CHAPTER 10

Elimination of Toxicants

GERALD A. LEBLANC

10.1 INTRODUCTION

The ability to efficiently eliminate toxic materials is critical to the survival of a species. The complexity of toxicant elimination processes has increased commensurate with the increased complexity associated with animal form. For unicellular organisms, passive diffusion can suffice for the elimination of toxic metabolic wastes produced by the organism. Similarly, as exogenous toxic materials derived from the environment diffuse into a unicellular organism, they can also readily diffuse out of the organism. The large surface area to mass ratio of these organisms ensures that a toxic chemical within the cell is never significantly distanced from a surface membrane across which it can diffuse.

As organisms evolved in complexity, several consequences of increased complexity compromised the efficiency of the passive diffusion of toxic chemicals:

1. They increased in size.
2. Their surface area to body mass decreased.
3. Their bodies compartmentalized (i.e., cells, tissues, organs).
4. They generally increased in lipid content.
5. They developed barriers to the external environment.

Size. With increased size of an organism, a toxic chemical has greater distance to traverse before reaching a membrane across which it can diffuse to the external environment. Thus overall retention of the chemical will increase as will propensity for the chemical to elicit toxicity.

Surface Area to Body Mass Ratio. Increased size of an organism is associated with a decrease in the surface area to body mass ratio. Accordingly, the availability of surface membranes across which a chemical can passively diffuse to the external environment decreases and propensity for retention of the chemical increases.

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Compartmentalization. With increased complexity comes increased compartmentalization. Cells associate to form tissues and tissues associate to form organs. Compartmentalization increases the number of barriers across which chemicals must traverse before sites of elimination are reached. As different compartments often have different physicochemical characteristics (i.e., adipose tissue is largely fat while blood is largely aqueous), chemicals are faced with the challenge to be mobile in these various environments.

Lipid Content. As a general but not universal rule, the ability of organisms to store energy as fat increases with increased size of the organism. Thus large organisms tend to have significant lipid stores into which lipophilic chemicals can be stored for extended periods of time. These stored chemicals tend to be largely immobile and difficult to release from the adipose tissue.

Barriers to the Environment. Through evolution, increased complexity of organisms led to increased exploitation of various environments. In order to survive in these environments, organisms developed barriers such as skin and scales that protect from harsh conditions on the outside and minimize loss of vital constituents such as water on the inside. Likewise these barriers impede the elimination of toxic constituents by the organisms, requiring the development of specialized membranes and organs through which toxic materials can be eliminated.

A consequence of this hindrance to elimination of toxic materials by complex organisms was the development of specialized routes of elimination. These routes generally evolved in concert (i.e., co-evolved) with biotransformation processes that render chemicals amenable to these modes of elimination (see Chapters 7–9).

Three major routes of elimination culminate in the specialized organs of elimination, the liver, kidneys, and lungs. The liver serves as a major organ at which lipophilic materials are collected from the blood, biotransformed to generally less toxic and more polar derivatives, then eliminated into the bile. The kidneys complement the liver in that these organs collect wastes and other chemicals in the blood through a filtration process and eliminate these wastes in the urine. The respiratory membranes of the lungs are ideal for the removal of volatile materials from the blood into expired air. In addition to these major routes of elimination, several quantitatively minor routes exist through which toxic materials can be eliminated from the body. These include the following:

1. *Skin.* Skin constitutes the largest organ in the human body, and it spans the interface between the body and the external environment. While the skin's epidermis constitutes a relatively impervious membrane across which chemical elimination is difficult, the shear surface area involved requires consideration of this organ as a route of elimination. Volatile chemicals are particularly adept at traversing the skin and exiting the body through this route.
2. *Sweat.* Humans lose an average of 0.7 L of water per day due to sweating. This loss of fluid provides a route for the elimination of water-soluble chemicals.
3. *Milk.* Mother's milk is rich in lipids and lipoproteins. Milk thus serves as an ideal route for the elimination of both water-soluble and fat-soluble chemicals from the mother's body. For example, the DDT metabolite DDE, the flame retardant mirex, and the polychlorinated biphenyls (PCBs) often have been detected in mother's

milk. While lactation may provide a benefit to the mother by the elimination of toxic chemicals, transfer of these toxicants to the suckling infant can have dire consequences.

4. *Hair.* Growing hair can serve as a limited route through which chemicals can escape the body. Pollutants such as mercury and drugs such as cocaine have been measured in human hair, and hair analyses is often used as a marker of exposure to such materials.

10.2 TRANSPORT

For a chemical to be eliminated from the body at a site of elimination (i.e., kidney) that is distant from the site of storage (i.e., adipose tissue) or toxicity (i.e., brain), the chemical must be transported from the site of origin to the site of elimination. Chemicals are transported to the site of elimination largely via the circulatory system. Sufficiently water-soluble chemicals can freely dissolve into the aqueous component of blood and be transported by both diffusion and blood circulation to sites of elimination. With decreasing water solubility and increasing lipid solubility, chemicals are less likely to freely diffuse into blood and extraction of these chemicals from sites of toxicity or storage can be more challenging. These materials generally associate with transport proteins in the blood, which either contain binding sites for chemical attachment or lipophilic cores (lipoproteins) into which lipophilic chemicals can diffuse. The blood contains various transport proteins that are typically suited for the transport of specific endogenous chemicals. These include albumin, sex steroid-binding globulin, and lipoproteins. Often xenobiotics can utilize these proteins, particularly the nonspecific transporters, to facilitate mobilization and transport in the aqueous environment of the blood. At the site of elimination, xenobiotics may diffuse from the transport protein to the membranes of the excretory organ, or the transport protein may bind to surface receptors on the excretory organ, undergo endocytosis and intracellular processing, where the xenobiotic is released and undergoes processing leading to elimination.

10.3 RENAL ELIMINATION

The kidneys are the sites of elimination of water-soluble chemicals that are removed from the blood by the process of reverse filtration. Two characteristics are primarily responsible for determining whether a chemical will be eliminated by the kidneys: size and water solubility.

Size. The reverse filtration process requires that chemicals to be removed from the blood are able to pass through 70 to 100 A pores. As a rule, chemicals having a molecular mass of less than 65,000 are sufficiently small to be subject to reverse filtration.

Water Solubility. Non-water-soluble chemicals will be transported to the kidneys in association with transport proteins. Thus, in association with these proteins, the chemicals will not be able to pass through the pores during reverse filtration. Lipophilic chemicals are generally subject to renal elimination after they

have undergone hydroxylation or conjugation reactions (Chapter 7) in the liver or elsewhere.

Blood is delivered to the human kidney by the renal artery. Blood flows to the kidneys of the adult human at a rate of roughly 1 L/min. The adult human kidney contains approximately 1 million functional units, called nephrons, to which the blood is delivered for removal of solutes. Collected materials are excreted from the body in the urine.

Blood entering the nephron passes through a network of specialized capillaries called the glomerulus (Figure 10.1). These capillaries contain the pores through which materials to be eliminated from the blood pass. Blood in the capillaries is maintained under high positive pressure from the heart coupled with the small diameter of the vessels. As a result these sufficiently small solutes and water are forced through the pores of the glomerulus. This filtrate is collected in the glomerular (or Bowman's) capsule in which the glomerulus is located (Figure 10.1). Included in this filtrate are water, ions, small molecules such as glucose, amino acids, urate, and foreign chemicals. Large molecules such as proteins and cells are not filtered and are retained in the blood.

Following glomerular filtration, molecules important to the body are re-absorbed from the filtrate and returned to the blood. Much of this re-absorption occurs in the proximal tubules (Figure 10.1). Cells lining the proximal tubules contain fingerlike projections that extent into the lumen of the tubule. This provides an expanse of cell surface area across which water and ions can diffuse back into the cells and, ultimately, be returned to the blood. The proximal tubules also contain active transport proteins that recover small molecules such as glucose and amino acids from the filtrate. From the proximal tubules, the filtrate passes through the Loop of Henle. Significant water

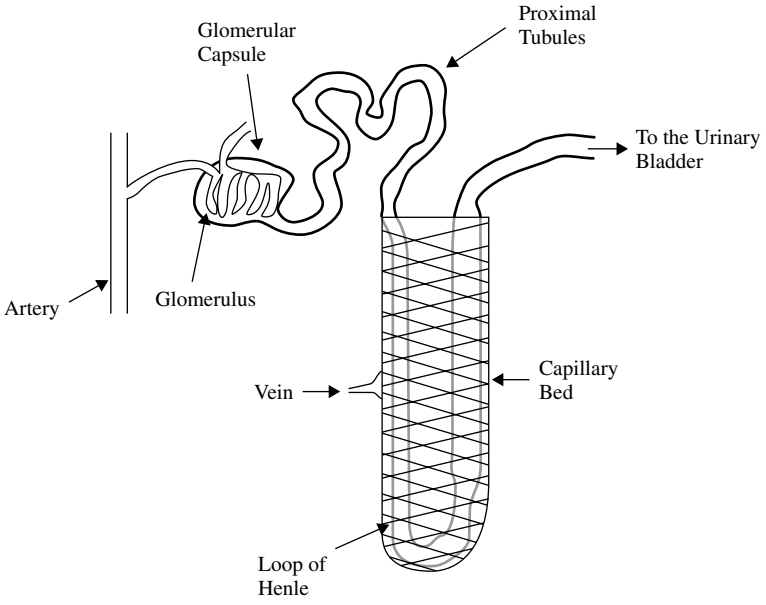

Figure 10.1 The nephron of the kidney. The nephron is the functional unit of the kidney that is responsible for the removal of water-soluble wastes and foreign compounds from the blood.

re-absorption occurs in the descending portion of the loop, resulting in concentration of the filtrate. Water re-absorption does not occur in the ascending portion of the loop. Rather, the remaining, concentrated ions such as sodium, chloride, and potassium are re-absorbed. Those materials retained in the filtrate during passage through the nephron constitute the urine. The urine is transported through the ureters to the bladder and retained until excretion occurs.

The kidneys are a common site of chemical toxicity since the nephron functions to concentrate the toxicant and thus increase levels of exposure to the materials. This increased exposure can result from the concentration of the toxicant in the tubules. It also can occur by concentration within the cells of the nephrons when a chemical is capable of utilizing one of the active transport proteins and is shuttled from the lumen of the tubules into the renal cells.

10.4 HEPATIC ELIMINATION

The liver serves many vital functions to the body. It has a large capacity to hold blood and thus serves as a blood storage site. The liver synthesizes and secretes many substances that are necessary for normal bodily function. It cleanses the blood of various endogenous and foreign molecules. It biotransforms both endogenous and exogenous materials, reducing their bioreactivity and preparing them for elimination. It eliminates wastes and foreign chemicals through biliary excretion. Three of these functions occur coordinately in a manner that makes the liver a major organ of chemical elimination: chemical uptake from blood, chemical biotransformation, and biliary elimination of chemicals.

Blood is delivered to the liver from two sources. Oxygen-rich blood is delivered through the hepatic artery. In addition blood is shunted from the capillaries that service the intestines and spleen to the liver by the hepatic portal vein. These two vessels converge, and the entire hepatic blood supply is passaged through sinusoids (Figure 10.2). Sinusoids are cavernous spaces among the hepatocytes that are the functional units of the liver. Hepatocytes are bathed in blood as the blood passes through the sinusoids, as 70% of the hepatocyte surface membrane contacts the blood in the sinusoid. This provides for a tremendous surface area across which chemicals can diffuse to gain entry into the hepatocytes. Chemicals may passively diffuse across the sinusoidal

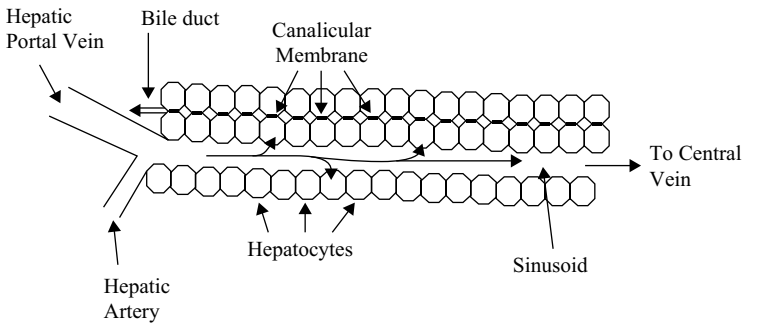

Figure 10.2 Diagrammatic representation of the basic architecture of the liver.

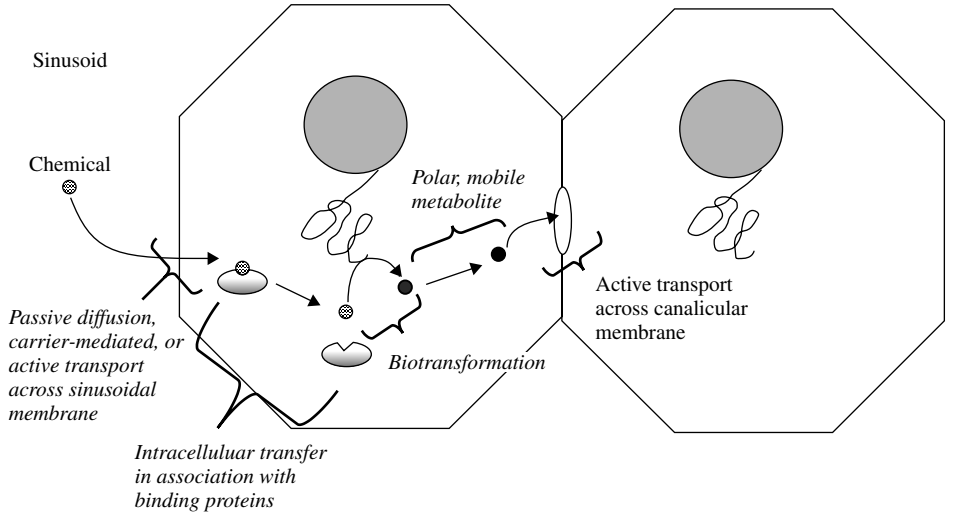

Figure 10.3 Vectorial transport of a chemical from the liver sinusoid, through the hepatocyte, to the canalicular space.

membrane of the hepatocytes, they may be exchanged between blood transport proteins and the sinusoidal membranes, or their carrier proteins may bind to sinusoidal membrane receptors and then undergo endocytosis (Figure 10.3).

Lipophilic materials require intracellular carrier proteins to be optimally mobilized, just as they required transport proteins in the blood (Figure 10.3). Several intracellular carrier proteins that mobilize specific endogenous chemical have been characterized, although less is known of which proteins typically mobilize xenobiotics. Some of the cytosolic glutathione *S*-transferase proteins have been shown to noncatalytically bind xenobiotics and to be coordinately induced along with xenobiotic biotransformation enzymes and efflux transporters, suggesting that these proteins may function to mobilize xenobiotics.

Once mobilized in the hepatocyte, chemicals can contact and interact with biotransformation enzymes (Chapter 7). These enzymes generally increase the polarity of the chemical, thus reducing its ability to passively diffuse across the sinusoidal membrane back into the blood. Biotransformation reactions also typically render the xenobiotics susceptible to active transport across the canalicular membrane into the bile canaliculus and, ultimately, the bile duct (Figure 10.3). The bile duct delivers the chemicals, along with other constituents of bile, to the gall bladder that excretes the bile into the intestines for fecal elimination.

10.4.1 Entero-hepatic Circulation

Once in the gastrointestinal tract, chemicals that have undergone conjugation reactions in the liver may be subject to the action of hydrolytic enzymes that de-conjugate the molecule. De-conjugation results in increased lipophilicity of the molecule and renders them once again subject to passive uptake. Re-absorbed chemicals enter the circulation via the hepatic portal vein, which shunts the chemical back to the liver where

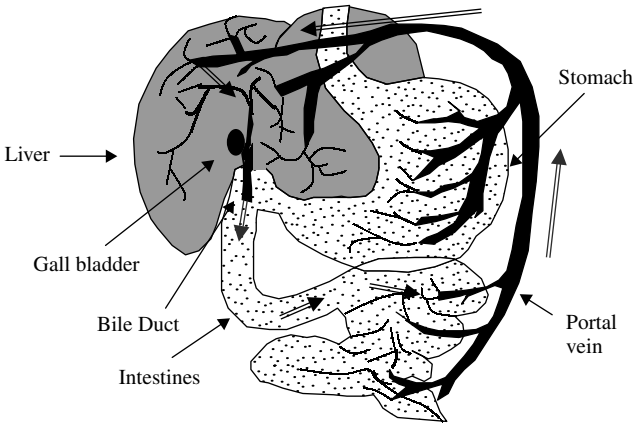

Figure 10.4 Enterohepatic circulation (as indicated by ⟹). Polar xenobiotic conjugates are secreted into the intestine via the bile duct and gall bladder. Conjugates are hydrolyzed in the intestines, released xenobiotics are reabsorbed, and transported back to the liver via the portal vein.

the chemical can be reprocessed (i.e., biotransformed) and eliminated. This process is called entero-hepatic circulation (Figure 10.4). A chemical may undergo several cycles of entero-hepatic circulation resulting in a significant increase in the retention time for the chemical in the body and increased toxicity.

The liver functions to collect chemicals and other wastes from the body. Accordingly, high levels of chemicals may be attained in the liver, resulting in toxicity to this organ. Biotransformation of chemicals that occur in the liver sometimes results in the generation of reactive compounds that are more toxic than the parent compound resulting in damage to the liver. Chemical toxicity to the liver is discussed elsewhere (Chapter 14).

10.4.2 Active Transporters of the Bile Canaliculus

The bile canaliculus constitutes only about 13% of the contiguous surface membrane of the hepatocyte but must function in the efficient transfer of chemical from the hepatocyte to the bile duct. Active transport proteins located on the canalicular membrane are responsible for the efficient shuttling of chemicals across this membrane. These active transporters are members of a multi-gene superfamily of proteins known as the ATP-binding cassette transporters. Two subfamilies are currently recognized as having major roles in the hepatic elimination of xenobiotics, as well as endogenous materials. The *P*-glycoprotein (ABC B) subfamily is responsible for the elimination of a variety of structurally diverse compounds. *P*-glycoprotein substrates typically have one or more cyclic structures, a molecular weight of 400 or greater, moderate to low lipophilicity ($\log K_{ow} < 2$), and high hydrogen (donor)-bonding potential. Parent xenobiotics that meet these criteria and hydroxylated derivatives of more lipophilic compounds are typically transported by *P*-glycoproteins.

The multidrug-resistance associated protein (ABC C) subfamily of proteins largely recognizes anionic chemicals. ABC C substrates are commonly conjugates of xenobiotics (i.e., glutathione, glucuronic acid, and sulfate conjugates). Thus conjugation

not only restricts passive diffusion of a lipophilic chemical but actually targets the xenobiotic for active transport across the canalicular membrane.

10.5 RESPIRATORY ELIMINATION

The lungs are highly specialized organs that function in the uptake and elimination of volatile materials (i.e., gasses). Accordingly, the lungs can serve as a primary site for the elimination of chemicals that have a high vapor pressure. The functional unit of the lung is the alveolus. These small, highly vascularized, membraneous sacs serve to exchange oxygen from the air to the blood (uptake), and conversely, exchange carbon dioxide from the blood to the air (elimination). This exchange occurs through passive diffusion. Chemicals that are sufficiently volatile also may diffuse across the alveolar membrane, resulting in removal of the chemical from the blood and elimination into the air.

10.6 CONCLUSION

Many processes function coordinately to ensure that chemicals distributed throughout the body are efficiently eliminated at distinct and highly specialized locations. This unidirectional transfer of chemicals from the site of origin (storage, toxicity, etc.) to the site of elimination is a form of vectorial transport (Figure 10.5). The coordinate action of blood binding proteins, active transport proteins, blood filtration units, intracellular binding proteins, and biotransformation enzymes ensures the unidirectional flow of chemicals, ultimately resulting in their elimination. The evolution of this complex interplay of processes results in the efficient clearance of toxicants and has provided the way for the co-evolution of complexity in form from unicellular to multi-organ organisms.

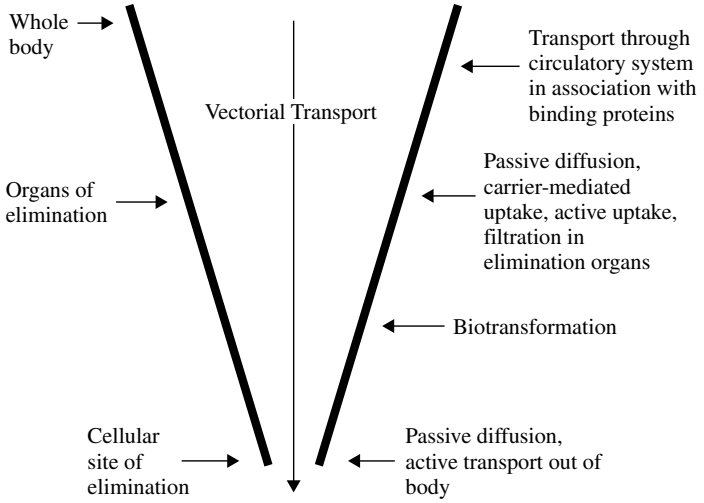

Figure 10.5 Processes involved in the vectorial transport of xenobiotics from the whole body point of origin to the specific site of elimination.

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PART IV

TOXIC ACTION

CHAPTER 11

Acute Toxicity

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11.1 INTRODUCTION

Acute toxicity of a chemical can be viewed from two perspectives. Acute toxicity may be the descriptor used as a qualitative indicator of an incident of poisoning. Consider the following statement: "methyl isocyanate gas, accidentally released from a chemical manufacturing facility in 1984, was *acutely toxic* to the residents of Bhopal, India." This statement implies that the residents of Bhopal were exposed to sufficiently high levels of methyl isocyanate over a relatively short time to result in immediate harm. High-level, short-term exposure resulting in immediate toxicity are all characteristics of acute toxicity. Alternatively, acute toxicity may represent a quantifiable characteristic of a material. For example, the statement: "the *acute toxicity* of methyl isocyanate, as measured by its LD50 in rats, is 140 mg/kg" defines the acute toxicity of the chemical. Again, the characterization of the quantified effects of methyl isocyanate as being acute toxicity implies that this quantification occurred during or following short-term dosing and that the effect measured occurred within a short time period following dosing. In terms of these qualitative and quantitative aspects, acute toxicity can be defined as *toxicity elicited immediately following short-term exposure to a chemical.* By this definition, two components comprise acute toxicity: acute exposure and acute effect.

11.2 ACUTE EXPOSURE AND EFFECT

In contrast to acute toxicity, chronic toxicity is characterized by prolonged exposure and sublethal effects elicited through mechanisms that are distinct from those that cause acute toxicity. Typically acute and chronic toxicity of a chemical are easily distinguished. For example, mortality occurring within two days of a single dose of a chemical would be a prime example of acute toxicity (Figure 11.1*a*). Similarly, reduced litter size following continuous (i.e., daily) dosing of the parental organisms would be indicative of chronic toxicity (Figure 11.1*b*). However, defining toxicity as being acute or chronic is sometimes challenging. For example, chronic exposure to a persistent, lipophilic chemical may result in sequestration of significant levels of the chemical

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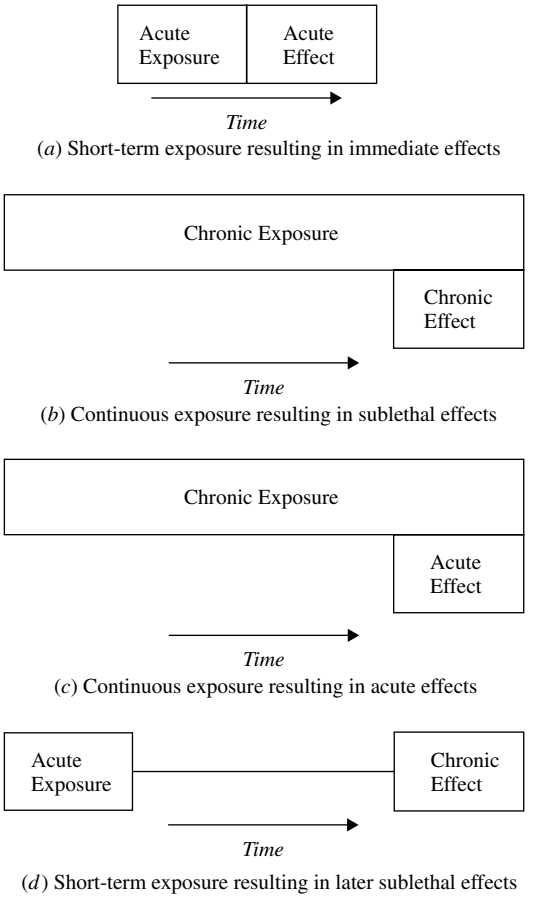

(*a*) Short-term exposure resulting in immediate effects

(*b*) Continuous exposure resulting in sublethal effects

(*c*) Continuous exposure resulting in acute effects

(*d*) Short-term exposure resulting in later sublethal effects

Figure 11.1 Examples of exposure/effect scenarios that result in either acute toxicity (*a*), chronic toxicity (*b*), or mixed acute/chronic toxicity (*c,d*). Examples for each scenario are provided in the text.

in adipose tissue of the organism with no resulting overt toxicity. Upon entering the reproductive phase, organisms may mobilize fatty stores, releasing the chemical into the blood stream resulting in overt toxicity including death (Figure 11.1*c*). One could argue under this scenario that chronic exposure ultimately resulted in acute effects. Lastly, acute exposure during a susceptible window of exposure (i.e., embryo development) may result in reproductive abnormalities and reduced fecundity once the organism has attained reproductive maturity (Figure 11.1*d*). Thus acute exposure may result in chronic toxicity.

An additional consideration is noteworthy when comparing acute and chronic toxicity. All chemicals elicit acute toxicity at a sufficiently high dose, whereas all chemicals do not elicit chronic toxicity. Paracelsus' often cited phrase "all things are poison ... the dose determines ... a poison" is clearly in reference to acute toxicity. Even the most benign substances will elicit acute toxicity if administered at a sufficiently high dose. However, raising the dose of a chemical does not ensure that chronic toxicity will ultimately be attained. Since chronic toxicity typically occurs at dosages below those

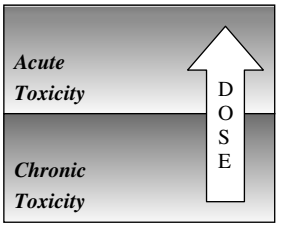

Figure 11.2 Relationships among chemical dose, acute toxicity and chronic toxicity. All chemicals elicit acute toxicity at a sufficiently high dose. However, chronic toxicity may not occur since dosage elevation may simply lead to acute toxicity.

that elicit acute toxicity, toxicity observed at the higher dosage may simply reflect acute, and not chronic, toxicity (Figure 11.2).

Effects encountered with acute toxicity commonly consist of mortality or morbidity. From a quantitative standpoint these effects are measured as the LD50, ED50, LC50, or EC50. The LD50 and ED50 represent the dose of the material that causes mortality (LD50) or some other defined effect (ED50) in 50% of a treated population. The LC50 and EC50 represent the concentration of the material to which the organisms were exposed that causes mortality (LC50) or some other defined effect (EC50) in 50% of an exposed population. LD50 and ED50 are normalize to the weight of the animal (i.e., mg chemical/kg body weight); whereas LC50 and EC50 are normalized to the environment in which the organisms were exposed (i.e., mg chemical/L water).

11.3 DOSE-RESPONSE RELATIONSHIPS

Acute toxicity of a chemical is quantified by its dose-response curve. This relationship between dose of the chemical administered and the resulting response is established by exposing groups of organisms to various concentrations of the chemical. Ideally doses are selected that will elicit $>0\%$ effect but $<100\%$ effect during the course of the experiment. At defined time periods following dosing, effects (e.g., mortality) are recorded. Results are plotted in order to define the dose-response curve (Figure 11.3*a*). A well-defined dose-response curve generated with a population of organisms whose susceptibility to the chemical is normally distributed will be sigmoidal in shape. The various segments (see Figure 11.3*a*) of the curve are represented as follows:

Segment I. This portion of the line has no slope and is represented by those doses of the toxicant that elicited no mortality to the treated population of organisms.

Segment II. This segment represents those dosages of the toxicant that affected only the most susceptible members of the exposed population. Accordingly, these effects are elicited at low doses and only a small percentage of the dosed organisms are affected.

Segment III. This portion of the line encompasses those dosages at which most of the groups of organisms elicit some response to the toxicant. Because most of the groups of exposed organisms respond to the toxicant within this range of dosages, segment III exhibits the steepest slope among the segments.

Segment IV. This portion of the line encompasses those dosages of the toxicant that are toxic to even the most tolerant organisms in the populations. Accordingly, high dosages of the toxicant are required to affect these organisms.

Segment V. Segment V has no slope and represents those dosages at which 100% of the organisms exposed to the toxicant have been affected.

A well-defined dose-response curve can then be used to calculate the LD50 for the toxicant. However, in order to provide the best estimate of the LD50, the curve is typically linearized through appropriate transformations of the data. A common transformation involves converting concentrations to logarithms and percentage effect to probit units (Figure 11.3*b*). Zero percent and 100% responses cannot be converted to probits; therefore data within segments I and V are not used in the linearization. A 95% confidence interval also can be determined for the linearize dose-response relationship (Figure 11.3*b*). As depicted in Figure 11.3*b*, the greatest level of confidence (i.e., the smallest 95% confidence interval) exists at the 50% response level, which is why LD50 values are favored over some other measure of acute toxicity (eg., LD05). This high level of confidence in the LD50 exists when ample data exist between the 51% and 99% response as well as between the 1% and 49% response.

Additional important information can be derived from a dose-response curve. The slope of the linearized data set provides information on the specificity of the toxicant. Steep slopes to the dose-response line are characteristic of toxicants that elicit toxicity by interacting with a specific target, while shallow slopes to the dose-response line are characteristic of toxicant that elicit more nonspecific toxicity such as narcosis.

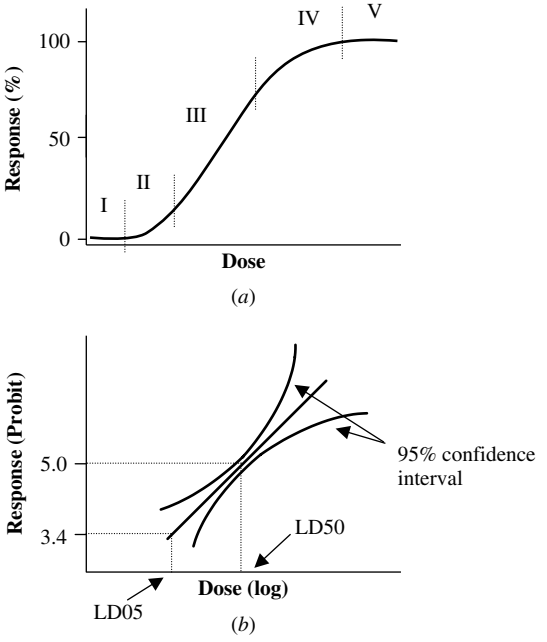

Figure 11.3 The dose-response relationship. (*a*) Five segments of the sigmoidal dose-response curve as described in the text. (*b*) Linearized dose-response relationship through log (dose)-probit (effect) transformations. Locations of the LD50 and LD05 are depicted.

The dose-response line also can be used to estimate the threshold dose. The threshold dose is defined as the lowest dose of the chemical that would be expected to elicit a response under conditions at which the assay was performed. The threshold dose is often empirically estimated as being a dose less that the lowest dose at which an effect was measured but higher than the greatest dose at which no effect was detected. Conceptually, the threshold dose is defined as the intercept of segments I and II of the dose-response curve (Figure 11.3*a*). Statistically, the threshold dose can be estimated from the linearized dose-response curve as the LC05. This value will closely approximate the threshold dose and can be statistically derived from the entire data set (i.e., the dose-response line). However, confidence in this value is greatly compromised, since it is derived from one end of the line (Figure 11.3*b*).

11.4 NONCONVENTIONAL DOSE-RESPONSE RELATIONSHIPS

The low-level effects of chemicals have received attention among pharmacologists for over 100 years. A current resurgence in interest among pharmacologists in low-level effects stems from use of homeopathic approaches to treating disease. Proponents of homeopathy maintain that low levels of toxic materials stimulate physiological responses that can target disease without eliciting adverse effects in the individual undergoing treatment. Homeopathic principles may have application in toxicology based on the premise that exposure to some chemicals at subthreshold levels, as defined by standard acute toxicity evaluations, can elicit toxicological as well as pharmacological effects. Both pharmacological and toxicological homeopathy may be the consequence of hormesis.

Hormesis is defined as an overcompensatory response to some disruption in homeostasis. Thus hormesis is typically evident at low doses of a chemical at which gross disruptions in homeostasis do not mask the hormetic response. Further, hormesis typically presents as an effect opposite to that elicited at higher levels of the chemical. For example, a chemical that stimulates corticosteroid secretion at high dosages resulting in hyperadrenocorticism might elicit a hormetic response at low dosages resulting in corticosteroid deficiency. A hypothetical nonconventional dose-response relationship resulting from such interactions is depicted in Figure 11.4. At the true threshold dose,

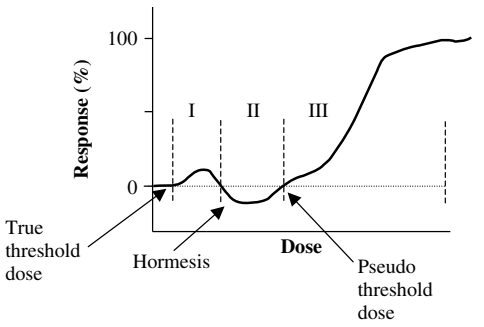

Figure 11.4 Nonconventional dose-response relationship involving low-dose effects and compensation. (I) True initiation of the response followed by a compensatory response that returns the effects to the 0% level. (II) A negative response due to overcompensation (hormesis) followed by recovery to the 0% effect level. (III) The standard sigmoidal dose-response relationship.

the organisms begin to exhibit increased stimulation in corticosteroid secretion. However, at slightly higher doses, a compensatory response occurs whereby corticosteroid secretion is decreased in order to maintain homeostasis within the organism. Overcompensation may actually result in a decrease in corticosteroid secretion at certain toxicant dosages. Finally the compensatory abilities of the organism are overcome by the high doses of the toxicant at the "pseudo" threshold dose, above which the standard dose-response relationship occurs. Nonconventional dose-response relationships have been observed with respect to both acute and chronic toxicity and are particularly relevant to the risk assessment process when establishing levels of exposure that are anticipated to pose no harm.

11.5 MECHANISMS OF ACUTE TOXICITY

An exhaustive review of the mechanisms by which chemicals cause acute toxicity is beyond the scope of this chapter. However, certain mechanisms of toxicity are relevant since they are common to many important classes of toxicants. Some of these mechanisms of acute toxicity are discussed.

11.5.1 Narcosis

Narcosis in toxicology is defined as toxicity resulting from chemicals associating with and disrupting the lipid bilayer of membranes. Narcotics are classified as either nonpolar (class 1) or polar (class 2) compounds. Members of both classes of compounds are lipid soluble. However, class 2 compounds possess constituents that confer some charge distribution to the compound (i.e., aliphatic and aromatic amines, nitroaromatics, alcohols). The aliphatic hydrocarbon (C5 through C8) are examples of powerful class 1 narcotics, whereas, ethanol is an example of a class 2 narcotic. The affinity of narcotics to partition into the nonpolar core of membranes (class 1 narcotics) or to distribute in both the polar and nonpolar components of membranes (class 2 narcotics) alters the fluidity of the membrane. This effect compromises the ability of proteins and other constituents of the membranes to function properly leading to various manifestation of narcosis. The central nervous system is the prime target of chemical narcosis and symptoms initially include disorientation, euphoria, giddiness, and progress to unconsciousness, convulsion, and death.

11.5.2 Acetylcholinesterase Inhibition

Acetylcholine is a neurotransmitter that functions in conveying nerve impulses across synaptic clefts within the central and autonomic nervous systems and at junctures of nerves and muscles. Following transmission of an impulse across the synapse by the release of acetylcholine, acetylcholinesterase is released into the synaptic cleft. This enzyme hydrolyzes acetylcholine to choline and acetate and transmission of the nerve impulse is terminated. The inhibition of acetylcholineasterase results in prolonged, uncoordinated nerve or muscle stimulation. Organophosphorus and carbamate pesticides (Chapter 5) along with some nerve gases (i.e., sarin) elicit toxicity via this mechanism.

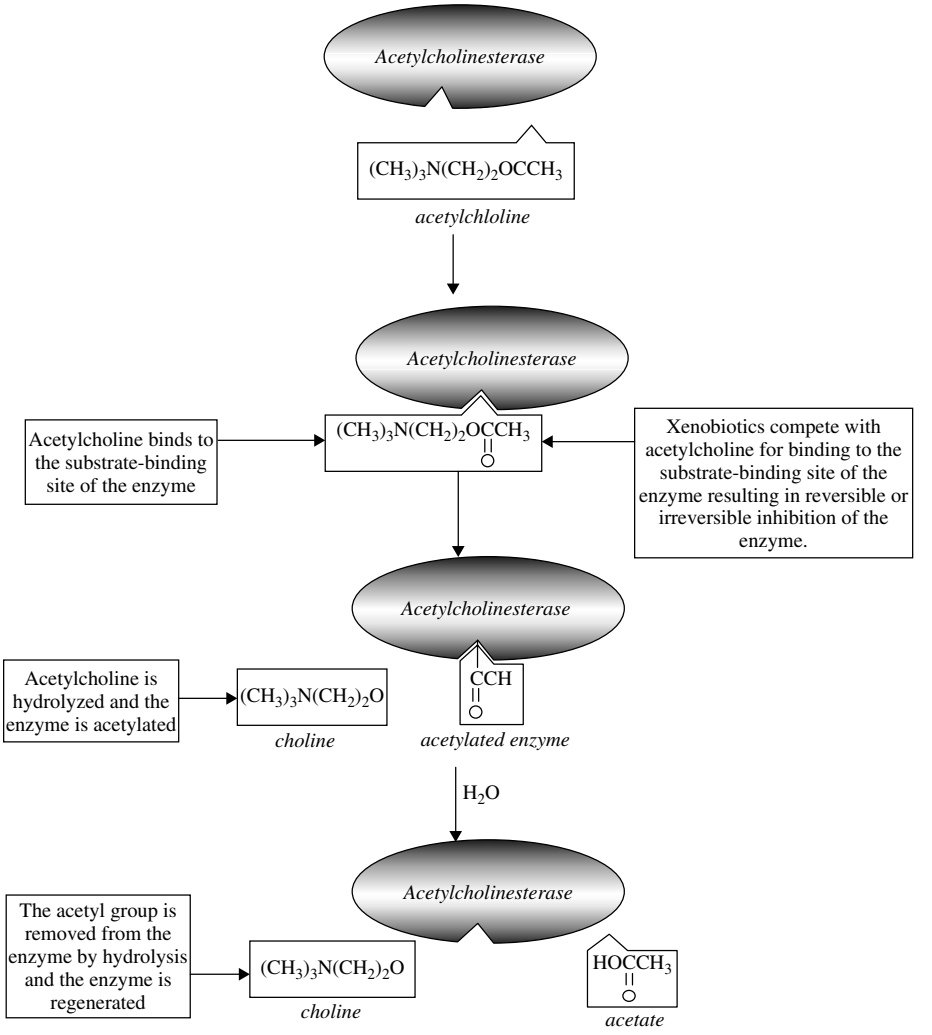

Figure 11.5 Hydrolysis of acetylcholine by the enzyme acetylcholinesterase and its inhibition by toxicants such as organophosphorus and carbamate insecticides.

Inhibitors of acetylcholinesterase function by binding to the substrate-binding site of the enzyme (Figure 11.5). Typically the inhibitor or a biotransformation derivative of the inhibitor (i.e., the phosphodiester component of organophosphorus compounds) covalently binds to the enzyme resulting in its inhibition. Inhibition persists until the bound inhibitor is hydrolytically cleaved from the enzyme. This inhibition may be range from minutes in duration to permanent. Toxic effects of cholinesterase inhibition typically are evident when the enzyme activity is inhibited by about 50%. Symptoms include nausea and vomiting, increased salivation and sweating, blurred vision, weakness, and chest pains. Convulsions typically occur between 50% and 80% enzyme inhibition with death at 80–90% inhibition. Death is most commonly due to respiratory failure.

11.5.3 Ion Channel Modulators

Ion transport is central to nerve impulse transmission both along the axon and at the synapse and many neurotoxicants elicit effects by interfering with the normal transport of these ions (Figure 11.6). The action potential of an axon is maintained by the high concentration of sodium on the outside of the cell as compared to the low concentration inside. Active transporters of sodium (Na^+K^+ ATPases) that actively transport sodium out of the cell establish this action potential. One action of the insecticide DDT resulting in its acute toxicity is the inhibition of these Na^+K^+ ATPases resulting in the inability of the nerve to establish an action potential. Pyrethroid insecticides also elicit neurotoxicity through this mechanism. DDT also inhibits $Ca^{2+}Mg^{2+}$ ATPases, which are important to neuronal repolarization and the cessation of impulse transmission across synapses.

The $GABA_A$ receptor is associated with chloride channels on the postsynaptic region of the neuron and binding of gamma-aminobutyric acid (GABA) to the receptor causes opening of the chloride channel. This occurs after transmission of the nerve impulse across the synaptic cleft and postsynaptic depolarization. Thus activation of $GABA_A$ serves to prevent excessive excitation of the postsynaptic neuron. Many neurotoxicants function by inhibiting the $GABA_A$ receptor, resulting in prolonged closure of the chloride channel and excess nerve excitation. Cyclodiene insecticides (i.e., dieldrin), the organochlorine insecticide lindane, and some pyrethroid insecticides all elicit acute neurotoxicity, at least in part, through this mechanism. Symptoms of $GABA_A$ inhibition include dizziness, headache, nausea, vomiting, fatigue, tremors, convulsions, and death. Avermectins constitute a class of pesticides that are used extensively in veterinary medicine to treat a variety of parasitic conditions. While the mode of toxicity of these compounds is not precisely known, they appear to bind a distinct subset of chloride channels (GABA-insensitive chloride channels) resulting in disruptions in normal chloride transport across nerve cell membranes. Barbituates (i.e., phenobarbital) and ethanol elicit central nervous system effects, at least in part, by binding to $GABA_A$ receptors. However, unlike the previously discussed chemicals, these compounds enhance the ability of gamma-aminobutyric acid to bind the receptor and open the chloride channel. Accordingly, these compounds suppress nerve transmission which contributes to the sedative action of the chemicals.

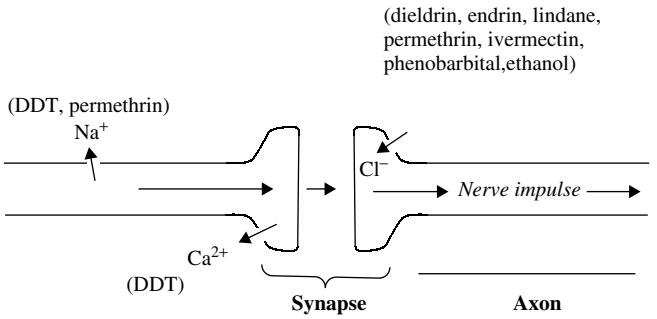

Figure 11.6 Ion channels that facilitate nerve impulse transmission and that are susceptible to perturbation by various toxicants and drugs. Ion transport inhibitors are indicated in parentheses.

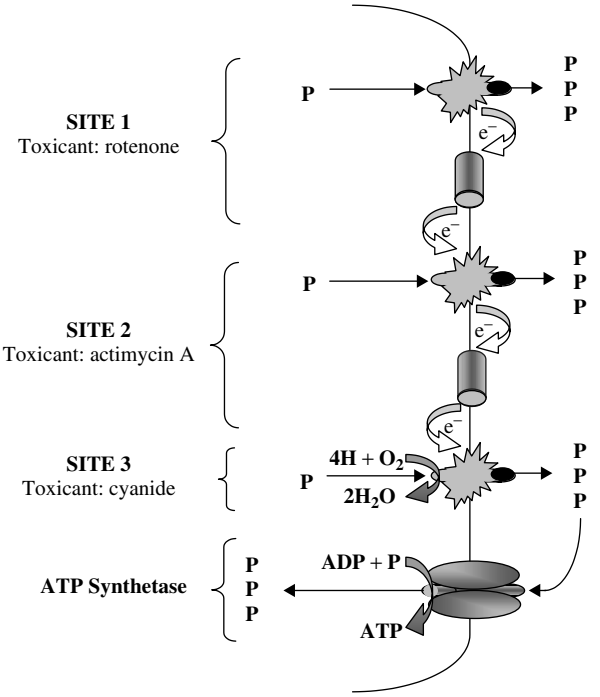

Figure 11.7 Electron (e^-) transport along the inner mitochondrial membrane resulting in the pumping of protons (P) out of the mitochondrial matrix. Protons are shuttled back into the matrix through the ATP synthetase complex where ATP is generated. Sites of toxicant action are indicated.

11.5.4 Inhibitors of Cellular Respiration

Cellular respiration is the process whereby energy, in the form of ATP, is generated in the cell while molecular oxygen is consumed. The process occurs along respiratory assemblies that are located in the inner mitochondrial membrane. Electrons derived from NADH or $FADH_2$ are transferred along a chain of electron carrier proteins. This step-by-step transfer leads to the pumping of protons out of the mitochondrial matrix, resulting in the generation of a membrane potential across the inner mitochrondrial membrane. Protons are pumped out of the mitochrondrial matrix at three locations along the respiratory chain. Site 1 consists of the NADH-Q reductase complex, site 2 consists of the QH_2-cytochrome c reductase complex, and site 3 is the cytochrome c oxidase complex. ATP is generated from ADP when protons flow back across the membrane through an ATP synthetase complex to the mitochrondrial matrix. The transfer of electrons culminates with the reduction of molecular oxygen to water.

Many chemicals can interfere with cellular respiration by binding to the cytochromes that constitute the electron transport chain and inhibiting the flow of electrons along this protein complex. The pesticide rotenone specifically inhibits electron transfer early in the chain with inhibition of proton transport beginning at site 1. Actimycin A inhibits electron transfer and proton pumping at site 2. Cyanide, hydrogen sulfide, and azide inhibit electron flow between the cytochrome oxidase complex and O_2 preventing the generation of a proton gradient at site 3. Symptoms of toxicity from the inhibition of

respiratory chain include excess salivation, giddiness, headache, palpitations, respiratory distress, and loss of consciousness. Potent inhibitors such as cyanide can cause death due to respiratory arrest immediately following poisoning.

Some chemicals do not interfere with electron transport leading to the consumption of molecular oxygen but rather interfere with the conversion of ADP to ATP. These uncouplers of oxidative phosphorylation function by leaking protons across the inner membrane back to the mitochondrial matrix. As a result a membrane potential is not generated, and energy required for the phosphorylation of ADP to ATP is lost. The uncoupling of oxidative phosphorylation results in increased electron transport, increased oxygen consumption, and heat production. The controlled uncoupling of oxidative phosphorylation is a physiologically relevant means of maintaining body temperature by hibernating animals, some newborn animals, and in some animals that inhabit cold environments. Chemicals known to cause uncoupling of oxidative phosphorylation include 2,4-dinitrophenol, pentachlorophenol, and dicumarol. Symptoms of intoxication include accelerated respiration and pulse, flushed skin, elevated temperature, sweating, nausea, coma, and death.

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CHAPTER 12

Chemical Carcinogenesis

ROBERT C. SMART

12.1 GENERAL ASPECTS OF CANCER

Carcinogenesis is the process through which cancer develops. Chemical carcinogenesis is the study of the mechanisms through which chemical carcinogens induce cancer and also involves the development/utilization of experimental systems aimed at determining whether a substance is a potential human carcinogen. An important aspect of toxicology is the identification of potential human carcinogens. To begin to appreciate the complexity of this subject, it is important to first have some understanding of cancer and its etiologies.

Cancer is not a single disease but a large group of diseases, all of which can be characterized by the uncontrolled growth of an abnormal cell to produce a population of cells that have acquired the ability to multiply and invade surrounding and distant tissues. It is this invasive characteristic that imparts its lethality on the host. Epidemiology studies have revealed that the incidence of most cancers increase exponentially with age (Figure 12.1). Epidemiologist have interpreted this exponential increase in cancer incidence to denote that three to seven critical mutations or "hits" within a single cell are required for cancer development. Molecular analyses of human tumors have confirmed the accumulation of mutations in critical genes in the development of cancer. These mutations can be the result of imperfect DNA replication/repair, oxidative DNA damage, and/or DNA damage caused by environmental carcinogens. Most cancers are monoclonal in origin (derived from a single cell) and do not arise from a single critical mutation but from the accumulation of sequential critical mutations in relevant target genes within a single cell (Figure 12.2). Initially a somatic mutation occurs in a critical gene, and this provides a growth advantage to the cell and results in the expansion of the mutant clone. Each additional critical mutation provides a further selective growth advantage resulting in clonal expansion of cells with mutations in multiple critical genes. It often requires decades for a cell clone to accumulate multiple critical mutations and for the progeny of this cell to clonally expand to produce a clinically detectable cancer. Thus the time required for accumulation of mutations in critical genes within a cell is likely related to the observation that cancer incidence increases exponentially with age.

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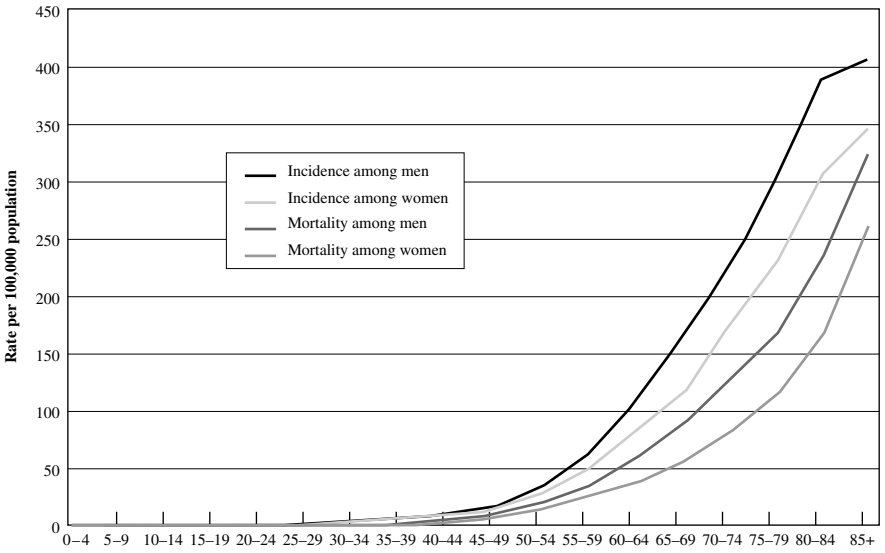


*Incidence and mortality rates are age-adjusted to the 1970 US standard.
Source: SEER Cancer Statistics Review, 1973–1998, Surveillance, Epidemiology, and End Results Program, Division of Cancer Control and Population Sciences, National Cancer Institute, 2001.

American Cancer Society, Surveillance Research, 2002

Figure 12.1 Colon/rectum cancer incidence and mortality rates (1994–1998) in the United States as related to age. (From *American Cancer Society's Facts and Figures—2002*, reprinted with permission of the American Cancer Society, Inc.)

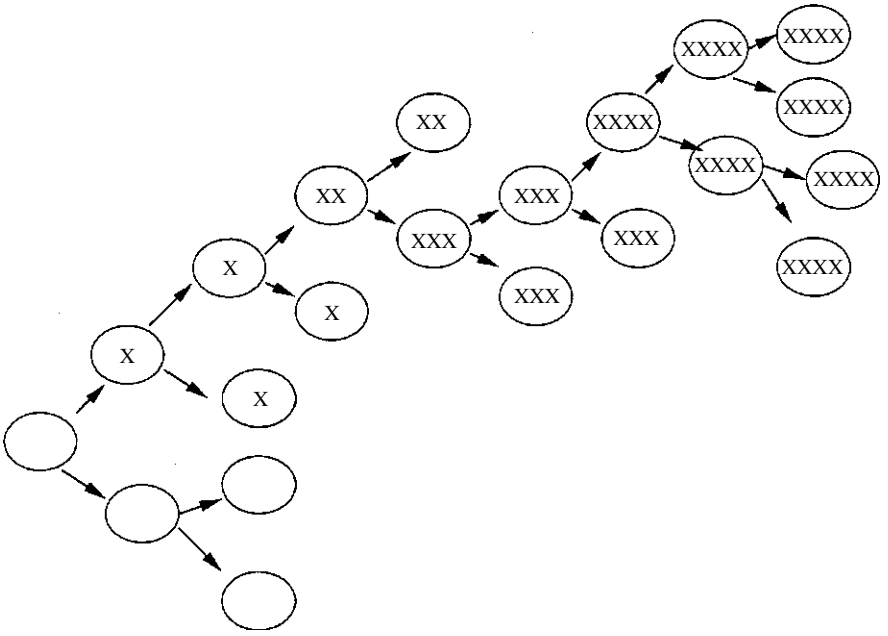


Figure 12.2 Monoclonal origin of cancer with the selection of cells with multiple mutations in critical genes. X designates the occurrence of a mutation in a critical gene.

Specific genes found in normal cells, termed proto-oncogenes, are involved in the positive regulation of cell growth and are frequently mutated in cancer. Mutational alteration of these proto-oncogenes can result in a gain of function, for example, the altered gene product can continually stimulate cell proliferation. Proto-oncogenes with gain-of-function mutations are now referred to as oncogenes. Another family of genes, known as tumor suppressor genes can be mutationally inactivated during carcinogenesis resulting in a loss of function. Tumor suppressor genes and the proteins they encode often function as negative regulators of cell growth. Tumor suppressor genes containing loss-of-function mutations encode proteins that are by and large inactive. Activation of oncogenes and inactivation of tumor suppressor genes within a single cell are important mutational events in carcinogenesis. A simple analogy can be made to the automobile; tumor suppressor genes are analogous to the brakes on the car while the proto-oncogenes are analogous to the accelerator pedal. Mutations within tumor suppressor genes inactivate the braking system while mutations in proto-oncogenes activate the acceleration system. Altering both the cellular brakes and cellular accelerator results in uncontrolled cell growth. In addition to the regulation in cell growth, some oncogenes and tumor suppressor genes can also impair the cells ability to undergo apoptosis or programmed cell death. Mutations in oncogenes and tumor suppressor genes provide a selective growth advantage to the cancer cell through enhanced cell growth and decreased apoptosis (Figure 12.3).

Cancer is a type of a neoplasm or tumor. While technically a tumor is defined as only a tissue swelling, the term is now used as a synonym for a neoplasm. A neoplasm or tumor is an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with the normal tissue, and persists after cessation of the stimuli that evoked it. There are two basic types of neoplasms, termed benign and malignant. The general characteristics of these tumors are defined in Table 12.1. Cancer is the general name for a malignant neoplasm. In terms of cancer nomenclature, most adult cancers are carcinomas that are derived from epithelial cells (colon, lung, breast, skin, etc). Sarcomas are derived from mesenchymal tissues, while leukemias and lymphomas

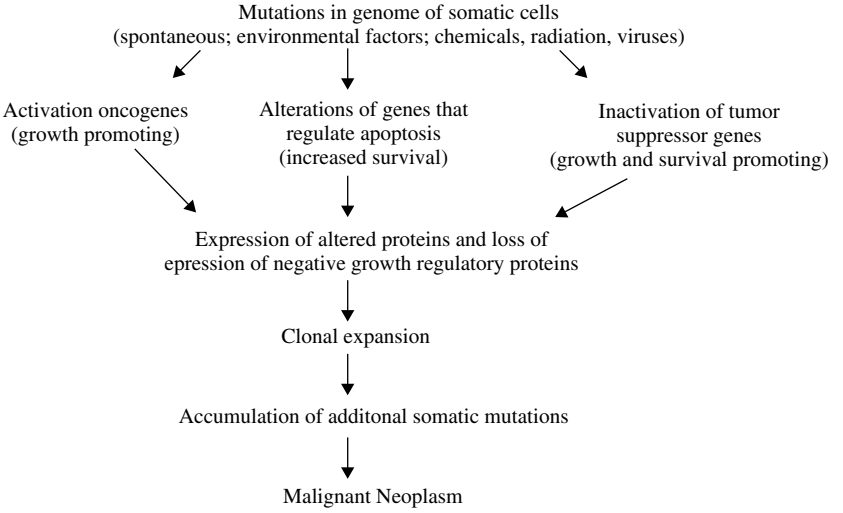


Figure 12.3 General overview of the cancer process.

Table 12.1 Some General Characteristics of Malignant and Benign Neoplasms

Benign	Malignant
Generally slow growing	May be slow to rapid growing
Few mitotic figures	Numerous mitotic figures
Well-differentiated architecture, resembles that of parent tissue	Some lack differentiation, disorganized; loss of parent tissue architecture
Sharply demarcated mass that does not invade surrounding tissue	Locally invasive, infiltrating into surrounding normal tissue
No metastases	Metastases

are derived from blood-forming cells and lymphoid tissue. Melanoma is derived from melanocytes and retinoblastoma, glioblastoma, and neuroblastoma are derived from the stem cells of the retina, glia, and neurons, respectively. According to the American Cancer Society, (1) the lifetime risk for developing cancer in the United States is about 1 in 3 for women and 1 in 2 for men, (2) in 2003 about 1.3 million new cancer cases are expected to be diagnosed not including carcinoma in situ or basal or squamous cell skin cancer, and (3) cancer is a leading cause of death in the United States and approximately 25% of all deaths are due to cancer.

12.2 HUMAN CANCER

Although cancer is known to occur in many groups of animals, the primary interest and the focus of most research is in human cancer. Nevertheless, much of the mechanistic research and the hazard assessment is carried out in experimental animals. A consideration of the general aspects of human carcinogenesis follows.

12.2.1 Causes, Incidence, and Mortality Rates of Human Cancer

Cancer cases and cancer deaths by sites and sex for the United States are shown in Figure 12.4. Breast, lung, and colon and rectum cancers are the major cancers in females while prostate, lung, and colon and rectum are the major cancer sites in males. A comparison of cancer deaths versus incidence for a given site reveals that prognosis for lung cancer cases is poor while that for breast or prostate cancer cases is much better. Age-adjusted cancer mortality rates (1930–1998) for selected sites in males are shown in Figure 12.5 and for females is shown in Figure 12.6. The increase in the mortality rate associated with lung cancer in both females and males is striking and is due to cigarette smoking. It is estimated that 87% of lung cancers are due to smoking. Lung cancer death rates in males and females began to increase in the mid-1930s and mid-1960s, respectively. These time differences are due to the fact that cigarette smoking among females did not become popular until the 1940s while smoking among males was popular in the early 1900s. Taking into account these differences along with a 20 to 25 year lag period for the cancer to develop explains the differences in the temporal increase in lung cancer death rates in males and female. Another disturbing statistic is that lung cancer, a theoretically preventable cancer, has recently surpassed breast cancer as the cancer responsible for the greatest number of cancer deaths in

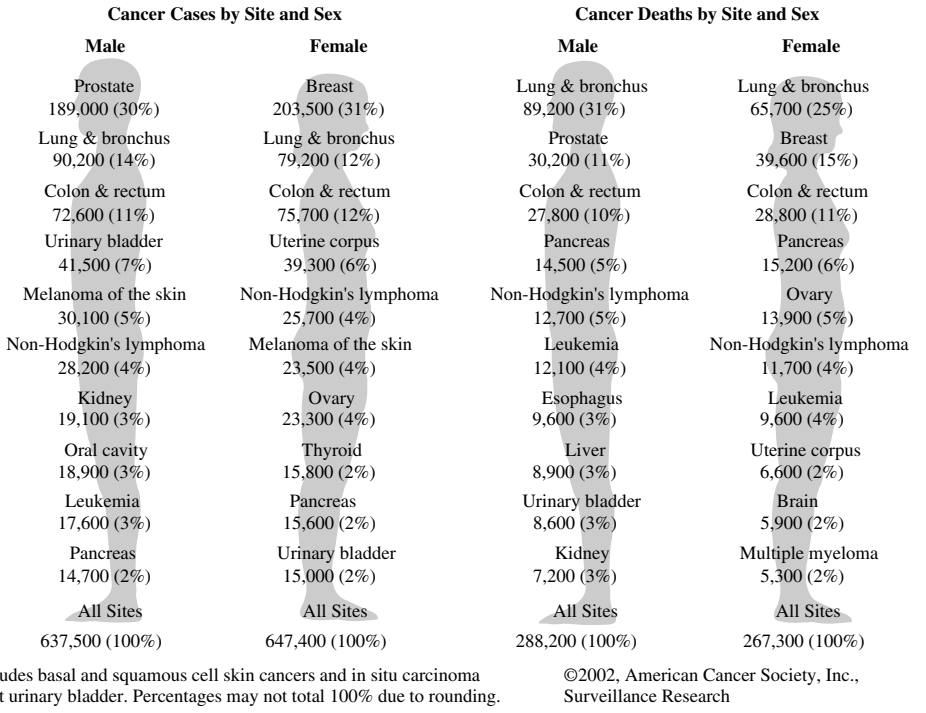
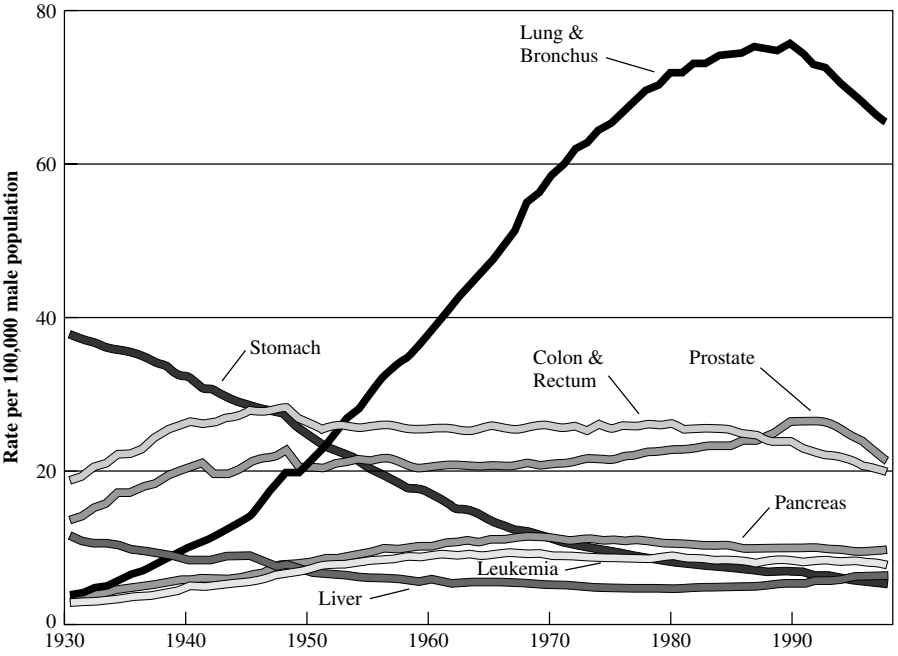


Figure 12.4 Cancer cases and cancer deaths by sites and sex: 2002 estimates. (From *American Cancer Society's Facts and Figures—2002*, reprinted with permission of the American Cancer Society, Inc.)

women. In addition to lung cancer, smoking also plays a significant role in cancer of the mouth, esophagus, pancreas, pharynx, larynx, bladder, kidney, and uterine cervix. Overall, the age-adjusted national total cancer death rate is increasing. In 1930 the number of cancer deaths per 100,000 people was 143. In 1940, 1950, 1970, 1984, and 1992 the rate had increased to 152, 158, 163, 170, and 172, respectively. According to the American Cancer Society, when lung cancer deaths due to smoking are excluded, the total age-adjusted cancer mortality rate had actually decreased by 16% between 1950 and 1993. However, it is important to realize that death and incidence rates for some types of cancers are increasing while the rates for others are decreasing or remaining constant.

Major insights into the etiologies of cancer have been attained through epidemiological studies that relate the roles of hereditary, environmental, and cultural influences on cancer incidence as well as through laboratory studies using rodent/cellular systems. Cancer susceptibility is determined by complex interactions between age, environment, and an individual's genetic makeup. It is estimated from epidemiological studies that 35–80% of all cancers are associated with the environment in which we live and work. The geographic migration of immigrant populations and differences in cancer incidence among communities has provided a great deal of information regarding the role of the environment and specific cancer incidences. For example, Japanese immigrants and the sons of Japanese immigrants living in California begin to assume a cancer death rate similar to the California white population (Figure 12.7). These results implicate



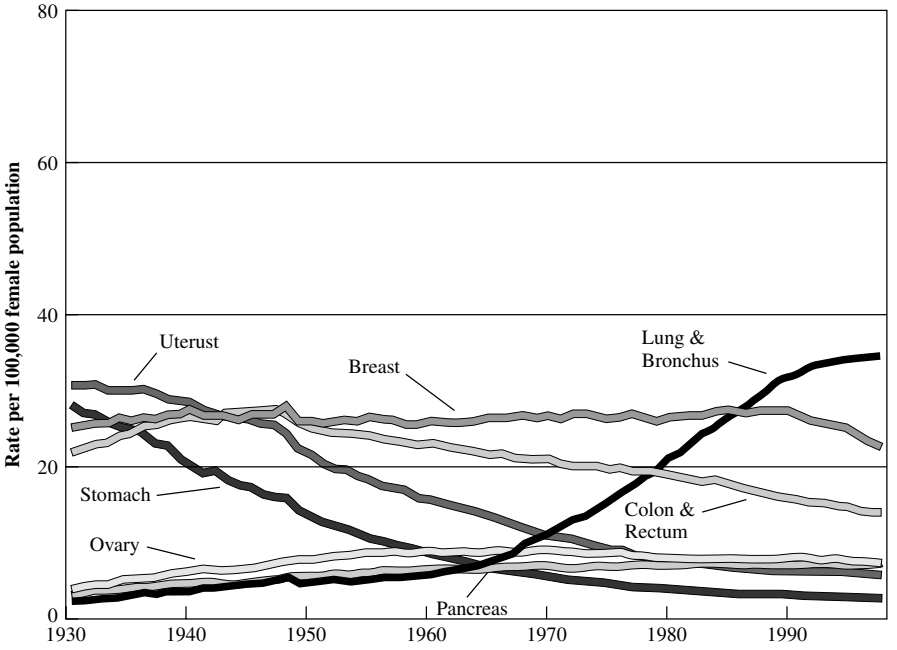
*Per 100,000, age-adjusted to the 1970 US standard population. **Note:** Due to changes in ICD coding, numerator information has changed over time. Rates for cancers of the liver, lung & bronchus, and colon & rectum are affected by these coding changes.

Source: US Mortality Public Use Data Tapes 1960-1998, US Mortality Volumes 1930-1959, National Center for Health Statistics, Centers for Disease Control and Prevention, 2001.

American Cancer Society, Surveillance Research, 2002

Figure 12.5 Age-adjusted mortality rates (1030–1998) for selected sites in males. (From *American Cancer Society's Facts and Figures—2002*, reprinted with permission of the American Cancer Society, Inc.)

a role of the environment in the etiology of cancer. It should be noted that the term environment is not restricted to exposure to human-made chemicals in the environment but applies to all aspects of our lifestyle including smoking, diet, cultural and sexual behavior, occupation, natural and medical radiation, and exposure to substances in air, water, and soil. The major factors associated with cancer and their estimated contribution to human cancer incidence are listed in Table 12.2. Only a small percentage of total cancer occurs in individuals with a hereditary mutation/hereditary cancer syndrome (ca. 5%). However, an individual's genetic background is the "stage" in which the cancer develops and susceptibility genes have been identified in humans. For example, genetic polymorphisms in enzymes responsible for the activation of chemical carcinogens may represent a risk factor as is the case for polymorphisms in the *N*-acetyl-transferase gene and the risk of bladder cancer. These types of genetic risk factors are of low penetrance (low to moderate increased risk); however, increased risk is usually associated with environmental exposure. While the values presented in Table 12.2 are a best estimate, it is clear that smoking and diet constitute the major factors associated with human cancer incidence. If one considers all of the categories that pertain to human-made chemicals, it is estimated that their contribution to human



*Per 100,000, age-adjusted to the 1970 US standard population. †Uterus cancer death rates are for uterine cervix and uterine corpus combined.
Note: Due to changes in ICD coding, numerator information has changed over time. Rates for cancers of the liver, lung & bronchus, and colon & rectum are affected by these coding changes.

Source: US Mortality Public Use Data Tapes 1960–1998, US Mortality Volumes 1930–1959, National Center for Health Statistics, Centers for Disease Control and Prevention, 2001.

American Cancer Society, Surveillance Research, 2002

Figure 12.6 Age-adjusted mortality rates (1030–1998) for selected sites in females. (From *American Cancer Society's Facts and Figures—2002*, reprinted with permission of the American Cancer Society, Inc.)

cancer incidence is approximately 10%. However, the factors listed in Table 12.2 are not mutually exclusive since there is likely to be interaction between these factors in the multi-step process of carcinogenesis.

12.2.2 Known Human Carcinogens

Two of the earliest observations that exposure of humans to certain chemicals or substances is related to an increased incidence of cancer were made independently by two English physicians, John Hill in 1771 and Sir Percival Pott in 1776. Hill observed an increased incidence of nasal cancer among snuff users, while Pott observed that chimney sweeps had an increased incidence of scrotal cancer. Pott attributed this to topical exposure to soot and coal tar. It was not until nearly a century and a half later in 1915 when two Japanese scientists, K. Yamagiwa and K. J. Itchikawa, substantiated Pott's observation by demonstrating that multiple topical applications of coal tar to rabbit skin produced skin carcinomas. This experiment is important for two major

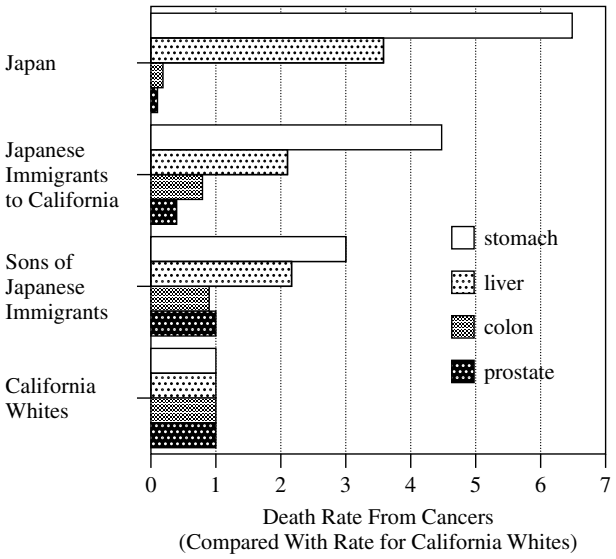


Figure 12.7 Change in incidence of various cancers with migration from Japan to the United States provides evidence that the cancers are caused by components of the environment that differ in the two countries. The incidence of each kind of cancer is expressed as the ratio of the death rate in the populations being considered to that in a hypothetical population of California whites with the same age distribution; the death rates for whites are thus defined as 1. (Adapted from J. Cairns, in *Readings from Scientific American-Cancer Biology*, W. H. Freeman, 1986, p. 13.)

Table 12.2 Proportions of Cancer Deaths Attributed to Various Different Factors

Major Factors	Best Estimate (%)	Range of Acceptable Estimates (%)
Diet	35	10–70
Tobacco	30	25–40
Infection	10	1–?
Reproductive and sexual behavior	7	1–13
Occupation	4	2–8
Geophysical factors	3	2–4
Alcohol	3	2–4
Pollution	2	<1–5
Food additives	1	−5–2
Medicines	1	0.5–3
Industrial products	1	<1–2
Unknown	?	?

Source: Adapted from R. Doll and R. Peto, *The Causes of Cancer*, Oxford Medical Publications, 1981.

reasons: (1) it was the first demonstration that a chemical or substance could produce cancer in animals, and (2) it confirmed Pott's initial observation and established a relationship between human epidemiology studies and animal carcinogenicity. Because of these important findings, Yamagiwa and Itchikawa are considered the fathers of experimental chemical carcinogenesis. In the 1930s Kennaway and coworkers isolated a single active carcinogenic chemical from coal tar and identified it as benzo[a]pyrene, a polycyclic aromatic hydrocarbon that results from the incomplete combustion of organic molecules. Benzo[a]pyrene has also been identified as one of the carcinogens in cigarette smoke. The p53 tumor suppressor gene can be mutationally inactivated by numerous carcinogens, including the carcinogenic metabolite of benzo[a]pyrene.

Epidemiological studies have provided sufficient evidence that exposure to a variety of chemicals, agents, or processes are associated with human cancer. For example, the following causal associations have emerged between exposure and the development of specific cancers: vinyl chloride and hepatic cancer, amine dyes and bladder cancer, benzene and leukemia, diethylstilbestrol and clear cell carcinoma of the vagina, and cigarette smoking and lung cancer. Naturally occurring chemicals or agents such as asbestos, aflatoxin B_1, betel nut, nickel, and certain arsenic compounds are also associated with an increased incidence of certain human cancers. Both epidemiological studies and rodent carcinogenicity studies are important in the identification and classification of potential human carcinogens. The strongest evidence for establishing whether exposure to a given chemical is carcinogenic in humans comes from epidemiological studies. However, these studies are complicated by the fact that it often takes 20 to 30 years after carcinogen exposure for a clinically detectable cancer to develop. This delay is problematic and can result in inaccurate historical exposure information and additional complexity due to the interference of a large number of confounding variables. This lag period can also prevent the timely identification of a putative carcinogen and result in unnecessary exposure. Therefore methods to identify potential human carcinogens have been developed. The long-term rodent bioassay also known as the two-year rodent carcinogenesis bioassay (see Chapter 21) is currently used in an attempt to identify potential human carcinogens. It is clear that almost all human carcinogens identified to date are rodent carcinogens; however, it is not known if all rodent carcinogens are human carcinogens. Indeed, identification of possible human carcinogens based on rodent carcinogenicity can be extremely complicated (see below). Table 12.3 contains the list of the known human carcinogens as listed by the International Agency for Research on Cancer (IARC). In addition Table 12.3 includes information on carcinogenic complex mixtures and occupations associated with increased cancer incidence. In vitro mutagenicity assays are also used to identify mutagenic agents that may have carcinogenic activity (see Chapter 21).

12.2.3 Classification of Human Carcinogens

Identification and classification of potential human carcinogens through the two-year rodent carcinogenesis bioassay is complicated by species differences, use of high doses (MTD, maximum tolerated dose), the short life span of the rodents, high background tumor incidence in some organs, sample size, and the need to extrapolate from high to low doses for human risk assessment. Although these problems are by no means trivial, the rodent two-year bioassay remains the "gold standard" for the classification

Table 12.3 List of Agents, Substances, Mixtures, and Exposure Circumstances Known to be Human Carcinogens

Aflatoxins
4-Aminobiphenyl
Arsenic and certain arsenic compounds
Asbestos
Azathioprine
Benzene
Benzidine
Beryllium and certain beryllium compounds
N,N-bis-(2-Chloroethyl)-2-naphthylamine (chlornaphazine)
Bis(chloromethyl) ether and chloromethyl methyl ether
1,4-Butanediol dimethylsulfonate (Myleran®)
Cadmium and certain cadmium compounds
Chlorambucil
1-(2-Chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea (MeCCNU)
Chromium and certain chromium compounds
Cyclophosphamide
Cyclosporin A (cyclosporin)
Diethylstilbestrol
Epstein-Barr virus
Erionite
Estrogen therapy
Estrogens, nonsteroidal
Estrogens, steroidal
Ethylene oxide
Etoposide in combination with cisplatin and bleomycin
Helicobacter pylori
Hepatitis B virus (chronic infection)
Hepatitis C virus (chronic infection)
Herbal remedies containing plant species of the genus *Aristolochia*
Human immunodeficiency virus, type 1
Human papillomavirus, type 16
Human papillomavirus, type 18
Human T-cell lymphotropic virus, type 1
Melphalan
Methoxsalen with ultraviolet A therapy (PUVA)
MOPP and other combined chemotherapy including alkylating agents
Mustard gas
2-Naphthylamine
Neutrons
Nickel compounds
Opisthorchis viverrini
Oral contraceptives
Radionuclides α-particle emitting
Radionuclides β-particle emitting
Radon
Schistosoma haematobium
Silica
Solar radiation
Talc containing asbestiform fibers

Table 12.3 (*continued*)

Tamoxifen
2,3,7,8-Tetrachlorodibenzo-*para*-dioxin
Thiotepa [*tris*(1-aziridinyl)phosphine sulfide]
Thorium dioxide
Treosulfan
Vinyl chloride
X and gamma(γ) Radiation
Mixtures
Alcoholic beverages
Analgesic mixtures containing phenacetin
Betel quid with tobacco
Coal tar and coal pitches
Mineral oils
Salted fish
Shale oils
Soots
Tobacco smoke and tobacco smokeless products
Wood dust
Exposure circumstances
Aluminium production
Auramine manufacture
Boot and show manufacture and repair
Coal gasification
Coke gasification
Furniture and cabinet making
Haematite mining with exposure to radon
Iron and steel founding
Isopropanol manufacture
Manufacture of magenta
Painter
Rubber industry
Strong inorganic acid mists containing sulfuric acid

of potential human carcinogens. Criteria for the classification of carcinogens used by the National Toxicology Program's *Tenth Report on Carcinogens*, 2002, are shown in Table 12.4; the criteria used by Environmental Protection Agency (EPA) and the International Agency for Research on Cancer (IARC) are shown in Table 12.5. Carcinogens are classified by the weight of evidence for carcinogenicity referred to as sufficient, limited, or inadequate based on both epidemiological studies and animal data. EPA is planning to change their guidelines for carcinogen risk assessment and their carcinogen classification scheme. New guidelines will emphasize the incorporation of biological mechanistic data in the analysis, and will not rely solely on rodent tumor data. In addition the six alphanumeric categories listed in Table 12.5 will be replaced by three descriptors for classifying human carcinogenic potential. Carcinogens will be classified by the EPA as (1) known/likely to be a human carcinogen, (2) cannot be determined to be a human carcinogen, and (3) not likely to be a human carcinogen.

Table 12.4 Carcinogen Classification System of the National Toxicology Program

Known to be a human carcinogen

There is sufficient evidence of carcinogenicity from studies in humans which indicates a causal relationship between exposure to the agent, substance, or mixture and human cancer.

Reasonably anticipated to be a human carcinogen

There is limited evidence of carcinogenicity from studies in humans which indicates a causal interpretation is credible, but alternate explanations, such as chance, bias, or confounding factors, cannot adequately be excluded;

or

There is sufficient evidence of carcinogenicity from studies in experimental animals which indicates there is an increased incidence of malignant and/or a combination of malignant and benign tumors: (1) in multiple species or at multiple tissue sites, or (2) by multiple routes of exposure, or (3) to an unusual degree with regard to incidence, site, or type of tumor, and age at onset;

or

There is less than sufficient evidence of carcinogenicity in humans or laboratory animals, however the agent, substance or mixture belongs to a well defined, structurally-related class of substances whose members are listed in a previous *Report on Carcinogens* as either a known to be human carcinogen or reasonably anticipated to be human carcinogen, or there is convincing relevant information that the agent acts through mechanisms indicating that it would likely cause cancer in humans.

Conclusions regarding carcinogenicity in humans or experimental animals are based on scientific judgment, with consideration given to all relevant information. Relevant information includes, but is not limited to, dose response, route of exposure, chemical structure, metabolism, pharmacokinetics, sensitive subpopulations, genetic effects, and other data relating to mechanism of action or factors that may be unique to a given substance. For example, there may be a substance for which there is evidence of carcinogenicity in laboratory animals but there are compelling data indicating that the agent acts through mechanisms that do not operate in humans and it would therefore not reasonably be anticipated to cause cancer in humans.

Source: From the *Tenth Report on Carcinogens*, US Department of Health and Human Services, Public Health Service, National Toxicology Program.

12.3 CLASSES OF AGENTS ASSOCIATED WITH CARCINOGENESIS

Chemical agents that influence cancer development can be divided into two major categories based on whether or not they are mutagenic in in vitro mutagenicity assay. DNA-damaging agents (genotoxic) are mutagenic in in vitro mutagenicity assays and are considered to produce permanent alterations in the genetic material of the host in vivo, and epigenetic agents (nongenotoxic) are not mutagenic in in vitro assays. These agents are not believed to alter the primary sequence of DNA but are considered to alter the expression or repression of certain genes and/or to produce perturbations in signal transduction pathways that influence cellular events related to proliferation, differentiation, or apoptosis. Many epigenetic/nongenotoxic agents contribute to the clonal expansion of cells containing an altered genotype (DNA alterations) to form tumors, however in the absence of such DNA alterations these epigenetic agents have no effect on tumor formation.

Table 12.5 IARC and EPA Classification of Carcinogens

IARC	EPA	
1	Group A	Human carcinogens Sufficient evidence from epidemiological studies to support a causal association between exposure to the agents and cancer
2A	Group B	Probable human carcinogens
	Group B1	Limited epidemiological evidence that the agent causes cancer regardless of animal data
	Group B2	Inadequate epidemiological evidence or no human data on the carcinogenicity of the agent and sufficient evidence in animal studies that the agent is carcinogenic
2B	Group C	Possible human carcinogens Absence of human data with limited evidence of carcinogenicity in animals
3	Group D	Not classifiable as to human carcinogenicity Agents with inadequate human and animal evidence of carcinogenicity or for which no data are available
4	Group E	Evidence of noncarcinogenicity for humans Agents that show no evidence for carcinogenicity in at least two adequate animal tests in different species or in both adequate epidemiologic and animal studies

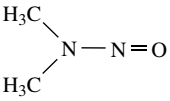
12.3.1 DNA-Damaging Agents

DNA-damaging agents can be divided into four major categories. (1) Direct-acting carcinogens are intrinsically reactive compounds that do not require metabolic activation by cellular enzymes to covalently interact with DNA. Examples include *N*-methyl-*N*-nitrosourea and *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine; the alkyl alkanesulfonates such as methyl methanesulfonate; the lactones such as beta propiolactone and the nitrogen and sulfur mustards. (2) Indirect-acting carcinogens require metabolic activation by cellular enzymes to form the ultimate carcinogenic species that covalently binds to DNA. Examples include dimethylnitrosamine, benzo[a]pyrene, 7,12-dimethylbenz[a]anthracene, aflatoxin B1 and 2-acetylaminofluorene (Figure 12.8). (3) Radiation and oxidative DNA damage can occur directly or indirectly. Ionizing radiation produces DNA damage through direct ionization of DNA to produce DNA strand breaks or indirectly via the ionization of water to reactive oxygen species that damage DNA bases. Ultraviolet radiation (UVR) from the sun is responsible for approximately 1 million new cases of human basal and squamous cell skin cancer each year. Reactive oxygen species can also be produced by various chemicals and cellular process including respiration and lipid peroxidation. (4) Inorganic agents such as arsenic, chromium and nickel are considered DNA-damaging agents although in many cases the definitive mechanism is unknown. DNA-damaging agents can produce three general types of genetic alterations: (1) gene mutations, which include point mutations involving single base pair substitutions that can result in amino acid substitutions in the encoded protein, and frame shift mutations

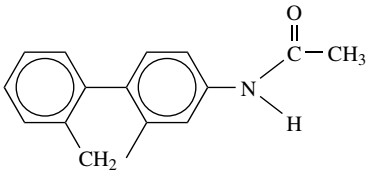
Procarcinogen

Proximate Carcinogen

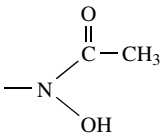
Putative Ultimate Carcinogen



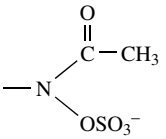
Dimethylnitrosamine



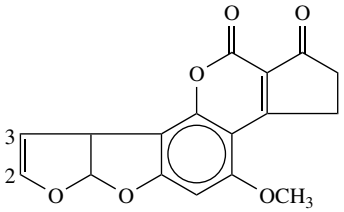
2-Acetylaminofluorene (AAF)



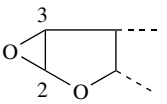
N-Hydroxy-AAF



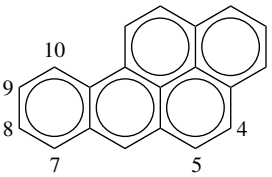
Sulfate ester Of N-hydroxy-AAF



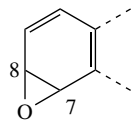
Aflatoxin B_1



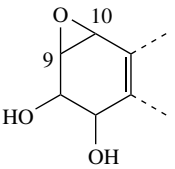
Aflatoxin B_1 2, 3-epoxide



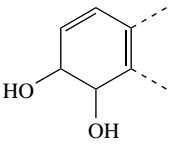
Benzo(a)pyrene (BP)



BP-7, 8-epoxide



BP-7, 8-dihydro-7, 8-diol-9, 10-epoxide



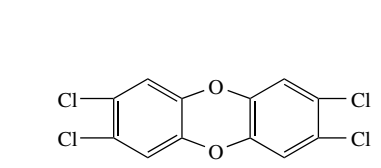
BP-7, 8-dihydro-7, 8-diol

Figure 12.8 Examples of DNA-damaging carcinogens.

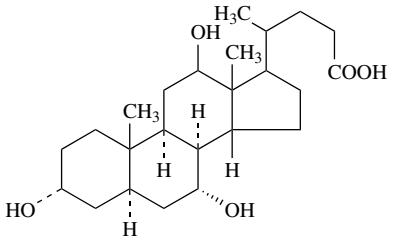
involving the loss or gain of one or two base pairs, resulting in an altered reading frame and gross alterations in the encoded protein; (2) chromosome aberrations, including gross chromosomal rearrangement such as deletions, duplications, inversions, and translocations; and (3) aneuploidy and polyploidy, which involve the gain or loss of one or more chromosomes.

12.3.2 Epigenetic Agents

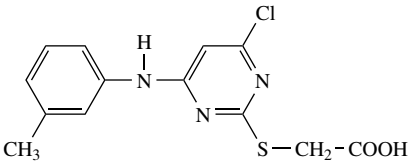
Epigenetic agents that influence carcinogenesis are not thought to alter the primary sequence of DNA, but rather they are considered to alter the expression or repression of certain genes and/or produce perturbations in signal transduction pathways that influence cellular events related to proliferation, differentiation, or apoptosis. Many epigenetic agents favor the proliferation of cells with an altered genotype (cells containing a mutated oncogene(s) and/or tumor suppressor gene(s)) and allow the clonal expansion of these altered or "initiated" cells. Epigenetic agents can be divided into four major categories: (1) hormones such as conjugated estrogens and diethylstilbestrol; (2) immunosuppressive xenobiotics such as azathioprine and cyclosporin A; (3) solid state agents, which include plastic implants and asbestos; and (4) tumor promoters in rodent models, which include 12-*O*-tetradecanoylphorbol-13-acetate, peroxisome proliferators, TCDD and phenobarbital (Figure 12.9). In humans, diet (including caloric,



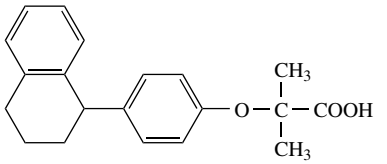
2, 3, 7, 8-Tetrachlorodibenzo-*p*-dioxin



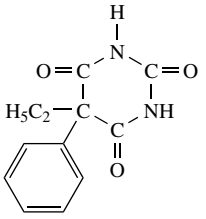
Cholic acid



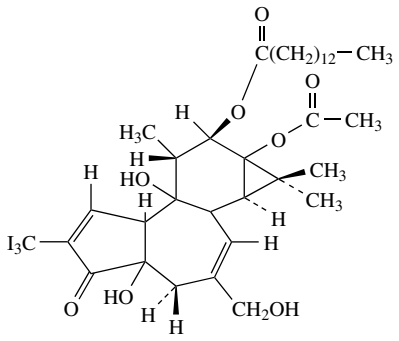
Wy-14,643



Nafenopin



Phenobarbital



Tetradecanoyl phorbol acetate (TPA)

Figure 12.9 Examples of tumor promoters.

fat, and protein intake), excess alcohol, and late age of pregnancy are considered to function through a promotion mechanism. While smoking and UVR have initiating activity, both are also considered to have tumor-promoting activity. By definition, tumor promoters are not classified as carcinogens since they are considered inactive in the absence of initiated cells. However, an altered genotype or an initiated cell can arise from spontaneous mutations resulting from imperfect DNA replication/repair oxidative DNA damage, or can result from environmental carcinogens. Theoretically, in the presence of a tumor promoter these mutant cells would clonally expand to form a tumor. Therefore the nomenclature becomes somewhat a matter of semantics as to whether the tumor promoter should or should not be classified as a carcinogen. Certain hormones and immunosuppressive agents are classified as human carcinogens, although it is generally considered that these agents are not carcinogenic in the absence of initiated cells. Rather, like tumor promoters, they may only allow for the clonal expansion of cells with an altered genotype.

Some nongenotoxic/epigenetic agents have been shown to induce DNA damage in vivo, for example, some "nongenotoxic/epigenetic agents" can induce oxidative DNA damage in vivo through the direct or indirect production of reactive oxygen species. For example, certain estrogens may possess this ability and such a characteristic may contribute to their carcinogenicity. Thus, as we gain a better understanding of chemical carcinogenesis, we find that there is functional and mechanistic overlap and interaction between these two major categories of chemical carcinogens.

12.4 GENERAL ASPECTS OF CHEMICAL CARCINOGENESIS

A great deal of evidence has accumulated in support of the somatic mutation theory of carcinogenesis, which simply states that mutations within somatic cells is necessary for neoplasia. As stated earlier, cancer development (carcinogenesis) involves the accumulation of mutations in multiple critical genes. These mutations can be the result of imperfect DNA replication/repair, oxidative DNA damage, and/or DNA damage caused by environmental carcinogens. Many chemical carcinogens can alter DNA through covalent interaction (DNA adducts) or direct and/or indirect oxidative DNA damage. Some chemical carcinogens are intrinsically reactive and can directly covalently bind to DNA, while others require metabolic via cytochromes P450 to produce reactive electrophilic intermediates capable of covalently binding to DNA (Figure 12.10). In the 1950s Elizabeth and James Miller observed that a diverse array of chemicals could produce cancer in rodents. In an attempt to explain this, they hypothesized that many carcinogens are metabolically activated to electrophilic metabolites that are capable of interacting with nucleophilic sites in DNA. The Millers termed this the electrophilic theory of chemical carcinogenesis. From this concept of metabolic activation, the important terms parent, proximate, and ultimate carcinogen were developed. A parent carcinogen is a compound that must be metabolized in order to have carcinogenic activity; a proximate carcinogen is an intermediate metabolite requiring further metabolism and resulting in the ultimate carcinogen, which is the actual metabolite that covalently binds to the DNA. The cell has many defense systems to detoxify the carcinogenic species, including cellular antioxidants and nucleophiles as well as a whole host of phase I and phase II enzymes. In addition reactive carcinogenic species may bind to noncritical sites in the cell, resulting in detoxification, or they can undergo spontaneous decomposition. If the carcinogenic species binds to DNA, the adducted DNA

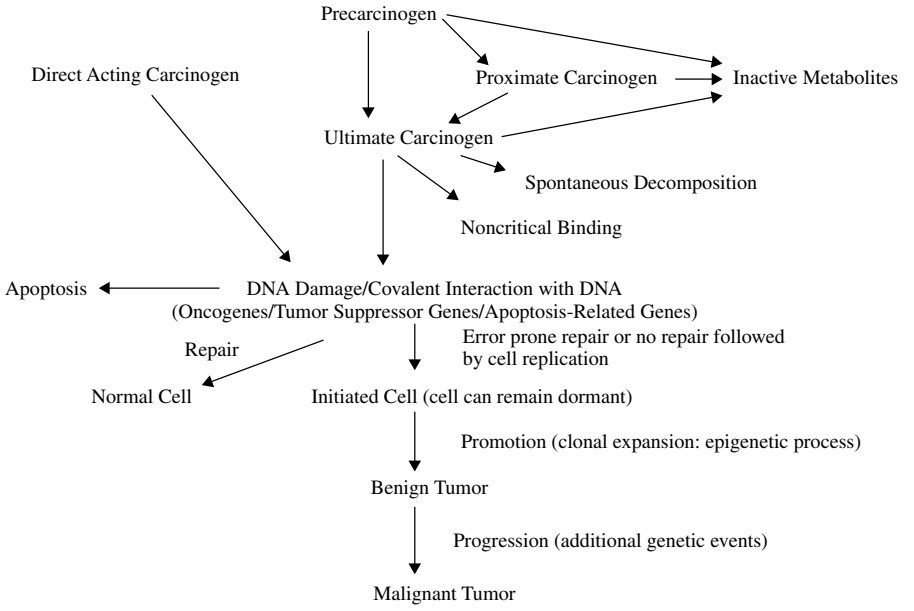


Figure 12.10 General aspects of chemically induced carcinogenesis.

can be repaired and produce a normal cell. If there is error in the repair of the DNA or the DNA adduct is not repaired before the cell replicates, an error in the newly synthesized DNA could occur, and if so, a mutation would occur in the DNA of the daughter cell. If this change has occurred in a critical gene, for example, in a proto-oncogene or tumor suppressor gene, it would represent an important mutagenic event(s) in carcinogenesis.

The mutationally altered cell or "initiated cell" has an altered genotype and may remain dormant (not undergo clonal expansion) for the lifetime of the animal. However, additional mutations or "hits" in critical genes followed by clonal expansion could lead to tumor development as described earlier in this chapter. In addition to this mechanism, chemical carcinogenesis in experimental models can be divided into at least three stages: termed initiation, promotion, and progression (Figure 12.10); this model is thus often referred to as the initiation/promotion model of chemical carcinogenesis. As mentioned above, the "initiated cell" may remain dormant (not undergo clonal expansion) for the lifetime of the animal. However, if the animal is repeatedly exposed to a tumor promoter, it will provide a selective growth advantage to the "initiated cell," which will clonally expand and eventually produce a benign tumor. This process is termed tumor promotion and is an epigenetic process favoring the growth of cells with an altered genotype. The development of a malignant tumor from a benign tumor encompasses a third step, termed progression and involves additional genetic changes.

12.5 INITIATION-PROMOTION MODEL FOR CHEMICAL CARCINOGENESIS

Experimentally, the initiation-promotion process has been demonstrated in several organs/tissues including skin, liver, lung, colon, mammary gland, prostate, and bladder

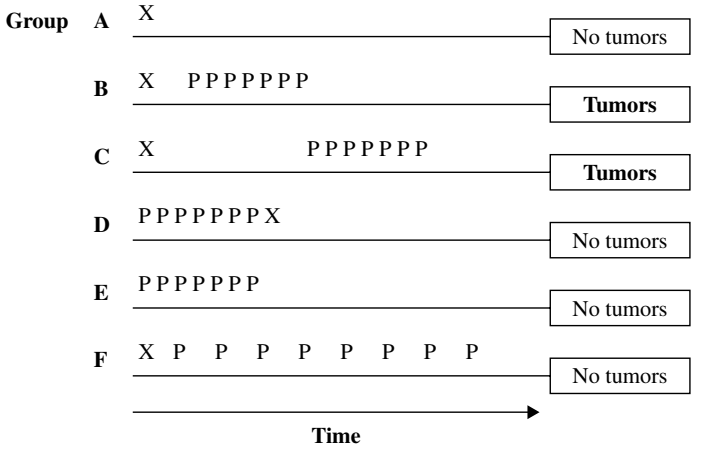


Figure 12.11 Initiation/promotion model. X = application of initiator, P = application of promoter.

as well as in variety of cells in culture. While tumor promoters have different mechanisms of action and many are organ specific, all have common operational features (Figure 12.11). These features include (1) following a subthreshold dose of initiating carcinogen, chronic treatment with a tumor promoter will produce many tumors; (2) initiation at a subthreshold dose alone will produce very few if any tumors; (3) chronic treatment with a tumor promoter in the absence of initiation will produce very few if any tumors; (4) the order of treatment is critical as it must be first initiated and then promoted; (5) initiation produces an irreversible change; and (6) promotion is reversible in the early stages, for example, if an equal number of promoting doses are administered but the doses are spaced further apart in time, tumors would not develop or would be greatly diminished in number. Many tumor promoters are organ specific. For example, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) also known as phorbol 12-myristate 13-acetate (PMA) belongs to a family of compounds known as phorbol esters. Phorbol esters are isolated from croton oil (derived from the seeds of the croton plant) and are almost exclusively active in skin. Phenobarbital, DDT, chlordane, TCDD and peroxisome proliferators Wy 24,643, clofibrate, and nafenopin are hepatic tumor promoters. TCDD is also a promoter in lung and skin. Some bile acids are colonic tumor promoters, while various estrogens are tumor promoters in the mammary gland and liver. There are multiple mechanisms of tumor promotion, and this may explain the organ specific nature of the many promoters. Under conditions in which the chemical produces tumors without tumor promoter treatment, the chemical agent is often referred to as a complete carcinogen.

It is generally accepted that tumor promoters allow for the clonal expansion of initiated cells by interfering with signal transduction pathways that are involved in the regulation of cell growth, differentiation, and/or apoptosis (Table 12.6). While the precise mechanisms of tumor promotion are not completely understood at the molecular/biochemical level, current research is providing new and promising mechanistic insights into how tumor promoters allow for the selective growth of initiated cells.

Table 12.6 Some General Mechanisms of Tumor Promotion

Selective proliferation of initiated cells
Increased responsiveness to and/or production of growth factors, hormones, and other active molecules
Decreased responsiveness to inhibitory growth signals
Perturbation of intracellular signaling pathways
Altered differentiation
Inhibition of terminal differentiation of initiated cells
Acceleration of differentiation of uninitiated cells
Inhibition of apoptosis in initiated cells
Toxicity/compensatory hyperplasia
Resistance to toxicity by initiated cells

12.6 METABOLIC ACTIVATION OF CHEMICAL CARCINOGENS AND DNA ADDUCT FORMATION

Having described the general aspects of chemical carcinogenesis including the initiation-promotion model, we now examine some aspects of chemical carcinogenesis in more detail. Metabolic activation of chemical carcinogens by cytochromes P450 is well documented. The metabolism of benzo[a]pyrene has been extensively studied and at least 15 major phase I metabolites have been identified. Many of these metabolites are further metabolized by phase II enzymes to produce numerous different metabolites. Extensive research has elucidated which of these metabolites and pathways are important in the carcinogenic process. As shown in Figure 12.12, benzo[a]pyrene is metabolized by cytochrome P450 to benzo[a]pyrene-7,8 epoxide, which is then hydrated by epoxide hydrolase to form benzo[a]pyrene-7,8-diol. Benzo[a]pyrene-7,8-diol is considered the proximate carcinogen since it must be further metabolized by cytochrome P450 to form the ultimate carcinogen, the bay region diol epoxide, (+)-benzo[a]pyrene-7,8-diol-9,10-epoxide-2. It is this reactive intermediate that binds covalently to DNA, forming DNA adducts. (+)-Benzo[a]pyrene-7,8-diol-9,10-epoxide-2 binds preferentially to deoxyguanine residues, forming *N*-2 adduct. (+)-Benzo[a]pyrene-7,8-diol-9,10-epoxide-2 is highly mutagenic in eukaryotic and prokaryotic cells and carcinogenic in rodents. It is important to note that not only is the chemical configuration of the metabolites of many polycyclic aromatic hydrocarbons important for their carcinogenic activity, but so is their chemical conformation/stereospecificity (Figure 12.12). For example, four different stereoisomers of benzo[a]pyrene-7,8-diol-9,10 epoxide are formed. Each one only differs with respect to whether the epoxide or hydroxyl groups are above or below the plane of the flat benzo[a]pyrene molecule, but only one, (+)-benzo[a]pyrene-7,8-diol-9,10-epoxide-2, has significant carcinogenic potential. Many polycyclic aromatic hydrocarbons are metabolized to bay-region diol epoxides. The bay-region theory suggests that the bay-region diol epoxides are the ultimate carcinogenic metabolites of polycyclic aromatic hydrocarbons.

DNA can be altered by strand breakage, oxidative damage, large bulky adducts, and alkylation. Carcinogens such as *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine and methyl

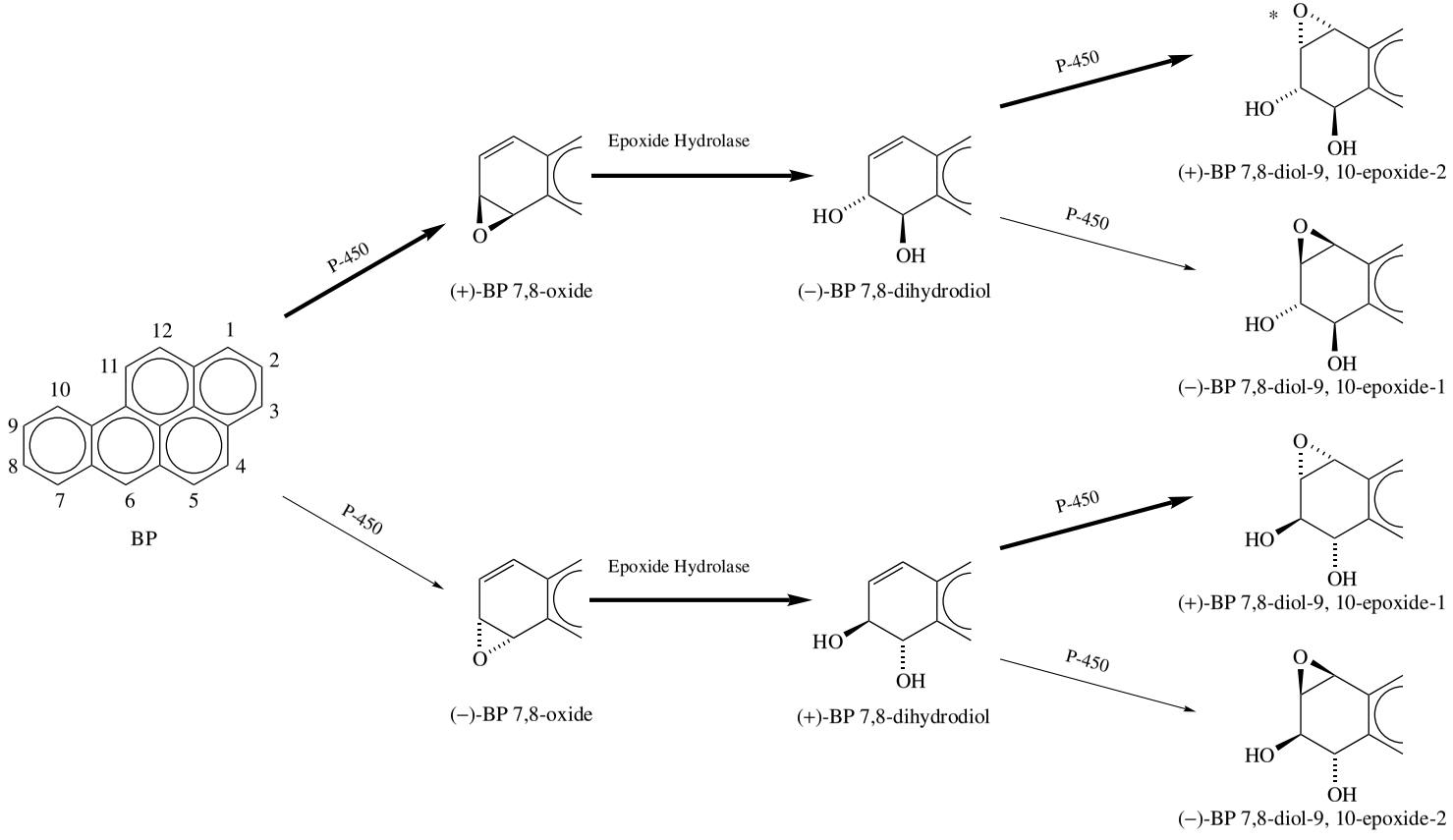


Figure 12.12 Benzo[*a*]pyrene metabolism to the ultimate carcinogenic species. Heavy arrows indicate major metabolic pathways, * represents ultimate carcinogenic species. (Adapted from A. H. Conney, *Cancer Res.* **42**: 4875, 1982.)

methanesulfonate alkylate DNA to produce *N*-alkylated and *O*-alkylated purines and pyrimidines. Ionizing radiation and reactive oxygen species commonly oxidize guanine to produce 8-oxoguanine. Formation of DNA adducts may involve any of the bases, although the *N*-7 position of guanine is one the most nucleophilic sites in DNA. Of importance is how long the adduct is retained in the DNA. (+)-Benzo[a]pyrene-7,8-diol-9,10-epoxide-2 forms adducts mainly at guanine *N*-2, while aflatoxin B1 epoxide, another well-studied rodent and human carcinogen, binds preferentially to the *N*-7 position of guanine. For some carcinogens there is a strong correlation between the formation of very specific DNA-adducts and tumorigenicity. Quantitation and identification of specific carcinogen adducts may be useful as biomarkers of exposure. Importantly, the identification of specific DNA-adducts has allowed for the prediction of specific point mutations that would likely occur in the daughter cell provided that there was no repair of the DNA-adduct in the parent cell. As will be discussed in a later section, some of these expected mutations have been identified in specific oncogenes and tumor suppressor genes in chemically induced rodent tumors, providing support that the covalent carcinogen binding produced the observed mutation. In several cases, specific base pair changes in p53 tumor suppressor gene in human tumors are associated with a mutational spectrum that is consistent with exposure of the individual to a specific carcinogen. For example, the mutation spectra identified in p53 in human tumors thought to result from the exposure of the individual to ultraviolet radiation (UVR), aflatoxin, and benzo[a]pyrene (from cigarette smoke), are consistent with the observed specific mutational damage in p53 induced by these agents in experimental cellular systems.

12.7 ONCOGENES

12.7.1 Mutational Activation of Proto-oncogenes

Much evidence has accumulated for a role of covalent binding of reactive electrophilic carcinogens to DNA in chemical carcinogenesis. It is known that chemical mutagens and carcinogens can produce point mutations, frameshift mutations, strand breaks, and chromosome aberrations in mammalian cells. If the interaction of a chemical carcinogen with DNA leading to a permanent alteration in the DNA is a critical event in chemical carcinogenesis, then the identification of these altered genes and the function of their protein products is essential to our understanding of chemical carcinogenesis. While specific DNA-carcinogen adducts were isolated in the 1970s and 1980s, it was not until the early to mid-1980s that the identification of specific genes that were mutationally altered by chemical carcinogens became known. Certain normal cellular genes, termed proto-oncogenes, can be mutated by chemical carcinogens providing a selective growth advantage to the cell. The mutational activation of proto-oncogenes is strongly associated with tumor formation, carcinogenesis, and cell transformation. Proto-oncogenes are highly conserved in evolution and their expression is tightly regulated. Their protein products function in the control of normal cellular proliferation, differentiation, and apoptosis. However, when these genes are altered by a mutation, chromosome translocation, gene amplification, or promoter insertion, an abnormal protein product or an abnormal amount of product is produced. Under these circumstances these genes have the ability to transform cells in vitro, and they are termed oncogenes.

Table 12.7 Oncogene Classification

Families	Genes
Growth factors	*sis, hst-1, int-2, wnt-1*
Growth factor receptor tyrosine kinases	*EGFR, fms, met/HGFR, ErbB2/neu/HER2, trk/NGFR*
Nonreceptor tyrosine kinases	*abl, src, fgr, fes, yes, lck*
Guanine nucleotide binding proteins	H-*ras*, K-*ras*, N-*ras, TC21*, GA_{12}
Serine/threonine kinases	*mos, raf, bcr, pim-1*
DNA-binding proteins	*myc, fos, myb, jun, E2F1, ets, rel*

Over a 100 oncogenes have been identified with approximately 30 oncogenes having a major role in human cancer.

Most oncogene protein products appear to function in one way or another in cellular signal transduction pathways that are involved in regulating cell growth, differentiation or apoptosis. Signal transduction pathways are used by the cells to receive and process information to ultimately produce a biological cellular response. These pathways are the cellular circuitry conveying specific information from the outside of the cell to the nucleus. In the nucleus, specific genes are expressed, and their encoded proteins produce the evoked biological response. Oncogenes encode proteins that are components of this cellular circuitry (Table 12.7). If a component of the circuit is altered, then the entire cellular circuit of which the component is a part is altered. It is not difficult to imagine how an alteration in a pathway that regulates cellular growth, differentiation, or apoptosis could have very profound effects on cellular homeostasis. Indeed, this is the molecular basis of how oncogenes contribute to the cancer process.

12.7.2 *Ras* Oncogene

Ras genes are frequently mutated in chemically induced animal tumors and are the most frequently detected mutated oncogenes in human tumors. Approximately 20–30% of all human tumors contain mutated *ras*. The Ras subfamily includes H-*ras*, K-*ras*, and N-*ras*, and all have been found to be mutationally activated in numerous types of tumors from a large variety of species including humans.

Activated *ras* oncogenes have been detected in a large number of animal tumors induced by diverse agents including physical agents, such as radiation, and a large number of chemical carcinogens. Some chemical carcinogens bind covalently to DNA, forming specific adducts which upon DNA replication yields characteristic alterations in the primary sequence of the Ha-*ras* proto-oncogene. The study of the *ras* oncogene as a target for chemical carcinogens has revealed a correlation between specific carcinogen-DNA adducts and specific activating mutations of *ras* in chemically induced tumors. For example, 7,12-dimethylbenz[a]anthracene, a polycyclic aromatic hydrocarbon carcinogen, is metabolically activated to a bay-region diol epoxide that binds preferentially to adenine residues in DNA. Skin tumors isolated from mice treated with DMBA contain an activated H-*ras* oncogene with an A to T transversion of the middle base in the 61st codon of H-*ras*. Therefore the identified mutation in *ras* is consistent with the expected mutation based on the DMBA-DNA adducts which have been identified. Likewise rat mammary carcinomas induced by nitrosomethylurea contain a G to A transition in the 12th codon of H-*ras*, and this mutation is consistent

with the modification of guanine residues by this carcinogen. Based on these events, the alteration of *ras* by specific chemical carcinogens appears to be an early event in carcinogenesis.

Ras proteins function as membrane-associated molecular switches operating downstream of a variety of membrane receptors. Ras is in the off position when it is bound to guanosine diphosphate (GDP). However, when stimulated by a growth factor receptor, Ras exchanges GTP guanosine triphosphate for GDP, and now Ras is in the on position. Ras communicates this "on" message to the next protein in the signaling circuitry, which through a kinase cascade ultimately results in the activation of several transcription factors. These transcription factors regulate the expression of genes involved in cell proliferation, for example. Once Ras has conveyed the "on" message, it turns itself off. Ras has intrinsic GTPase activity that hydrolyzes GTP to form GDP, and Ras is once again in the off position. Another protein, termed GAPp120 (GTPase activating protein), aids Ras in GTP hydrolysis. When *ras* is mutated in certain codons, including the 12th, 13th, or 61st codon, the intrinsic GTPase activity of Ras is greatly diminished as is its ability to interact with GAP. The net effect is that mutated Ras is essentially stuck in the "on" position continually sending a proliferative signal to the downstream circuitry.

12.8 TUMOR SUPPRESSOR GENES

12.8.1 Inactivation of Tumor Suppressor Genes

Activation of oncogenes results in a gain of function while inactivation of tumor suppressor genes results in a loss of function. Tumor suppressor genes have also been termed anti-oncogenes, recessive oncogenes, and growth suppressor genes. Tumor suppressor genes encode proteins that generally function as negative regulators of cell growth or regulators of cell death. In addition some tumor suppressor genes function in DNA repair and cell adhesion. The majority of tumor suppressor genes were first identified in rare familial cancer syndromes, and some are frequently mutated in sporadic cancers through somatic mutation. There are approximately 18 known tumor suppressor genes (e.g., p53, Rb, APC, p16, and BRCA1) that have been shown to have a role in cancer and another 12 putative tumor suppressors have been identified. When tumor suppressor genes are inactivated by allelic loss, point mutation, or chromosome deletion, they are no longer capable of negatively regulating cellular growth leading to specific forms of cancer predisposition. Generally, if one copy or allele of the tumor suppressor gene is inactivated, the cell is normal, and if both copies or alleles are inactivated, loss of growth control occurs. In some cases a single mutant allele of certain tumor suppressor genes, such as p53, can give rise to an altered intermediate phenotype. However, inactivation of both alleles is required for full loss of function and the transformed phenotype.

12.8.2 p53 Tumor Suppressor Gene

p53 encodes a 53 kDa protein. *p53* is mutated in 50% of all human cancer and is the most frequently known mutated gene in human cancer. The majority (ca. 80%) of *p53* mutations are missense mutations and *p53* is mutated in approximately 70% of

colon cancers, 50% of breast and lung cancers, and 97% of primary melanomas. In addition to point mutations, allelic loss, rearrangements, and deletions of *p53* occur in human tumors. p53 is a transcription factor and participates in many cellular functions, including cell cycle regulation, DNA repair, and apoptosis. The p53 protein is composed of 393 amino acids, and single missense mutations can inactivate the p53. Unlike *ras* genes, which have a few mutational codons that result in its activation, the p53 protein can be inactivated by hundreds of different single-point mutations in *p53*. It has been proposed that the mutation spectrum of *p53* in human cancer can aid in the identification of the specific carcinogen that is responsible for the genetic damage; that is to say, different carcinogens cause different characteristic mutations in *p53*. Some of the mutations in *p53* reflect endogenous oxidative damage, while others such as the mutational spectrum in *p53* in hepatocellular carcinomas from individuals exposed to aflatoxin demonstrate a mutation spectrum characteristic aflatoxin. In sun-exposed areas where skin tumors develop, the mutations found in *p53* in these tumors are characteristic of UV light induced pyrimidine dimers, and finally the mutation spectrum induced by (+)-benzo[a]pyrene-7,8-diol-9,10-epoxide-2 in cells in culture is similar to the mutational spectrum in *p53* in lung tumors form cigarette smokers. Thus certain carcinogens produce a molecular signature that may provide important information in understanding the etiology of tumor development.

p53 has been termed the "guardian of genome" because it controls a G1/S checkpoint, regulates DNA repair, and apoptosis. DNA damage results in the activation of p53 function and p53 prevents cells with damaged DNA from entering the S-phase of the cell cycle until the DNA damage is repaired. If the DNA damage is severe, p53 can cause the cell to undergo apoptosis. Mutation of *p53* disrupts these functions leading to the accumulation of mutations as cells enter S-phase with damaged DNA (mutator phenotype, genetic instability) and further development of malignant clones.

12.9 GENERAL ASPECTS OF MUTAGENICITY

Mutagens are chemical and physical agents that are capable of producing a mutation. Mutagens include agents such as radiation, chemotherapeutic agents, and many carcinogens. A mutation is a permanent alteration in the genetic information (DNA) of the cell. DNA-damaging agents/mutagens can produce (1) point mutations involving single base pair substitutions that can result in amino acid substitutions in the encoded protein and frame-shift mutations involving the loss or gain of one or two base pairs, resulting in an altered reading frame and gross alterations in the encoded protein, (2) chromosome aberrations including gross chromosomal rearrangement such as deletions, duplications, inversions, and translocations, and (3) aneuploidy and polyploidy, which involve the gain or loss of one or more chromosomes. Point mutations are classified as missense or nonsense mutations. A missense mutation produces an altered protein in which an incorrect amino acid has been substituted for the correct amino acid. A nonsense mutation is an alteration that produces a stop codon and results in a truncated protein. A point mutation can also be characterized based on the mutagen-induced substitution of one base for another within the DNA. When a point mutation produces a substitution of a purine for another purine (i.e., guanine for adenine) or a pyrimidine for another pyrimidine (i.e., thymine for cytosine), the mutation is referred

as a transition. If a purine is substituted for a pyrimidine, and vice versa (i.e., thymine for adenine or guanine for cytosine), the mutation is referred to as a transversion.

12.10 USEFULNESS AND LIMITATIONS OF MUTAGENICITY ASSAYS FOR THE IDENTIFICATION OF CARCINOGENS

As mentioned earlier in this chapter, the two-year rodent carcinogenesis bioassay is considered the "gold standard" and is utilized to determine whether a test compound has carcinogenic potential. Identification and classification of potential human carcinogens through the two-year rodent carcinogenesis bioassay is complicated by species differences, use of high doses (MTD, maximum tolerated dose), the short life span of the rodents, sample size, and the need to extrapolate from high to low doses for human risk assessment. In addition the two-year rodent bioassay to is costly to conduct (>2 million dollars) and takes two to four years before complete results can be obtained. Since many carcinogens are mutagens, short-term test systems to evaluate the mutagenicity or genetic toxicity of compounds were developed with the idea that these tests could be used to quickly and inexpensively detect/identify chemical carcinogens. Short-term genotoxicity/mutagenicity assays were developed in a variety of organisms including bacteria, yeast, Drosophila, and human and rodent cells. These mutagenic assays or short-term genotoxicity tests directly or indirectly measure point mutations, frame-shift mutations, chromosomal damage, DNA damage and repair, and cell transformation.

In the 1970s it was reported that mutagenicity could predict rodent carcinogenicity 90% of the time. However, after extensive evaluation, it is now considered that mutagenicity can predict rodent carcinogenicity approximately 60% of the time. For certain classes of carcinogens such as the polycyclic aromatic hydrocarbons, short-term mutagenicity tests are generally highly accurate at predicting rodent carcinogenicity. For other classes of carcinogens such as the halogenated hydrocarbons, short-term genotoxicity tests often fail to detect these rodent carcinogens. Many of these halogenated hydrocarbons probably function through an epigenetic mechanism/tumor promoting mechanism.

In an important study published in 1987 by Tennant et al. (*Science* 236, pp. 933–941) 73 chemicals previously tested in the rodent two-year carcinogenesis bioassay were examined in four widely used short-term tests for genetic toxicity. The short-term assays measured mutagenesis in the Salmonella assay (Ames Assay) and mouse lymphoma assay, and chromosome aberrations and sister chromatid exchanges in Chinese hamster ovary cells. The concordance (% agreement between short-term genotoxicity test and rodent bioassay results) of each assay with the rodent bioassay data was approximately 60%. Within the limits of the study there was no evidence of complementarity among the four tests, and no battery of tests constructed from these assays improved substantially on the overall performance of the Salmonella assay. When interpreting the results of short-term test for genetic toxicity assays, it is important to consider (1) the structure and physical properties of the test compound, (2) the 60% concordance between the short-term test for genetic toxicity and rodent carcinogenicity, (3) epigenetic versus genetic mechanisms of carcinogenesis, and (4) the existence noncarcinogenic mutagens. It is also important to keep in mind that there is accumulating evidence that some compounds that are negative in

short-term tests for mutagenicity can induce oxidative DNA damage in vivo through the direct or indirect production of reactive oxygen species. These compounds are in vivo mutagens but are negative in the short-term test of genetic toxicity.

Several bacterial and mammalian short-term tests for genetic toxicity as well as their biochemical and genetic rationale are described in Chapter 21 on toxicity testing. They include the salmonella assay, the Chinese hamster ovary cell/hypoxanthine-guanine phosphoribosyl transferase assay, the mouse lymphoma assay, the mammalian transformation assay, sister chromatid exchange, and the chromosome aberration assay.

SUGGESTED READING

10th Report on Carcinogens, US Department of Health and Human Services, Public Health Service, National Toxicology Program. http : //ehp.niehs.nih.gov/roc.toc10.html

Cancer Facts and Figures, American Cancer Society. www.cancer.org

International Agency for Research on Cancer. www.iarc.fr

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