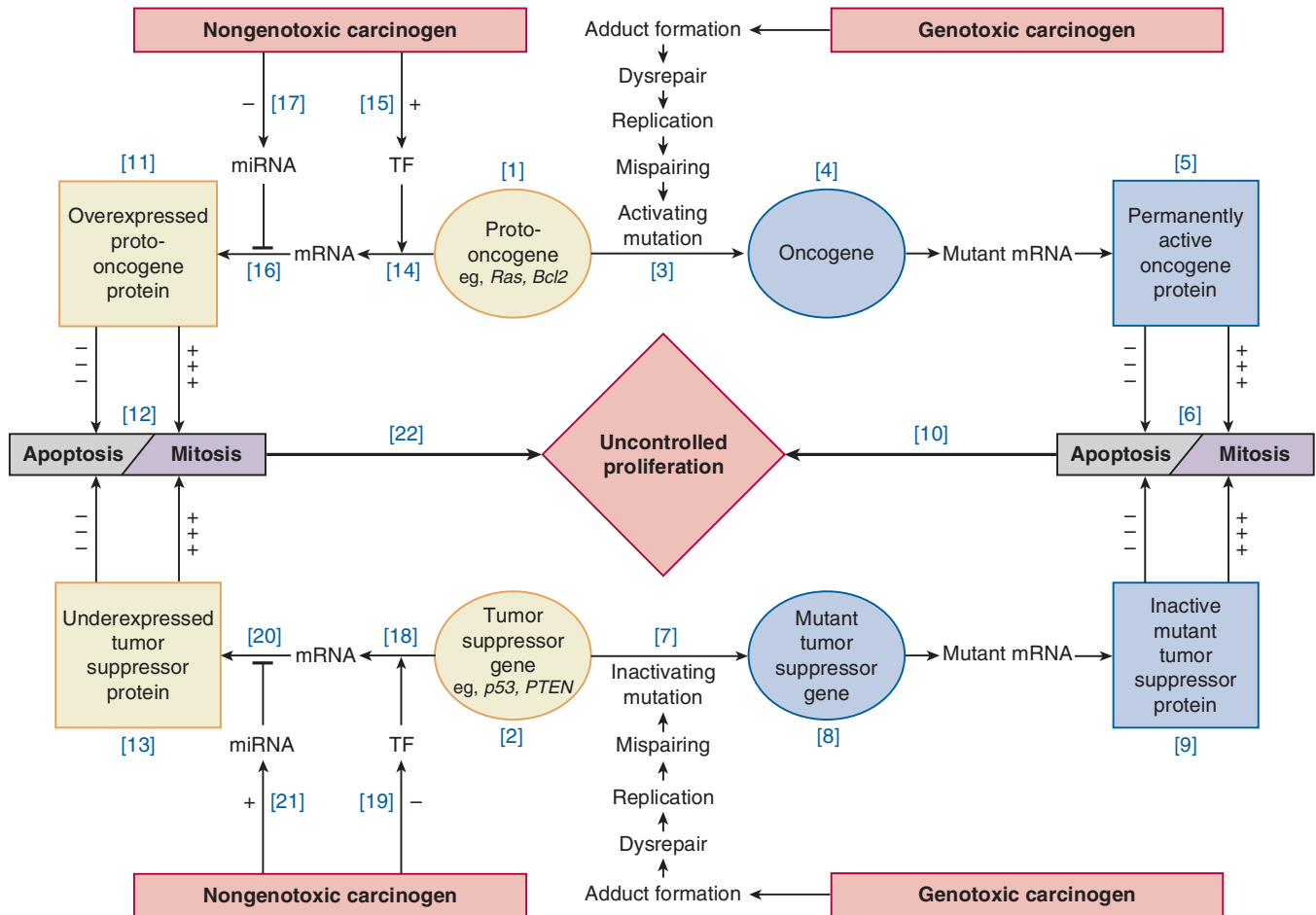
replication, postreplication repair is error-prone. In addition, activation of the cell division cycle increases the proportion of cells that replicate their DNA at any given time. During replication, DNA becomes unpacked and its amount doubles, greatly increasing the effective target size for DNA-reactive mutagenic chemicals, including ROS.

2. **Enhanced mitotic activity may compromise DNA methylation,** which occurs in the early postreplication period and is carried out by DNMTs that copy the methylation pattern of the parental DNA strand to the daughter strand (maintenance methylation). Limitation of DNMTs by shortened G<sub>2</sub> phase or by presence of other transacting factors might impair methylation and may contribute to overexpression of proto-oncogenes, starting a vicious cycle.
3. **During cell division, the cell-to-cell communication through gap junctions (constructed from connexins) and AJ (constructed from cadherins) is temporarily disrupted.** Several tumor promoters, such as phenobarbital, phorbol esters, and peroxisome proliferators, decrease gap junctional intercellular communication. It has been hypothesized that this contributes to neoplastic transformation. Increased susceptibility of connexin-knockout mice to spontaneous and chemically induced liver tumors supports this hypothesis (Chipman *et al.*, 2003). Lack of the AJ contributes to the invasiveness of tumor cells (see below).
4. Another mechanism by which proliferation promotes the carcinogenic process is **through clonal expansion of the initiated cells to form nodules (foci) and tumors.**

In summary, transformation of normal cells with controlled proliferative activity to malignant cells with uncontrolled proliferative activity is driven by 3 major forces: (1) accumulation of genetic damage in the form of mutant proto-oncogenes (which encode permanently activated cell cycle accelerator and/or antiapoptotic proteins) and mutant tumor suppressor genes (which encode permanently inactivated cell cycle decelerator and/or proapoptotic proteins); (2) increased transcription and/or translation of normal proto-oncogenes (causing expression of more cell cycle accelerator and/or antiapoptotic proteins); and (3) silencing of normal tumor suppressor genes at transcriptional and/or translational level (causing expression of less cell cycle decelerator and/or proapoptotic proteins). Uncontrolled proliferation results from offset of the balance between mitosis and apoptosis (Fig. 3-34).

Genotoxic carcinogens appear to induce cancer primarily by inducing activating mutations in proto-oncogenes or inactivating mutations in tumor suppressor genes, and secondarily by causing inappropriate expression rate of these critical genes. In contrast, nongenotoxic carcinogens cause cancer primarily by transcriptional or translational upregulation of the synthesis of proto-oncogene proteins or downregulation of the synthesis of tumor suppressor proteins. Secondarily, however, nongenotoxic carcinogens may also increase mutation of critical genes, which is initiated by genotoxic agents or spontaneous DNA damage. In human cells, spontaneous DNA damage may give rise to mutation at a frequency of 1 out of 10<sup>8</sup> to 10<sup>10</sup> base pairs (Barrett, 1992). Genotoxic carcinogens increase this frequency 10- to 1000-fold. Nongenotoxic carcinogens also increase the rate of spontaneous mutations through a mitogenic effect (see above) and increase the number of cells with DNA damage and mutations by facilitating their division and inhibiting their apoptosis. Both enhanced mitotic activity and decreased apoptotic activity expand the population of transformed cells.

According to an emerging theory, cancers may form by genotoxic and/or epigenetic mechanisms in pluripotent stem cell



**Figure 3-34.** A model depicting the modes of action of genotoxic and nongenotoxic carcinogens and the cooperation between proto-oncogenes and tumor suppressor genes in transformation of normal cells with controlled proliferation into neoplastic cells with uncontrolled proliferation. When produced in appropriate quantities, the normal proteins encoded by proto-oncogenes [1] and tumor suppressor genes [2] reciprocally influence mitosis and apoptosis and thus ensure controlled cell proliferation. However, the balance between the effects of these 2 types of proteins on the fate of cells is offset by carcinogens via genotoxic and nongenotoxic mechanisms resulting in uncontrolled proliferation.

A genotoxic (or DNA-reactive) carcinogen may induce cell proliferation in 2 ways. In the first way, it inflicts DNA damage (eg, by forming DNA adducts) that ultimately brings about an activating mutation [3] in a proto-oncogene [1] and the mutant proto-oncogene (then called an oncogene) [4] in turn encodes a constitutively (ie, permanently) active oncogene protein [5] that continuously signals for mitosis or against apoptosis [6], depending on its function. In the second way, the DNA-reactive chemical produces DNA damage that eventually yields an inactivating mutation [7] in a tumor suppressor gene [2], with the mutant gene [8] encoding an inactive tumor suppressor protein [9] that cannot restrain mitosis or evoke apoptosis (eg, in response to DNA damage). In both instances, the rate of mitosis will exceed the rate of apoptosis [6] and uncontrolled proliferation of the affected cells will ensue [10]. Such a scenario may underlie the carcinogenicity of aflatoxin B<sub>1</sub>, which induces mutation sometimes in the *Ras* proto-oncogene and often in the *p53* tumor suppressor gene (see text for details).

Nongenotoxic (epigenetic) carcinogens may also induce cell proliferation by 2 modes of action: first, by causing the overexpression of normal proto-oncogenes [1], yielding increased quantity of their protein products [11], which in turn excessively stimulate mitosis or inhibit apoptosis [12]. The second mode involves the underexpression of normal tumor suppressor genes [2], yielding diminished quantity of their protein products [13], which thus fail to restrain mitosis or promote apoptosis appropriately [12]. Nongenotoxic carcinogens may induce the synthesis of proto-oncogene proteins [11] at transcriptional and/or translational levels. They may facilitate the transcription of a proto-oncogene [1] into its mRNA [14] either by increasing the abundance of active transcription factors (TFs) at the promoter region of the gene or by facilitating the accessibility of TFs to the promoter (eg, by hypomethylation of this region) [15]. Nongenotoxic carcinogens may promote the translation of the mRNA into proto-oncogene protein [16] by decreasing the expression of microRNA (miRNA) [17] that normally represses the translation of this protein [16]. (Such miRNAs have tumor suppressor roles.) Nongenotoxic (also called epigenetic) carcinogens also may reduce the synthesis of tumor suppressor proteins [13] at transcriptional and/or translational levels. They may diminish the transcription of a tumor suppressor gene [2] into its mRNA [18] either by decreasing the abundance of active TFs at the promoter region of the gene or by impeding the accessibility of TFs to the promoter (eg, by hypermethylation of this region) [19]. Nongenotoxic carcinogens may downregulate the translation of the mRNA into a tumor suppressor protein [20] by increasing the expression of a miRNA [21] that normally represses the translation of this protein [20]. (Such miRNAs have oncogenic roles.) Eventually, overexpression of proto-oncogene proteins and/or underexpression of tumor suppressor proteins produce mitosis rate that exceeds the rate of apoptosis [12], thereby leading to uncontrolled proliferation of the affected cells [22]. Examples for nongenotoxic carcinogens acting by these mechanisms are given in the text.

In effect, the modes of action of these 2 types of chemical carcinogens are more complex: genotoxic carcinogens may also exert epigenetic effects and nongenotoxic carcinogens may increase the frequency of spontaneous mutations as well as the division and survival of cells carrying mutations (see the text for details).

populations. Such cells are characterized by quiescence, self-renewal, and conditional immortality, thus would potentially supply a lifelong, latent neoplastic population after carcinogen attack. Stem cells may be an especially likely target for transplacental carcinogens, such as DES or arsenic (Tokar *et al.*, 2011), because they are abundant during fetal development and have the longevity to carry the molecular lesion to the adult period of life.

Finally, further changes in gene expression may occur in these proliferating cells making them capable of invading the tissue and forming metastasis. These alterations confer the cells capacity for increased mobility by (1) their transdifferentiation into a mobile fibroblastoid phenotype (called epithelial-to-mesenchymal transition [EMT]), (2) disruption of the AJ that anchor cells to their neighbor (by downregulation of E-cadherin expression), and (3) degradation of the surrounding ECM (by upregulation of matrix metalloproteinases). Signaling systems that orchestrate EMT include those triggered by TGF- $\beta$  (Fig. 3-12), the Wnt ligand (see Fig. 3-13), and the Hh ligand (see Fig. 3-14) (Katoh and Katoh, 2008).

## CONCLUSIONS

This overview systematically surveys the mechanisms of the potential events that follow toxicant exposure and contribute to toxicity.

This approach is also useful in the search for mechanisms responsible for (1) selective toxicity, that is, differences in the sensitivity to toxicants of various organisms, such as different species and strains of animals, organs, and cells, and (2) alteration of toxicity by exogenous factors such as chemicals and food and physiologic or pathologic conditions such as aging and disease. To identify the mechanisms that underlie selective toxicity or alterations in toxicity, all steps where variations might occur must be considered systematically. Selective or altered toxicity may be due to different or altered (1) exposure; (2) delivery, thus resulting in a different concentration of the ultimate toxicant at the target site; (3) target molecules; (4) biochemical processes triggered by the reaction of the chemical with the target molecules; (5) repair at the molecular, cellular, or tissue level; or (6) altered gene expression-based stress responses as well as circulatory and thermoregulatory reflexes by which the affected organism can adapt to some of the toxic effects.

In this chapter, a simplified scheme has been used to give an overview of the development of toxicity (Fig. 3-1). In reality, the route to toxicity can be considerably more diverse and complicated. For example, one chemical may yield several ultimate toxicants, one ultimate toxicant may react with several types of target molecules, and reaction with one type of target molecule may have a number of consequences. Thus, the toxicity of one chemical may involve several mechanisms that can interact with and influence each other in an intricate manner.

This chapter has emphasized the significance of the chemistry of a toxicant in governing its delivery to and reaction with the target molecule as well as the importance of the biochemistry, molecular and cell biology, immunology, and physiology of the affected organism in its response to the action of the toxicant. An organism has mechanisms that (1) counteract the delivery of toxicants, such as detoxication; (2) reverse the toxic injury, such as repair mechanisms; and (3) offset some dysfunctions, such as adaptive responses. Thus, toxicity is not an inevitable consequence of toxicant exposure because it may be prevented, reversed, or compensated for by such mechanisms. Toxicity develops if the toxicant exhausts or impairs the protective mechanisms and/or overrides the adaptability of biological systems.

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# chapter 4

## Risk Assessment

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### Introduction and Historical Context

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## INTRODUCTION AND HISTORICAL CONTEXT

In the 1970s Congress established a basic plan for environmental laws that authorized regulatory actions to protect public health and the environment. These science-based actions provided the foundation for environmental and human health risk assessment. Toxicological research and toxicity testing constitute the scientific core of risk assessment, which is used for evaluating potential adverse health impacts from chemical exposures. However, such considerations for risk evaluation were not new, since for decades the American Conference of Governmental Industrial Hygienists (ACGIH) set threshold limit values for occupational exposures and the US Food and Drug Administration (FDA) established acceptable daily intakes (ADIs) for pesticide residues and food additives. In 1958, the US Congress instructed the FDA in the Delaney Clause to prohibit the addition of all substances found to cause cancer in animals or humans to the food supply. Pragmatically, this policy allowed food sources that had nondetectable levels of these additives to be declared “safe.” As advances in analytical chemistry revealed that “nondetects” were not equivalent to “not present,” regulatory agencies were forced to develop “tolerance levels” and “acceptable risk levels.” Risk assessment methodologies blossomed in the 1970s with the rising need to address these issues and statutes and to provide a common framework for considering human and ecological health effects (Albert, 1994).

Risk assessment was defined as a specific activity in 1983 in the National Research Council (NRC) publication *Risk Assessment*

*in the Federal Government: Managing the Process (Red Book)*. This book detailed steps for hazard identification, dose–response assessment, exposure analysis, and characterization of risks (NRC, 1983). These 4 basic framework steps now form the foundation framework for risk assessment and risk management approaches for many contexts such as children’s health and ecological health and they provide a consistent context for risk assessment frameworks across agencies nationally and worldwide. Risk assessment frameworks have been used by the Health and Safety Executive Risk Assessment Policy Unit of the United Kingdom (Great Britain and Health and Safety Executive, 1999) and by the World Health Organization (NRC, 2009; WHO, 2010) and all of these build from these common components. Fig. 4-1 illustrates a framework with bidirectional arrows demonstrating an ideal situation where mechanistic research feeds directly into risk assessments and critical data uncertainty drives research. The common elements of the basic risk assessment framework are shown in red highlight. Initially, attention in risk assessment was focused on cancer risks; in subsequent years, noncancer end points were examined with similar methods and risk assessments were performed across life stages and for ecosystems. Continuing advances in toxicology, epidemiology, exposure assessment, biologically based modeling of adverse responses, and modeling of variability and uncertainty, as well as the rapidly growing area of genomics, have contributed to improvements in risk assessment. Risk assessment links with the public policy objectives from risk management often require extrapolations that go far beyond the observation of actual effects. Extrapolating data to address risks continues to generate controversy.

The National Academy of Sciences (NAS) 1994 report entitled *Science and Judgment in Risk Assessment* captured in its title the combination of qualitative and quantitative approaches essential for effective assessment of risks (NRC, 1994). The report discussed in detail the challenges and provided approaches for incorporating new scientific findings into the risk assessment process. It also highlighted ways to deal with uncertainty when insufficient scientific information was available. To address these challenges, the Presidential/Congressional Commission on Risk Assessment and Risk Management (Risk Commission, 1997) formulated a comprehensive framework that (1) placed each environmental problem or issue into public health and/or ecological context and (2) proactively called for engaging relevant stakeholders, often affected or potentially affected community groups, from the very beginning of the 6-stage process shown in Fig. 4-2. This report emphasized that particular exposures and potential health effects must be evaluated across sources and exposure pathways and in light of multiple end points, not just one chemical, in one environmental medium (air, water, soil, food, products), for one health effect at a time. The single-chemical, single-exposure approach had been the general approach up to this time. The importance of defining the risk problem based on this initial input is critical to risk assessment. Problem formulation and scoping has now been defined as Phase 1 for risk-based decision making and has been outlined by the US Environmental Protection Agency (EPA) (US EPA, 2011b) and reinforced by Science and Decisions, the *Silver Book* (NRC, 2009).

## DEFINITIONS

Risk assessment is the systematic scientific evaluation of potential adverse health effects resulting from human exposures to hazardous agents or situations (NRC, 1983, 1994; Omenn, 2000). Risk is defined as the probability of an adverse outcome based

Table 4-1

**Objectives of Risk Assessment**

1. Protect human and ecological health
  - Toxic substances
2. Balance risks and benefits
  - Drugs
  - Pesticides
3. Set target levels of risk
  - Food contaminants
  - Water pollutants
4. Set priorities for program activities
  - Regulatory agencies
  - Manufacturers
  - Environmental/consumer organizations
5. Estimate residual risks and extent of risk reduction after steps are taken to reduce risks

toxic properties, whereas exposure becomes an essential consideration along with hazard for risk. Risk assessment requires an integration of both qualitative and quantitative scientific information. For example, qualitative information about the overall evidence and nature of the end points and hazards is integrated with quantitative assessment of the exposures, host susceptibility factors, and the magnitude of the hazard. A description of the uncertainties and variability in the estimates is a significant part of risk characterization and an essential component of risk assessment. Analogous approaches are applied to ecological risks, as demonstrated by EPA's ecological risk assessment guidelines (US EPA, 2011b). The objectives of risk assessment are outlined in Table 4-1.

The phrase “characterization of risk” reflects the combination of qualitative and quantitative analysis. Increasingly, the use of the term “hazard characterization” versus “hazard identification” has been seen in both the US EPA (2012a) and WHO (1999) documents. This shift recognizes the difficulties in evaluating only hazard information without dose–response information. Unfortunately, many users equate risk assessment with quantitative risk assessment, generating a number for an overly precise risk estimate, while ignoring crucial information about the uncertainties of the 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 stage of the framework (Fig. 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 Commission, 1997).

Risk communication is the challenging process of making risk assessment and risk management information comprehensible to the public, community groups, lawyers, local elected officials, judges, business people, labor, environmentalists, etc (Morgan, 1993; Sandman, 1993; Fischhoff *et al.*, 1996; NRC, 1996). Such communication requires an understanding of level of risk response needed, how and which individuals and organizations should be informed, and what the audience hears versus what was intended as the risk communication message. Involving stakeholders in the initial problem formulation, as well as in the translation of the bottom line has become an essential part of risk management. Part of this analytical–deliberative process is the crucial, too-often neglected or delayed need for considering the fears, perceptions, priorities, and

proposed remedies of these stakeholders (Risk Commission, 1997; Drew *et al.*, 2003, 2006; Judd *et al.*, 2005; Kramer *et al.*, 2006; Cullen *et al.*, 2008). Underestimating potential public and media attitudes toward local polluters, other responsible parties, and relevant government agencies may lead to what has been labeled “the outrage factor” (Sandman, 1993), greatly influencing the communication process and the choices for risk management. Sometimes the decision makers and stakeholders simply want to know the “bottom line”: whether a substance or a situation is “safe” or not. Others will be keenly interested in knowing how and why the risk estimates are uncertain and may be well prepared to challenge underlying assumptions about context and methodology. Stakeholders can also be part of a risk management solution (Judd *et al.*, 2005; Drew *et al.*, 2006). Perception of risk is further discussed at the end of this chapter.

## DECISION MAKING

Risk management decisions are reached under diverse statutes in the United States. Table 4-2 lists examples of major environmental statutes and the year of initial enactment. Some statutes specify reliance on risk alone, whereas others require a balancing of risks and benefits of the product or activity. 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. Similar approaches for risk assessment have been developed in many other countries and through such international organizations as the International Programme on Chemical Safety (IPCS) within the WHO. Currently within the IPCS, there are significant efforts toward a global harmonization of risk assessment methodology (WHO, 2006). The *WHO Human Health Risk Assessment Toolkit: Chemical Hazards* is an example (WHO, 2010). Their goals include use of a common risk assessment framework and development of confidence and acceptance of different risk assessment approaches through a common understanding and agreement on basic principles of testing and evaluation.

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 epidemiologic studies, animal bioassays, short-term in vitro and in vivo tests, and structure–activity 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 potential action in target organs and to host health impacts (NRC, 1989a,b, 1992a,b; Adami *et al.*, 2011). Mechanistic investigations of the actions of specific chemicals can help us penetrate the “black box” approach of simply counting tumors, for example, in exposed animals in routine bioassays. Greater appreciation of the mechanisms and extent of individual variation in susceptibility to adverse health impacts among humans can improve protection of susceptible populations and better relate findings in animals to the characterization of risk for humans. Identifying individual behavioral and social risk factors can be critically important both to the characterization of risk and to the reduction of risk.

## HAZARD IDENTIFICATION

### Assessing Toxicity of Chemicals—Introduction

In order to assess the toxicity of chemicals, information from four types of studies is used: structure–activity relationships (SAR), in vitro or short-term studies, in vivo animal bioassays, and