PART III

TOXICANT PROCESSING *IN VIVO*

CHAPTER 5

Absorption and Distribution of Toxicants

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5.1 INTRODUCTION

As illustrated in the previous chapter, the human body can be exposed to a variety of toxicants which may be present in various environmental media such as air, soil, water, or food. However, just simply being exposed to these hazardous chemicals does not necessarily translate into a toxicological response. The mammalian body has several inherent defense mechanisms and membrane barriers which tend to prevent the entry or absorption and distribution of these toxicants once an exposure event has occurred. However, if the toxicant is readily absorbed into the body, there are still other anatomical and physiological barriers which may prevent distribution to the target tissue to elicit a toxic response. As the toxicological response is often related to the exposed dose, interactions between the toxicant and the body's barriers and defense mechanisms will have an effect on toxicant movement in the body, and ultimately modulate the rate and extent of toxicant absorption and distribution to the target tissue.

The skin represents the largest organ in the human body, and one of its primary functions can be seen as a physical barrier to absorption of toxicants. The other major routes of toxicant entry into the body are through the respiratory and gastrointestinal tract (GIT) which can be seen to offer less resistance to toxicant absorption than the skin. In general, the respiratory tract offers the most rapid route of entry and the dermal the least rapid. One reason for this major difference is primarily because membrane thickness, which is really the physical distance between the external environment (skin surface, air in the lung, or lumen of the gut) and the blood capillaries, varies across these portals of entry. The overall entry depends on both the amount present and the saturability of the transport processes involved.

Liver metabolism will have the most significant effect on toxicant bioavailability following gastrointestinal absorption, but microbial activity and various enzymes in the GIT and the skin can play a significant role in oral and dermal absorption, respectively. Physicochemical characteristics of the toxicant such as the chemical form can be a useful indicator of whether the toxicant will be absorbed and distributed in the body. In this regard, toxicant molecular weight, ionization (pKa), and

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octanol/water partition coefficient (logP) are useful indices of predicting chemical transport from an environmental media across biological membranes to the bloodstream. The reader should also be aware that for those toxicants that are readily ionized, the pH gradient across membranes can determine the extent of toxicant transport and accumulation in tissues.

Once the toxicant has been absorbed, the toxicant molecules can move around the body in two ways: (1) by bulk flow transfer (i.e., in the bloodstream) and (2) by diffusional transfer (i.e., molecule by molecule over short distances). Disposition is the term often used to describe the simultaneous effects of distribution and elimination processes subsequent to absorption. The cardiovascular system provides distribution of all toxicant irrespective of their chemical nature to various organs and tissues with various levels of affinities for toxicants. It should be remembered that organ mass and blood perfusion can vary, which can account for differential distribution of toxicants. Toxicant disposition can also be influenced by plasma protein binding in the bloodstream. The nature of this toxicant-protein interaction is dependent on the chemical nature of the toxicant, the presence of other toxicants or drugs in the bloodstream, as well as plasma protein levels. However, what distinguishes one toxicant pharmacokinetically from another is its diffusional characteristics. That is, its ability to cross nonaqueous diffusional barriers (e.g., cell membranes) from an aqueous compartment. This usually involves movement across several compartments separated by lipid membranes. It is therefore important to understand the *mechanisms by which drugs cross membranes* and the *physiochemical properties* of molecules and membranes that influence the movement of drugs from the environment to the body via either oral, inhalation, or dermal routes. These factors also influence movement from one compartment to another within the body during distribution as well as metabolism and excretion.

We can quantitate this movement or transport from one compartment to another using mathematical models to describe transport rates. This in fact is what we do in pharmacokinetic analysis and modeling. *Pharmaco- or toxicokinetics* is therefore the quantitation of the time course of toxicants in the body during the various processes of absorption, distribution, and elimination or clearance (metabolism and/or excretion) of the toxicant. Stated differently, this is a study of how the body "handles" the toxicant as it is reflected in the plasma concentration at various time points. The two most important pharmacokinetic parameters that describe the disposition of a chemical are volume of distribution (V_d) and systemic (body) clearance. *Pharmaco- and toxicodynamics* is the study of the biochemical and physiological effects of drugs and toxicants and determines their mechanism of action. Physiologically based pharmaco- or toxicokinetic models are used to integrate this information and to predict disposition of toxicants for a given exposure scenario. These concepts will be introduced at the end of this chapter.

5.2 CELL MEMBRANES

During absorption, distribution, and elimination processes, the toxicant will encounter various cell membranes before interacting with the target tissue. Each step of these processes involves translocation of the chemical across various membrane barriers, from the skin or mucosa through the capillary membranes, and through

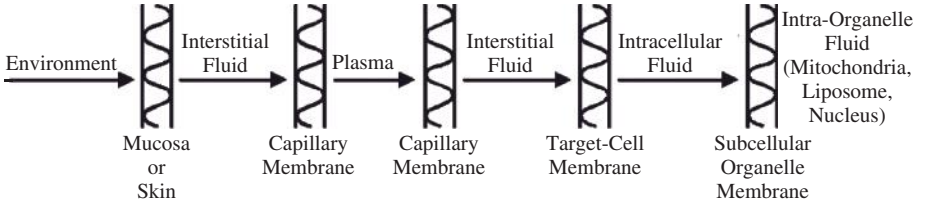


Figure 5.1 Schematic showing membranes that a chemical may need to cross during passage from the environment to the site of action. Redrawn from Hodgson and Levi, eds. *Introduction to Biochemical Toxicology*, 2nd ed. Norwalk, CT: Appleton & Lange, 1994, p. 12.

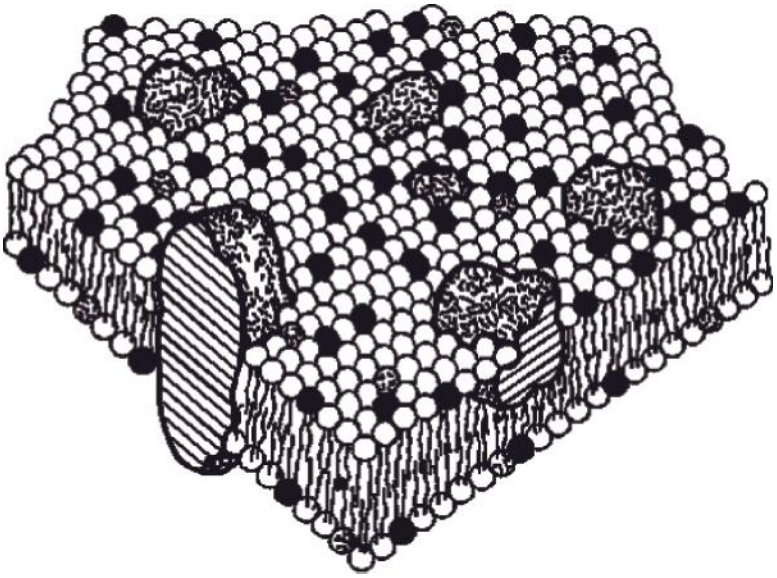


Figure 5.2 Schematic diagram of biological membrane. Head groups of lipids represented by spheres, tail ends by zigzag lines. Black, white, or stippled spheres indicate different kinds of lipids and illustrate asymmetry in certain cases. Large bodies are membrane-associated proteins. Modified from Singer and Nicolson. *Science* **175**:720, 1972.

the cellular and organelle membranes (Figure 5.1). These membrane barriers vary from the relatively thick areas of the skin to the relatively thin lung membranes. In all cases, however, the membranes of tissue, cell, and cell organelle are relatively similar.

The cell membranes are predominantly a lipid matrix or can be considered a lipid barrier with an average width of a membrane being approximately 75 Å. The membrane is described as the fluid mosaic model (Figure 5.2) which consists of: (1) a bilayer of phospholipids with hydrocarbons oriented inward (hydrophobic phase); (2) hydrophilic heads oriented outward (hydrophilic phase); and (3) associated intra- and extracellular proteins that transverse the membrane. The ratio of lipid to

protein varies from 5:1 for the myelin membrane to 1:5 for the inner structure of the mitochondria. However, 100% of the myelin membrane surface is lipid bilayer, whereas the inner membrane of the mitochondria may have only 40% lipid bilayer surface. In this example, the proportion of membrane surface that is lipid will clearly influence distribution of toxicants of varying lipophilicity.

The lipid constituents in the membrane permit considerable movement of macromolecules, and membrane constituents may move appreciably within membranes. Membrane fluidity, a function of lipid composition, can be altered by temperature and chemicals (e.g., anesthetics). Several types of lipids are found in membranes, with phospholipids and cholesterol predominating. Sphingolipids comprise the primary minor component. Phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine are the primary phosphatides, and their two fatty acid hydrocarbon chains (typically from 16 to 18, but varying from 12 to 22) comprise the nonpolar region. Some of the fatty acids are unsaturated and contribute appreciably to the fluidity of the membrane.

Proteins, which have many physiological roles in normal cell function, are intimately associated with lipids and may be located throughout lipid bilayers. These proteins may be located on either the surface or traverse the entire structure. Hydrophobic forces are responsible for maintaining the structural integrity of proteins and lipids within membranes, but movement within the membranes may occur. External and internal membrane proteins can function as receptors. Many proteins that traverse the membrane are transport proteins and are involved in translocation of ligands; that is, they are involved in active and facilitated transport.

Complexes of intrinsic membrane proteins and lipids can form hydrophilic or hydrophobic channels that allow transport of molecules with different physicochemical characteristics. The amphipathic nature of the membrane creates a barrier for ionized, highly polar drugs, although it does not completely exclude them. The presence of pores of approximately 4 Å are believed to allow for ready movement of small molecules such as water. Thus, certain molecules that ordinarily would be excluded can rapidly traverse the highly lipid membrane barrier.

It is worth noting that differences between membranes, such as the presence of different lipids, the amount of surface lipid, differences in size and shape of proteins, or physical features of bonding may cause differences in permeability between membranes. These biochemical and biophysical differences are thought to be responsible for permeability differences in skin from different anatomical regions of the body.

5.3 MECHANISMS OF TRANSPORT

In general, there are four main ways by which small molecules cross biological lipid membranes:

1. *Passive diffusion*, that is, by diffusing through the lipid membrane
2. *Filtration*, that is, by diffusing through aqueous pores
3. *Special transport*, that is, by combination with a carrier molecule, which acts as a "ferryboat"
4. *Endocytosis*, that is, by pinocytosis for liquids and phagocytosis for solids

The first and third routes are important in relation to pharmacokinetic mechanisms. The aqueous pores are too small in diameter for diffusion of most drugs and toxicant, although important for movement of water and small polar molecules (e.g., urea). Pinocytosis is important for some macromolecules (e.g., insulin crossing the blood–brain barrier [BBB]).

5.3.1 Passive Diffusion

Most drugs and toxicant pass through membranes by *simple diffusion* down a concentration gradient. The driving force being the concentration gradient across the membrane.

This diffusion process can continue until equilibrium, although in reality there is always movement, but the net flux is zero. Eventually, the concentration of unionized or unbound (free) toxicant is the same on either side of the membrane. Please note that there is no competition of molecules, and there is generally a lack of saturation. Solubility in the lipid bilayer is important, and the greater the partition coefficient, the higher the concentration in the membrane, and the greater the rate of diffusion across the membrane. For ionized toxicants, the steady state concentration is dependent on the differences in pH across the membrane. Most membranes are relatively permeable to water either by diffusion or by flow that results from hydrostatic or osmotic differences across the membrane, and bulk flow of water can also carry with it small and water-soluble molecules by this mechanism. These substances generally have a molecular weight of less than 200. Although inorganic ions are small and will readily diffuse across some membranes, their hydrated ionic radius is relatively large. In such cases, active transport is required (see below). Specific ion fluxes are also controlled by specific channels that are important in nerves, muscles, and signal transduction.

We can now quantitate the *rate* at which a toxicant can be transported by passive diffusion, and this can be described by *Fick's Law of Diffusion* in the following equation:

$$\text{Rate of Diffusion} = \frac{D \times \text{Sa} \times \text{Pc}}{d}(C_{\text{H}} - C_{\text{L}})$$

D is the diffusion coefficient; Sa is the surface area of the membrane, Pc is the partition coefficient, d is the membrane thickness, and C_{H} and C_{L} are the concentrations at both sides of the membrane (high and low, respectively). The first part of this equation ($D\text{Pc}/d$) represents the permeability coefficient of the drug. The permeability expresses the ease of penetration of a chemical and has units of velocity, distance/time (cm/hr).

The diffusion coefficient or diffusivity of the toxicant, D, is primarily dependent on solubility of the toxicant in the membrane and its molecular weight and molecular conformation. Depending on the membrane, there is a functional molecular size and/or weight cutoff that prevents very large molecules from being passively absorbed across any membrane. One would expect small molecular weight molecules to diffuse more rapidly than larger molecular weight toxicants. Therefore, the magnitude of a toxicant's diffusion coefficient really reflects the ease with which it is able to diffuse through the membrane. The reader should also be aware that as

a toxicant crosses from the donor or aqueous medium and through the membrane medium, there are really two diffusion environments and thus two diffusion coefficients to consider. Another important factor that can influence the diffusion coefficient is membrane viscosity. This physicochemical characteristic should remain constant in biological systems, but can be modified in skin membranes exposed to various pharmaceutical or pesticide formulations. Formulation additives or excipients may enter the membrane barrier and reversibly or irreversibly change viscosity and thus diffusion coefficient of the drug or pesticide in the barrier membranes of the skin.

The partition coefficient that will be described in more detail later in this chapter is the relative solubility of the compound in lipid and water, and really reflects the ability of the toxicant to move from a relatively aqueous environment across a lipid membrane. It is this factor that is often manipulated in pesticide and drug formulations to create a vehicle. Membrane permeability is therefore strongly correlated to the lipid solubility of the toxicant in the membrane as well as the aqueous environment surrounding the membrane. Please be aware that there are instances where partition coefficient or lipid solubility of the toxicant may be very large, and there may be a tendency for the drug to sequester in the membrane. Membrane surface area and membrane thickness can also vary across different organs in the body, but one does not expect these two factors in Fick's equation to vary considerably. The final component of Fick's equation is the concentration gradient (C_H–C_L) across the membrane, which is the *driving force for diffusion*, and as will be demonstrated below in our discussion on first-order kinetics, is the most important factor dictating the rate of transport across most biological membranes.

First-Order Kinetics: When the rate of a process is dependent upon a *rate constant* and a concentration gradient, a linear or first-order kinetic process will be operative. The reader should be aware that there are numerous deviations from the first-order process when chemical transport *in vivo* is analyzed, and this can be deemed an *approximation* since in many barriers, penetration is slow, and a long period of time is required to achieve steady state.

The rate of movement of a toxicant across a membrane may be expressed as the *change in amount of toxicant, A, (dA) or toxicant concentration, C, (dC) per unit of time (dt) which equals dA/dt.* Calculus can be used to express instantaneous rates over very small time intervals (*dt*). Thus, rate processes may then be generally expressed as:

$$dA/dt = K\ A^n$$

where dA/dt is the rate of chemical (X) movement (e.g., absorption, distribution, elimination); k is the rate constant of the process; and n is the kinetic order of the transport process (e.g., absorption). The "n" either equals 1 (first order) or 0 (zero order). Thus, a "first-order" rate equation is:

$$dA/dt = k\ A^1 = KA$$

and a "zero-order" rate equation is:

$$dA/dt = K\ A^0 = K.$$

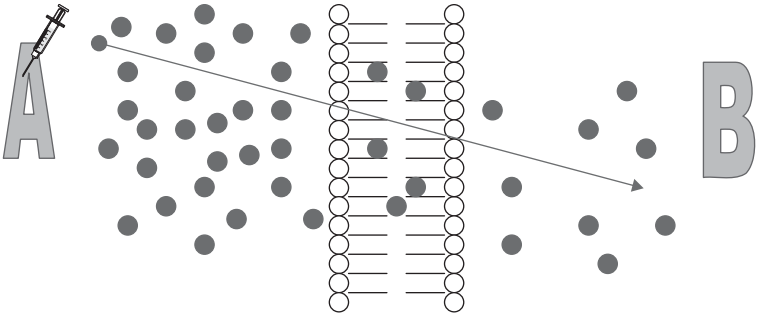


Figure 5.3 Illustration of concentration gradient generated by administration of a drug that can travel down this gradient from area A and across a biological membrane to area B.

We know from Fick's Law that the rate of diffusion (now expressed as dA/dt) is:

$$dA/dt = D \bullet \mathrm{Sa} \bullet \mathrm{Pc}(A_1 - A_2)/d.$$

Once a toxicant crosses a membrane, it is rapidly removed from the "receiving side" (compartment B in Figure 5.3) either by uptake into the bloodstream or elimination from the organism. Thus, it is A_1 that is the primary driving force, and if we replace this with "A" in all equations, then:

$$dA/dt = (D \bullet \mathrm{Sa} \bullet \mathrm{Pc}/d)\,A$$

If we let **K** = (D•Sa•Pc/d) and realize that since A is present in this equation, n must equal 1, we have a first-order rate process. Fick's Law of Diffusion, which is so important for quantitating rates of absorption, distribution, and elimination, is thus the basis for using first-order kinetics in most pharmacokinetic models.

Therefore, in a first-order process, the rate of drug movement is directly proportional to the amount of drug (A) in the body, which is usually a function of the dose. K is the *first-order fractional rate constant with units of l/time (time^{-1})* and *represents the fraction of drug that is transported per unit of time*. Thus, in a *first-order* process, *the rate of drug movement is proportional to dose, but the fraction moved per unit of time is constant and independent of dose.*

When first-order kinetics hold, a simple relationship exists between the penetration rate constant, K, and $t_{0.5}$ (time necessary for one-half of the applied dose to penetrate):

$$K = \frac{0.693}{t_{0.5}} \quad \text{and the units of } K \text{ are percentage of change/time unit.}$$

We can also derive the concentration of the toxicant if we know the volume or V_d of the toxicant compartment as:

$$V_\mathrm{d}\,(\text{volume}) = A\,(\text{mass})/C\,(\text{mass/volume})$$

V_d is discussed in more detail later in this chapter.

5.3.2 Carrier-Mediated Membrane Transport

This mechanism is important for compounds that lack sufficient lipid solubility to move rapidly across the membrane by simple diffusion. A membrane-associated protein is usually involved; specificity, competitive inhibition, and the saturation phenomenon and their kinetics are best described by *Michaelis–Menten enzyme kinetic models*. Membrane penetration by this mechanism is more rapid than simple diffusion and, in the case of active transport, may proceed beyond the point at which concentrations are equal on both sides of the membrane. Generally, there are two types of specialized carrier-mediated transport processes:

Passive facilitated diffusion involves movement down a concentration gradient without an input of energy. However, this mechanism, which may be highly selective for specific conformational structures, is necessary for transport of endogenous compounds whose rate of transport by simple diffusion would otherwise be too slow. The classical example of the latter is transport of glucose into red blood cells.

Active transport requires energy and transport is against a concentration. Maintenance against this gradient often requires energy. It is often coupled to energy-producing enzymes (e.g., ATPase) or to the transport of other molecules (e.g., Na^+, Cl, H^+) that generate energy as they cross the membranes. Carrier-mediated drug transport can occur in only a few sites in the body, and the main sites are:

1. BBB, neuronal membranes, choroid plexus;
2. renal tubular cells; and
3. hepatocytes, biliary tract.

There are instances in which toxicants have chemical or structural similarities to endogenous chemicals that rely on these special transport mechanisms for normal physiological uptake and can thus utilize the same system for membrane transport. Useful examples of drugs known to be transported by this mechanism include levodopa, which is used in treating Parkinson's disease, and fluorouracil, a cytotoxic drug. Levodopa is taken up by the carrier that normally transports phenylalanine, and fluorouracil is transported by the system that carries the natural pyrimidines, thymine, and uracil. Iron is absorbed by a specific carrier in the mucosal cells of the jejunum, and calcium by a vitamin D-dependent carrier system. Lead may be more quickly moved by a transport system that is normally involved in the uptake of calcium.

For carrier-mediated transport, the rate of movement across a membrane will now be *constant*, since flux is dependent upon the capacity of the membrane carriers and not the mass of the chemical to be transported. These processes are described by *zero-order* kinetic rate equations of the form:

$$dX/dt = K\ X^0 = K_0$$

K_0 is now the *zero-order* rate constant and is expressed in terms of *mass/time*. In an active carrier-mediated transport process following zero-order kinetics, the rate of drug transport is always equal to K once the system is fully loaded or saturated. At subsaturation levels, the rate is *initially first order* as the carriers become loaded

TABLE 5.1 An Example of Differences between First-Order and Zero-Order Processes although the System Is Exposed to the Same Initial Mass of Toxicant of 1000, 100, or 10 mg

Initial Toxicant Mass (mg)	Amount Transported/min (First Order)	Amount Transported/min (Zero Order)
1000	100	10
100	10	10
10	1	10

with the toxicant, but at concentrations normally encountered in pharmacokinetics, the rate becomes constant. Thus, as dose increases, the rate of transport does *not* increase in proportion to dose as it does with the fractional rate constant seen in first-order process. This is illustrated in Table 5.1 below, where it is assumed that the *first-order* rate constant is 0.1 (10% per min) and the *zero-order* rate is 10 mg/min.

In the case of first order, these amounts will subsequently diminish (10% of 900 is 90, and so on). In the case of zero order, the amount transported does not vary with time (constant rate of transport).

The plot below illustrates the differences in passive (linear) versus carrier-mediated (nonlinear) transport. At relatively low concentrations of drug, carrier-mediated processes may appear to be first order since the protein carriers are not saturated. However, at higher concentrations, zero-order behavior becomes evident. It is in plots such as this that the terms *linear* (first order) and *nonlinear* (zero order) come into existence.

5.4 PHYSICOCHEMICAL PROPERTIES RELEVANT TO DIFFUSION

The following physicochemical properties are important for chemical diffusion, and we have discussed several of these in previous sections of this chapter as it relates to passive diffusion mechanism and how it impacts rate of toxicant transport across membranes.

1. Molecular size and shape
2. Solubility at site of absorption
3. Degree of ionization
4. Relative lipid solubility of ionized and unionized forms

Although molecular weight is important, it is less important than the drug's *lipid solubility* when it comes to assessing the rate of passive diffusion across membranes. The permeability, P ($P = \mathrm{Pc} \times D$), of a nonpolar substance through a cell membrane is dependent on two physicochemical factors: (1) *solubility in the membrane* (Pc), which can be expressed as a partition coefficient of the drug between the aqueous phase and membrane phase, and (2) *diffusivity or diffusion coefficient* (D), which is a measure of mobility of the drug molecules within the lipid. The latter may vary only slightly between toxicants, but the former is more important. Lipid solubility

is therefore one of the most important determinants of the pharmacokinetic characteristics of a chemical, and it is important to determine whether a toxicant is readily ionized or not influenced by pH of the environment. If the toxicant is readily ionized, then one needs to understand its chemical behavior in various environmental matrices in order to adequately assess its transport mechanism across membranes.

5.4.1 Ionization

For the purposes of this discussion on membrane transport, chemicals can be broadly categorized into those that are ionized and those that are not ionized. Many drugs (e.g., antibiotics) and several toxicants (e.g., strychnine) are either weak acids or weak bases and can exist in solution as a mixture of nonionized and ionized forms. Generally, these drugs and toxicants must be in the uncharged or nonionized form to be transported by passive diffusion across biological membranes. This is because biological membranes are of a lipid nature and are less permeable to the ionized form of the chemical. The pH of the environment (e.g., lumen of the GIT and renal tubules) can influence transfer of toxicant that is ionizable by increasing or decreasing the amount of nonionized form of the toxicant. Aminoglycosides (e.g., gentamicin) are the exception to this general rule in that the uncharged species is insufficiently lipid soluble to cross the membrane appreciably. This is due to a preponderance of hydrogen-bonding groups in the sugar moiety that render the uncharged molecule hydrophilic. Note that some amphoteric drugs (e.g., tetracyclines) may be absorbed from both acidic and alkaline environments. In essence, the amount of drug or toxicant in ionized or nonionized form depends on the pKa (pH at which 50% of the drug is ionized) of the drug and the pH of the solution in which the drug is dissolved. *The pKa, which is the negative logarithm of the dissociation constant of a weak acid or weak base, is a physicochemical characteristic of the drug or toxicant.* When the pH of the solution is equal to the pKa, then 50% of the toxicant is in the ionized form and 50% is in the nonionized form. The ionized and nonionized fractions can be calculated according to the *Henderson–Hasselbach* equations listed below:

$$\textit{For weak acids} \quad \mathrm{pKa} - \mathrm{pH} = \log[\text{nonionized form}/\text{ionized form}]$$

$$\textit{For weak bases} \quad \mathrm{pKa} - \mathrm{pH} = \log[\text{ionized form}/\text{nonionized form}]$$

For an organic acid ($RCOOH \Leftrightarrow RCOO^- + H^+$), acidic conditions (pH less than the pKa of the compound) will favor the formation of the nonionized RCOOH, whereas alkaline conditions (pH greater than pKa) will shift the equilibrium to the right. For an organic base ($RNH_2 + H^+ \Leftrightarrow RNH_3^+$), the reverse is true, and decreasing the pH (increasing the concentration of H^+) will favor formation of the ionized form, whereas increasing the pH (decreasing the concentration of H^+) will favor formation of the nonionized form.

Memory aid. In general, weak organic acids readily diffuse across a biological membrane in an acidic environment, and organic bases can similarly diffuse in a basic environment. This is illustrated quite well in Table 5.2 below for chemical in the rat intestine. There are the usual exceptions to the generalizations concerning

TABLE 5.2 Effect of pH on Absorption of Weak Organic Acids and Bases from Rat Intestine

Compound	Percent Absorbed at Various pH Values				
	pKa	3.6–4.3	4.7–5.0	7.0–7.2	7.8–8.0
Acids					
Nitrosalicyclic	2.3	40	27	<02	<02
Salicyclic	3.0	64	35	30	10
Benzoic	4.2	62	36	35	05
Bases					
Aniline	4.6	40	48	58	61
Aminopyrene	5.0	21	35	48	52
Quinine	8.4	09	11	41	54

ionization and membrane transport, and some compounds, such as pralidoxime (2-PAM), paraquat, and diquat, are absorbed to an appreciable extent even in the ionized forms. The mechanisms allowing these exceptions are not well understood.

Ion trapping can occur when at equilibrium the total (ionized + nonionized) concentration of the drug will be different in each compartment, with an acidic drug or toxicant being concentrated in the compartment with the relatively high pH, and vice versa. The pH partition mechanism explains some of the qualitative effects of pH changes in different body compartment on the pharmacokinetics of weakly basic or acidic drugs or toxicant as it relates to renal excretion and penetration of the BBB. Alkalization of urine in the lumen of renal tubules can enhance elimination of weak acids. However, this phenomenon is not the main determinant of absorption of drugs or toxicants from the GIT. In the GIT, the enormous absorptive surface area of the villi and microvilli in the ileum compared to the smaller absorptive area of the stomach is of overriding importance.

5.4.2 Partition Coefficients

A second physicochemical parameter influencing chemical penetration through membranes is the relative lipid solubility of the potential toxicant which can be ascertained from its known partition coefficient. The partition coefficient is a measure of the ability of a chemical to separate between two immiscible phases. The phases consist of an organic phase (e.g., octanol or heptane) and an aqueous phase (e.g., water). The lipid solvent used for measurement is usually octanol because it best mimics the carbon chain of phospholipids, but many other systems have been reported (chloroform/water, ether/water, olive oil/water). The lipid solubility and the water solubility characteristics of the chemical will allow it to proportionately partition between the organic and water phase. The partition coefficients can be calculated using the following equation:

$$P = V_w / V_o \, [C_{wo} - C_w / C_w]$$

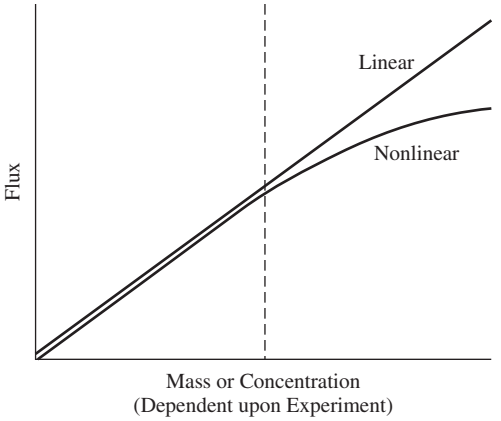


Figure 5.4 Plot depicting a linear relationship (first order) and nonlinear relationship (zero order) between chemical flux across a membrane and the initial mass or concentration of the chemical.

where P is the partition coefficient and is usually expressed in terms of its logarithmic value (log P); V_w and V_o are the volumes of aqueous and oil or organic phase, respectively; and C_{wo} and C_w are drug or toxic concentrations in the aqueous phase before and after shaking, respectively.

The lower the partition coefficient, the more water soluble, and the least permeable the toxicant is across a membrane. Regarding dermal absorption, partition coefficients can be predictive of absorption. However, toxicants with extremely high partition coefficients tend to remain in the membrane or skin. This explains why a strong correlation between permeability and partition coefficient can exist for a hypothetical series of analogous chemicals for a specific range of partition coefficients, but the correlation does not exist for log P greater values greater than 6 in many instances. A log P of around 1 is often taken as desirable for skin penetration. The reader should also recall that this parameter is operative as the chemical diffuses across membranes (Figure 5.1) of varying lipid content during absorption, distribution, and elimination processes.

5.5 ROUTES OF ABSORPTION

Primary routes of entry of toxicants to the human body are dermal, gastrointstinal, and respiratory. Methods for studying these different routes are numerous, but they are perhaps best developed for the study of dermal absorption because this route is subject to more direct methodology, whereas methods for studying respiratory or gastrointestinal absorption require more highly specialized instrumentation. Additional routes encountered in experimental studies include intraperitoneal, intramuscular, and subcutaneous routes. When direct entry into the circulatory system is desired, intravenous (IV) or intra-arterial injections can be used to bypass the absorption phase. Information from this more direct route of entry (e.g., IV)

should, however, be used in addition to data from the extravascular route of interest to adequately assess the true extent of absorption of a toxicant.

5.5.1 Extent of Absorption

It is often useful to determine *how much of the drug* actually penetrates the membrane barrier (e.g., skin or GIT) and gets into the bloodstream. This is usually determined experimentally for oral and dermal routes of administration. The *area under the curve (AUC)* of the concentration-time profiles for oral or dermal routes is compared with the AUC for IV routes of administration. The AUC is determined by breaking the curve up into a series of trapezoids and summing all of the areas with the aid of an appropriate computer program (Figure 5.5).

The intravenous correction is *very* important if absolute bioavailability is desired. The ratio of these AUC values is absolute bioavailability, F

$$F = \frac{(\mathrm{AUC})_{\mathrm{route}}}{(\mathrm{AUC})_{\mathrm{IV}}}$$

The above relationship holds if the same doses were used with both routes; however, the bioavailability should be corrected if different doses were used.

$$F = \frac{\mathrm{AUC}_{\mathrm{route}} \times \mathrm{Dose}_{\mathrm{IV}}}{\mathrm{AUC}_{\mathrm{IV}} \times \mathrm{Dose}_{\mathrm{route}}}$$

Another technique is to monitor drug or toxicant excretion rather than blood concentrations, especially when blood or plasma concentrations are very low. Using the same equations, the AUC is now replaced by chemical concentrations in urine, feces, and expired air. Some chemicals are primarily excreted by the kidney, and

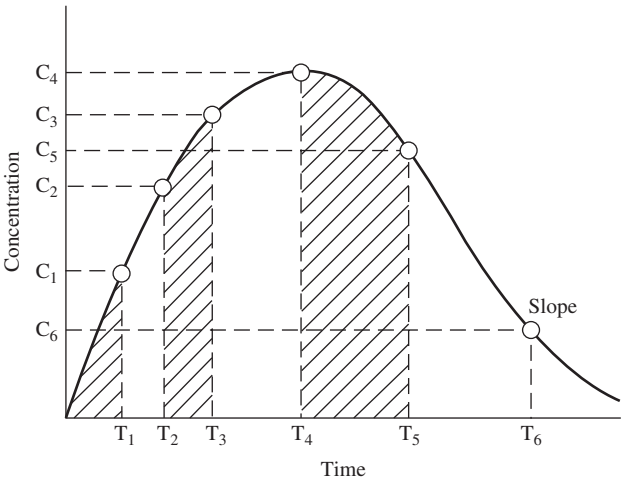


Figure 5.5 Plasma concentration time profile for oral exposure to a toxicant and depiction of AUCs determined by summation of trapezoids at several time periods.

urine data alone may be necessary. The rate and extent of absorption are clearly important for therapeutic and toxicological considerations. For example, different formulations of the same pesticide can change the absorption rate in skin or GIT, and not bioavailability, but can result in blood concentrations near the toxic dose. Also, different formulations can result in similar absorption rates, but different bioavailability.

5.5.2 Gastrointestinal Absorption

The GIT is a hollow tube (Figure 5.6a) lined by a layer of columnar cells, and is usually protected by mucous that offers minimal resistance to toxicant penetration. The distance from the outer membrane to the vasculature is about 40 μm, from which point further transport can easily occur. However, we should recognize that the cornified epithelium of the esophagus prevents absorption from this region of the GIT. Most of the absorption will therefore occur in the intestine (pH = 6) and, to some extent, in the stomach (pH = 1–3). Buccal and rectal absorption can occur in special circumstances. Note that secretions from the lachrymal duct, salivary gland, and nasal passages can enter the GIT via the buccal cavity. Therefore, following IV administration, a toxicant can enter the GIT if the drug is in these secretions.

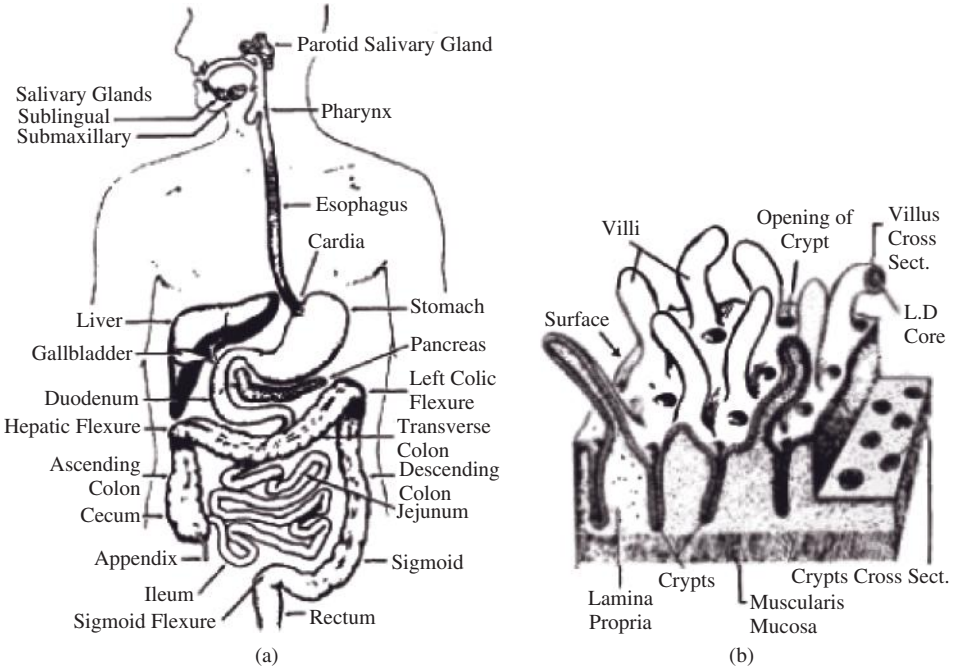


Figure 5.6 Schematic showing (a) alimentary canal and associated structures and (b) lining of the small intestine. From (a) Scholtelius and Scholtelius in *Textbook of Physiology*, St. Louis: Mosby, 1973, and (b) Ham and Cormack in *Histology*, 8th ed, Philadelphia: Lippincott, 1979.

The intestine can compensate the 2.5 log units difference between it and the stomach by the increased surface area in the small intestines. The presence of microvilli (Figure 5.6b) in the intestine is an increase of 600-fold in surface area compared to a hollow tube of comparable length. Note that there is no absorption, except for water, in the large intestine.

Most of the absorption in the GIT is by passive diffusion, except for nutrients; glucose, amino acids, and drugs that look like these substances are taken up by active transport. For toxicants with structural similarities to compounds normally taken up by these active transport mechanisms, entry is enhanced. For example, cobalt is absorbed by the same active transport mechanism that normally transports iron, and 5-bromouracil is absorbed by the pyrimidine transport system.

Very lipid-soluble toxicants and drugs, which are not miscible in the aqueous intestinal fluid, are presented as emulsions, and brought into solution through the action of detergent-like bile acids. The product of this mixing is large surface area micelles (hydrophobic interior) that deliver the lipids to the brush border of the intestine for diffusion across the membrane. As stated previously, the rate of passive transfer will be dependent on ionization and lipid solubility. Very strong bases (e.g., tubocurarine, succinylcholine) and strong acids are not readily absorbed in the GIT. These muscle relaxants therefore are given intravenously. The smaller the particle size of the toxicant, the greater the absorption, and a chemical must be in aqueous solution for it to be absorbed in the GIT. A feature of the GIT that seems to contradict basic assumptions of absorption is the penetration of certain very large molecules. Compounds such as bacterial endotoxins, large particles of azo dyes, and carcinogens are apparently absorbed by endocytotic mechanisms.

GIT motility has a significant effect on GIT absorption of a toxicant. For example, excessively rapid movement of gut contents can reduce absorption by reducing residence time in the GIT, while the presence of food in the stomach can delay the progress of drugs from the stomach to the small intestine where most of the absorption will occur. Increased splanchnic blood flow after a meal can result in absorption of several drugs (e.g., propranolol), but in hypovolemic states, absorption can be reduced.

Biotransformation in the GIT prior to absorption can have a significant impact on bioavailability of a toxicant. The resident bacterial population can metabolize drugs in the GIT. Because of microbial fermentation in the rumen of ruminants and large intestine and cecum of horses and rabbits, it is often difficult to compare drug absorption profiles with carnivores (e.g., dogs) and omnivores (e.g., humans, pigs). Acid hydrolysis of some compounds can also occur, and enzymes in the intestinal mucosa can also have an effect on oral bioavailability. If the toxicant survive these microbial and chemical reactions in the stomach and small intestine, it is absorbed in the GIT and carried by the hepatic portal vein to the liver, which is the major site of metabolism. Chapters 7, 8, and 9 will discuss liver metabolism of toxicants in more detail. In brief, this activity in the liver can result in detoxification and/or bioactivation. Some drugs and toxicant that are conjugated (e.g., glucuronidation) in the liver are excreted via the biliary system back into the GIT. Once secreted in bile by active transport and excreted from the bile duct into the small intestine, this conjugated toxicant can be subjected to microbial beta-glucuronidase activity which can result in regeneration of the parent toxicant that is more lipophilic than the conjugate. The toxicant can now be reabsorbed by the GIT, prolonging the presence

of the drug or toxicant in the systemic circulation. This is called *enterohepatic circulation*, which will be covered in greater detail in subsequent chapters.

5.5.3 Dermal Absorption

The skin is a complex multilayered tissue with a large surface area exposed to the environment. Skin anatomy, physiology, and biochemistry vary between species, within species, and even between anatomic sites within an individual animal or human. Logically, these biological factors alone can influence dermal absorption. However, what is consistent is that the outer layer, the *stratum corneum (SC)*, can provide as much as 80% of the resistance to absorption to most ions as well as aqueous solutions. However, the skin is permeable to many toxicants, and dermal exposure to agricultural pesticides and industrial solvent can result in severe systemic toxicity.

The anatomy of the skin is depicted in the schematic diagram below (Figure 5.7a). In mammalian skin there are really three distinct layers which are the epidermis, dermis, and hypodermis or subcutaneous fat layer. Human skin is 3 mm thick, but it is the epidermis which is only 0.1–0.8 mm that provides the greatest resistance to toxicant penetration. The five layers of the epidermis starting from the outside are the SC, stratum lucidum, stratum granulosum, stratum spinosum, and stratum basale. The basal cells of the epidermis proliferate and differentiate as they migrate outward toward the surface of the skin. It requires about 2~28 days for cells to migrate from the basal layer to the SC, where they are eventually sloughed off. These dead, keratinized cells are, however, very water absorbant (hydrophilic), a property that keeps the skin soft and supple. Sebum, a natural oil covering the skin, functions in maintaining the water-holding ability of the epidermis. The SC is the primary barrier to penetration, and it consists primarily of these dead keratin-filled keratinocytes embedded in an extracellular lipid matrix. The lipids are primarily sterols, other

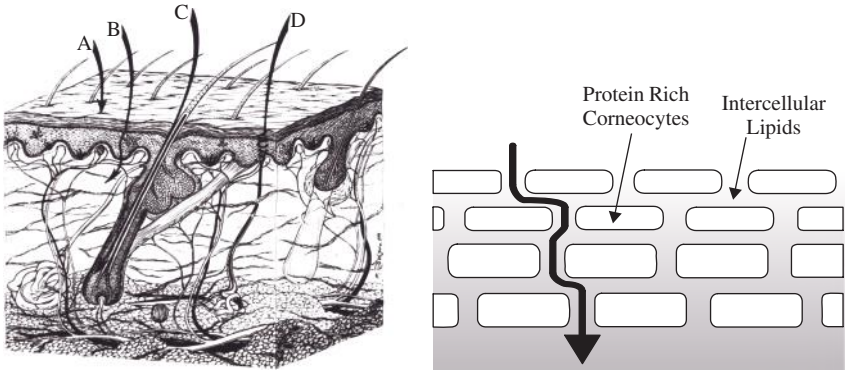


Figure 5.7 (a) Schematic diagram of the microstructure of mammalian skin and potential pathways for absorption by (A) intercellular, (B) transcellular, (C) transfollicular, or (D) sweat pore routes. (b) "Brick and Mortar" model of the stratum corneum depicting intercellular pathway (i.e., route A) between keratinocytes through the lipid domain of the stratum corneum.

neutral lipids, and ceramides. This association between lipids and dead keratinized cells is often referred to as the "Brick and Mortar" model as depicted in Figure 5.7b and is often used to simplify the composition of the SC that are integral to chemical transport through skin.

A number of appendages are associated with the skin, including hair follicles, sebaceous glands, eccrine and apocrine sweat glands, and nails. Recently, it was found that removal of the SC does not allow complete absorption; thus, it is apparent that some role, although of lesser importance, is played by other parts of the skin. The dermis and subcutaneous areas of the skin are less important in influencing penetration, and once a toxicant has penetrated the epidermis, the other layers are traversed rather easily. The dermis is highly vascular, a characteristic that provides maximal opportunity for further transport once molecules have gained entry through the epidermis or through skin appendages. Most of the systemic absorption occurs at the capillary loops located at the epidermis–dermis junction. The blood supply of the dermis is under neural and humoral influences whose temperature-regulating functions could thus affect penetration and distribution of toxicants. Vasoactive drugs or environmental temperature can also influence absorption by altering blood flow to these capillaries. The subcutaneous layer of the skin is highly lipid in nature and serves as a shock absorber, an insulator, and a reserve depot of energy. The pH of the skin varies between 4 and 7 and is markedly affected by hydration.

Cutaneous biotransformation is mostly associated with the stratum basale layer where there can be Phase I and Phase II metabolism. However, the skin is not very efficient, compared to the liver. The epidermal layer accounts for the major portion of biochemical transformations in the skin, although the total skin activity is low (2–6% that of the liver). If activity is based on epidermis alone, however, that layer is as active as the liver or, in the case of certain toxicants, several times more active. For some chemicals, metabolism can influence absorption, and transdermal delivery systems of drugs utilize this activity. For example, prodrug such as lipid esters are applied topically, and cutaneous esterases liberate the free drug. These basal cells and extracellular esterases have been shown to be involved in detoxification of several pesticides and bioactivation of carcinogens such as benzo(a)pyrene. For rapidly penetrating substances, metabolism by the skin is not presently considered to be of major significance; however, skin may have an important first-pass metabolic function, especially for compounds that are absorbed slowly.

The *intercellular pathway* is now accepted as the major pathway for absorption. Recall that the rate of penetration is often correlated with the partition coefficient. In fact, this is a very tortuous pathway, and the "h" (skin thickness) in Fick's First Law of Diffusion is really 10× the measured distance. By placing a solvent (e.g., ether, acetone) on the surface or a tape stripping the surface, the SC is removed, and absorption can be significantly increased by removing this outer barrier. This may not be the case for very lipophilic chemical. This is because the viable epidermis and dermis are regarded as aqueous layers compared to the SC. Note that the more lipophilic the drug, the more likely it will form depot in the SC, and slowly absorbed over time, leading to a prolonged half-life.

The *transcellular pathway* has been discredited as a major pathway, although some polar substances can penetrate the outer surface of the protein filaments of hydrated SC. The *transfollicular pathway* is really an invagination of the epidermis

into the dermis, and the chemical still has to penetrate the epidermis to be absorbed into the bloodstream. This is also regarded as a *minor* route. *Sweat pores* are not lined with the SC layer, but the holes are small, and this route is still considered a minor route for chemical absorption. In general, the epidermal surface is 100–1000 times the surface area of skin appendages, and it is likely that only very small and/or polar molecules penetrate the skin via these appendages.

Variations in areas of the body cause appreciable differences in penetration of toxicants. The rate of penetration is in the following order:

Scrotal > Forehead > Axilla > = Scalp > Back = Abdomen > Palm and Plantar

The palmar and plantar regions are highly cornified and are 100–400 times thicker than other regions of the body. Note that there are differences in blood flow and, to a lesser extent, hair density, which may influence absorption of more polar toxicants.

Formulation additives used in topical drug or pesticide formulations can alter the SC barrier. Surfactants are least likely to be absorbed, but they can alter the lipid pathway by fluidization and delipidization of lipids, and proteins within the keratinocytes can become denatured. This is mostly likely associated with formulations containing anionic surfactants than nonionic surfactants. Similar effects can be observed with solvents. Solvents can partition into the intercelluar lipids, thereby changing membrane lipophilicity and barrier properties in the following order: ether/acetone > DMSO (dimethyl sulfoxide) > ethanol > water. Higher alcohols and oils do not damage the skin, but they can act as a depot for lipophilic drugs on the skin surface. The presence of water in several of these formulations can hydrate the skin. Skin occlusion with fabric or transdermal patches, creams, and ointments can increase epidermal hydration which can increase permeability.

The reader should be aware of the animal model being used to estimate dermal absorption of toxicants in humans. For many toxicants, direct extrapolation from a rodent species to human is not feasible. This is because of differences in skin thickness, hair density, lipid composition, and blood flow. Human skin is the least permeable compared to skin from rats, mice, and rabbits. Pig skin is, however, more analogous to human skin anatomically and physiologically, and pig skin is usually predictive of dermal absorption of most drugs and pesticides in human skin. Human skin is the best model, followed by skin from pigs, primates, and hairless guinea pigs, and rats, mice, and rabbits. In preliminary testing of a transdermal drug, if the drug does not cross rabbit or mice skin, it is very unlikely that it will cross human skin. There are several *in vitro* experimental techniques such as static diffusion (Franz) cells or flow-through diffusion (Bronough) cells. There are several *ex vivo* methods, including the isolated perfused porcine skin flap (IPPSF), which with its intact microvasculature makes this model unique. *In vivo* methods are the golden standard, but they are very expensive, and there are human ethical and animal rights issues to be considered.

There are other factors that can influence dermal absorption, and these can include environmental factors such as air flow, temperature, and humidity. Preexisting skin disease and inflammation should also be considered. The topical dose this is usually expressed in per unit surface area can vary, and relative absorption usually decreases with increase in dose.

5.5.4 Respiratory Penetration

As observed with the GIT and skin, the respiratory tract can be regarded as an external surface. However, the lungs, where gas/vapor absorption occurs, are preceded by protective structures (e.g., nose, mouth, pharynx, trachea, and bronchus) which can reduce the toxicity of airborne substances, especially particles. There is little or no absorption in these structures, and residual volume can occur in these sites. However, cells lining the respiratory tract may absorb agents which can cause a toxicological response. The absorption site which is the alveoli–capillary membrane is very thin (0.4–1.5 μm). The membranes to cross from the alveolar air space to the blood will include *Type I cells to basement membrane to capillary endothelial cells* (Figure 5.8). This short distance allows for rapid exchange of gases/vapors. The analogous absorption distance in skin is 100–200 μm, and in GIT it is about 30 μm. There is also a large surface area (50 times the area of skin) available for absorption as well as significant blood flow which makes it possible to achieve rapid adjustments in plasma concentration. Gases/vapors must get into solution in the thin fluid film in the alveoli for systemic absorption to occur. Please note that doses are often a measurement of partial pressures, which is important for gases/vapors.

The process of respiration involves the movement and exchange of air through several interrelated passages, including the nose, mouth, pharynx, trachea, bronchi, and successively smaller airways terminating in the alveoli, where gaseous exchange occurs. These alveoli consist mainly of type I pneumocytes that represent 40% of all cells but cover >90% of surface area, and type II pneumocytes that represent 60% of all cells but cover 5% of surface area. Macrophages make up 90% of cells in alveolar space. The amount of air retained in the lung despite maximum expiratory effort is known as the residual volume. Thus, toxicants in the respiratory air may not be cleared immediately because of slow release from the residual volume.

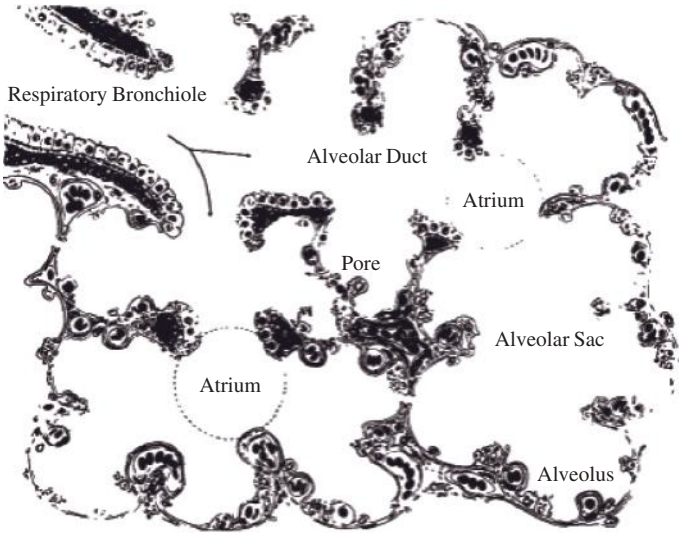


Figure 5.8 Schematic representation of the respiratory unit of the lung. From Bloom and Fawcett, *A Textbook of Histology*, Philadelphia: Saunders, 1975.

The rate of entry of vapor-phase toxicants is controlled by the alveolar ventilation rate, with the toxicant being presented to the alveoli in an interrupted fashion approximately 20 times/min.

Airborne toxicants can be simplified to two general types of compounds, namely, gases and aerosols. Compounds such as gases, solvents, and vapors are subject to gas laws and are carried easily to alveolar air. Much of our understanding of xenobiotic behavior is with anesthetics. Compounds such as aerosols, particulates, and fumes are not subject to gas laws because they are in particulate form.

The transfer of gas from alveoli to blood is the actual absorption process. Among the most important factor that will determine rate and extent of absorption of a gas in lungs is the solubility of that gas. Therefore, it is not membrane partition coefficient that necessarily affects absorption as has been described for skin and GIT membranes, but rather the blood:gas partition coefficient or blood/gas solubility of the gas. A high blood:gas partition coefficient indicates that the blood can hold a large amount of gas. Keeping in mind that it is the *partial pressure* at equilibrium that is important, and the more soluble the gas is in blood, the greater the amount of gas that is needed to dissolve in the blood to raise the partial pressure or tension in blood. For example, anesthetics such as diethyl ether and methoxyflurane which are soluble (Table 5.3), require a longer period for this partial pressure to be realized. Again, the aim is to generate the same tension in blood as in inspired air. Because these gases are very soluble, detoxification is a prolonged process. In practice, anesthetic induction is slower, and so is recovery from anesthesia. For less soluble gases (e.g., NO, isoflurane, halothane), the partial pressure or tension in blood can be raised a lot easier to that of inspired gases, and detoxification takes less time than those gases that are more soluble.

There are several other important factors that can determine whether the gas will be absorbed in blood and then transported from the blood to the perfused tissue. The concentration of the gas in inspired air influences gas tension, and partial pressure can be increased by overventilation. In gas anesthesiology, we know that the effects of respiratory rate on speed of induction are transient for gases that have low solubility in blood and tissues, but there is a significant effect for agents that are more soluble and take a longer time for gas tensions to equilibrate. In determining how much of the gas is absorbed, it is important to consider what fraction of the lung is ventillated and what fraction is perfused. However, one should be aware that due to diseased lungs, there can be differences between these fractions. For example, decreased perfusion will decrease absorption although there is agent in the alveoli and vice versa. The rate at which a gas passes into tissues is also

TABLE 5.3 Blood:Gas Partition Coefficient of Various Anesthetic Gases

Agent	Blood:Gas Partition Coefficient in Humans
Methoxyflorane	13.0–15.0
Halothane	2.3–2.5
Isoflurane	1.4
NO	0.5

dependent on gas solubility in the tissues, rate of delivery of the gas to tissues, and partial pressures of gas in arterial blood and tissues. After uptake of the gas, the blood takes the gas to other tissues. The mixed venous blood returned to the lungs progressively begins to have more of the gas, and differences between arterial (or alveolar) and mixed venous gas tensions decrease continuously.

While gases are more likely to travel freely through the entire respiratory tract to the alveoli, passage of aerosols and particles will be affected by the upper respiratory tract which can act as an effective filter to prevent particulate matter from reaching the alveoli. Mucous traps particles to prevent entry to alveoli, and the mucociliary apparatus in the trachea traps and pushes particles up the trachea to the esophagus where they are swallowed and possibly absorbed in the GIT.

In addition to upper pathway clearance, lung phagocytosis is very active in both upper and lower pathways of the respiratory tract and may be coupled to the mucus cilia. Phagocytes may also direct engulfed toxicants into the lymph, where the toxicants may be stored for long periods. If not phagocytized, particles $\leq 1\,\mu m$ may penetrate to the alveolar portion of the lung. Some particles do not desquamate but instead form a dust node in association with a developing network of reticular fibers. Overall, removal of alveolar particles is markedly slower than that achieved by the directed upper pulmonary mechanisms. This defense mechanism is not important for vapors/gases. The efficiency of the system is illustrated by the fact than on average, only 100 g of coal dust is found postmortem in the lungs of coal miners although they inhale approximately 6000 g during their lifetime.

The deposition site of particles in the respiratory tract is primarily dependent on the *aerodynamic behavior* of the particles. The particle size, density, shape, hygroscopicity, breathing pattern, and lung airway structure are also important factors influencing the deposition site and efficiency. The *aerodynamic-equivalent diameter* (for particle $>0.5\,\mu m$) and *diffusion-equivalent diameter* ($<0.5\,\mu m$) are *defined* as the diameter of a *unit density sphere* having the same *settling velocity* (aerodynamic-equivalent) or the same *diffusion rate* (diffusion-equivalent) as the *irregularly shaped particle of interest*. Deposition occurs by five possible mechanisms: electrostatic precipitation, interception, impaction, sedimentation, impaction, and diffusion. The latter three are most important. Only particle sizes less than 10–20 μm that get past the nasopharyngeal regions and reach the alveoli are of medical concern. As particle size decreases below 0.5 μm, the aerosol begins to behave like a gas (Figure 5.9). For these particles, diffusion becomes the primary mechanism of deposition in the respiratory tract before it finally reaches the alveoli.

5.6 TOXICANT DISTRIBUTION

5.6.1 Physicochemical Properties and Protein Binding

Absorption of toxicants into the blood needs to be high enough so that it will have a significant effect at the site of action in other areas of the body. The distribution process that takes the absorbed drug to other tissues is dependent on various physiological and physicochemical properties of the drug. This process is therefore a reversible movement of the toxicant between blood and tissues or between extracellular and intracellular compartments. There are, however, several complicating

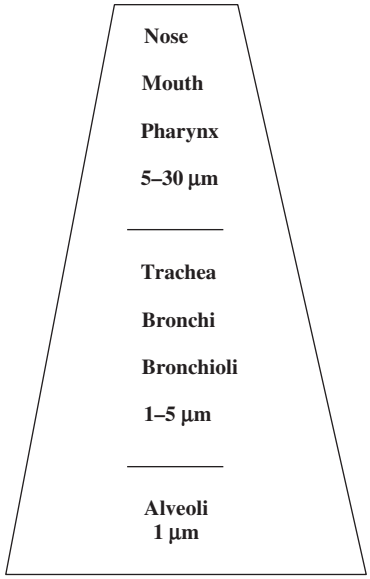


Figure 5.9 Schematic illustration of the regions where absorption may occur in the respiratory tract.

factors that can influence the distribution of a toxicant. For example, *perfusion* of tissues is an important physiological process, as some organs are better perfused (e.g., heart, brain) than others (e.g., fat). There can also be significant *protein binding* that affects delivery of drug to tissues. To further complicate the issue, elimination processes such as excretion and biotransformation (discussed at a later time) are occurring simultaneously to remove the toxicant from the blood as well as the target site.

There are several physiochemical properties of the toxicant that can influence its distribution. These include lipid solubility, pKa, and molecular weight that have been described earlier in this chapter (Section 5.4) and will not be described here. For many toxicants, distribution from the blood to tissues is by simple diffusion down a concentration gradient, and the absorption principles described earlier also apply here. The concentration gradient will be influenced by the partition coefficient or rather the ratio of toxicant concentrations in blood and tissue. Tissue mass and blood flow will also have a significant effect on distribution. For example, a large muscle mass can result in increased distribution to muscle, while limited blood flow to fat or bone tissue can limit distribution. The ratio of blood flow to tissue mass is also a useful indicator of how well the tissue is perfused. The well-perfused tissues include liver, kidney, and brain, and the low-perfused tissues include fat and bone where there is slow elimination from these tissues. Initial distribution to well-perfused tissues (e.g., heart, brain) occurs within the first few minutes, while delivery of drug to other tissues (e.g., fat, skin) is slower.

If the affinity for the target tissue is high, then the chemical will accumulate or form a depot. The advantage here is that if this were a drug, there is no need to load up the central compartment to get to the active site. However, if the reservoir

for the drug has a large capacity and fills rapidly, it so alters the distribution of the drug that larger quantities of the drug are required initially to provide a therapeutic effective concentration at the target organ. If this were a toxicant, this may be an advantageous feature as toxicant levels at the target site will be reduced. In general, lipid-insoluble toxicants stay mainly in the plasma and interstitial fluids, while lipid-soluble toxicants reach all compartments and may accumulate in fat. There are numerous examples of cellular reservoirs for toxicants and drugs to distribute. Tetracycline antibiotics have a high affinity for calcium-rich tissues in the body. The bone can become a reservoir for the slow release of chemicals such as lead, and effects may be chronic or there may be acute toxicity if the toxicant is suddenly released or mobilized from these depots. The antimalaria drug quinacrine accumulates due to reversible intracellular binding, and the concentration in the liver can be several thousand times that of plasma. Another antimalaria drug, chloroquine, has a high affinity for melanin, and this drug can be taken up by tissues such as the retina that are rich in melanin granules and can cause retinitis with a drug overdose. Lipophilic pesticides and toxicants (e.g., polychlorinated biphenyls [PCBs]) and lipid-soluble gases can be expected to accumulate in high concentration in fat tissue.

There are unique anatomical barriers that can limit distribution of toxicants. A classical example of such a unique barrier is the BBB which can limit the distribution of toxicants into the central nervous system (CNS) and cerebrospinal fluid (CSF). There are three main processes or structures that keep drug or toxicant concentrations low in this region: (1) the BBB, which consists of capillary endothelial tight junctions and glial cells, surround the precapillaries, reduce filtration, and requires that the toxicant cross several membranes in order to get to the CSF. Please note that endothelial cells in other organs can have intercellular pores and pinocytotic vesicles; (2) active transport systems in the choroid plexus allow for transport of organic acids and bases from the CSF into blood; and (3) the continuous process of *CSF* production in the ventricles and venous drainage continuously dilutes toxicant or drug concentrations. Disease processes such as meningitis can disrupt this barrier and can allow for penetration of antibiotics (e.g., aminoglycosides) that would not otherwise readily cross this barrier in a healthy individual. Other tissue/blood barriers include prostate/blood, testicles/blood, and globe of eye/blood; inflammation or infection can also increase permeability of these barriers. Toxicants can cross the placenta primarily by simple diffusion, and this is most easily accomplished if the toxicants are lipid soluble (i.e., nonionized weak acids or bases). The view that the placenta is a barrier to drugs and toxicants is inaccurate. The fetus is, at least to some extent, exposed to essentially all drugs even if those with low lipid solubility are taken by the mother.

As indicated earlier, the circulatory system and components in the bloodstream are primarily responsible for transport of toxicants to target tissues or reservoirs. Erythrocytes and lymph can play important roles in transport of toxicants, but compared to plasma proteins, their role in toxicant distribution is relatively minor for most toxicants. Plasma protein binding can affect distribution because only the unbound toxicant is free or available to diffuse across the cell membranes. The toxicant–protein binding reaction is reversible and obeys the laws of mass action:

$$\underset{\text{(free)}}{\text{Toxicant}} + \text{Protein} \underset{k_2}{\overset{k_1}{\Leftrightarrow}} \underset{\text{(bound)}}{\text{Toxicant} - \text{Protein}}$$

Usually, the ratio of unbound plasma concentration (Cu) of the toxicant to total toxicant concentration in plasma (C) is the fraction of drug unbound, *fu*, that is,

$$\boldsymbol{fu} = \mathrm{Cu/C}$$

The constants k_1 and k_2 are the specific rate constants for association and dissociation, respectively. The association constant K_a will be the ratio k_1/k_2, and conversely, the dissociation constant, K_d will be k_2/k_1. The constants and parameters are often used to describe and more importantly compare the relative affinity of xenobiotics for plasma proteins.

There are many circulating proteins, but those involved in binding xenobiotics include albumin, α_1-acid glycoprotein, lipoproteins, and globulins. Because many toxicants are lipophilic, they are more likely to bind to plasma α- and β-lipoproteins. There are mainly three classes of lipoproteins, namely, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and very low-density lipoprotein (VLDL). Iron and copper are known to interact strongly with the metal-binding globulins transferin and ceruloplasmin, respectively. Acidic drugs bind primarily to albumin, and basic drugs are bound primarily to α_1-acid glycoprotein and β-globulin. Albumin makes up 50% of total plasma proteins, and it reacts with a wide variety of drugs and toxicants. The α_1-acid glycoprotein does not have as many binding sites as albumin, but has one high-affinity binding site. The amount of toxicant drug that is bound depends on free drug concentration, and its affinity for the binding sites, and protein concentration. Plasma protein binding is nonselective, and therefore, toxicants and drugs with similar physicochemical characteristics can compete with each other and endogenous substances for binding sites. Binding to these proteins does not necessarily prevent the toxicant from reaching the site of action, but slows the rate at which the toxicant reaches a concentration sufficient to produce a toxicological effect. Again, this is related to what fraction of the toxicant is free or unbound (*fu*).

Toxicants complex with proteins by various mechanisms. Covalent binding may have a pronounced effect on an organism due to the modification of an essential molecule, but such binding is usually a very minor portion of the total dose. Because covalently bound molecules dissociate very slowly, if at all, they are not considered further in this discussion. However, we should recognize that these interactions are often associated with carcinogenic metabolites. Noncovalent binding is of primary importance to distribution because the toxicant or ligand can dissociate more readily than it can in covalent binding. In rare cases, the noncovalent bond may be so stable that the toxicant remains bound for weeks or months, and for all practical purposes, the bond is equivalent to a covalent one. Types of interactions that lead to noncovalent binding under the proper physiological conditions include ionic binding, hydrogen bonding, van der Waals forces, and hydrophobic interactions. There are, however, some transition metals that have high association constants and dissociation is slow.

We know more about ligand–protein interactions today because of the numerous protein binding studies performed with drugs. The major difference between drugs and most toxicants is the frequent ionizability and high water solubility of drugs as compared with the nonionizability and high lipid solubility of many toxicants. Thus, experience with drugs forms an important background, but one that may not always be relevant to other potentially toxic compounds.

TABLE 5.4 Distribution of Insecticides into Albumin and Lipoproteins

Insecticide	Percent Distribution of Bound Insecticide			
	Percent Bound	Albumin	LOL	HDL
DDT	99.9	35	35	30
Deildrin	99.9	12	50	38
Lindane	98.0	37	38	25
Parathion	98.7	67	21	12
Diazinon	96.6	55	31	14
Carbaryl	97.4	99	<1	<1
Carbofuran	73.6	97	1	2
Aldicarb	30.0	94	2	4
Nicotine	25.0	94	2	4

Source: Adapted from Maliwal, B. P. and F. E. Guthrie. *Chem. Biol. Interact.* **35**:177–188, 1981.
LOL, low-density lipoprotein; HDL, high-density lipoprotein.

Variation in chemical and physical features can affect binding to plasma constituents. Table 5.4 shows the results of binding studies with a group of insecticides with greatly differing water and lipid solubilities. The affinity for albumin and lipoproteins is inversely related to water solubility, although the relation may be imperfect. Chlorinated hydrocarbons bind strongly to albumin but even more strongly to lipoproteins. Strongly lipophilic organophosphates bind to both protein groups, whereas more water-soluble compounds bind primarily to albumin. The most water-soluble compounds appear to be transported primarily in the aqueous phase. Chlordecone has partitioning characteristics that cause it to bind in the liver whereas dichlorodiphenyldichloroethylene (DDE), the metabolite of dichlorodiphenyltrichloroethane (DDT), partitions into fatty depots. Thus, the toxicological implications for these two compounds may be quite different.

Although highly specific (high-affinity, low-capacity) binding is more common with drugs, examples of specific binding for toxicants seem less common. It seems probable that low-affinity, high-capacity binding describes most cases of toxicant binding. The number of binding sites can only be estimated, often with considerable error, because of the nonspecific nature of the interaction. The number of ligand or toxicant molecules bound per protein molecule, and the maximum number of binding sites, n, define the definitive capacity of the protein. Another consideration is the binding affinity K_{binding} (or $1/K_{\text{diss}}$). If the protein has only one binding site for the toxicant, a single value, K_{binding}, describes the strength of the interaction. Usually more than one binding site is present, each site having its intrinsic binding constant, $k_1, k_2, \ldots k_n$. Rarely does one find a case where $k_1 = k_2 = \ldots = k_n$, where a single value would describe the affinity constant at all sites. This is especially true when hydrophobic binding and van der Waals forces contribute to nonspecific, low-affinity binding. Obviously, the chemical nature of the binding site is of critical importance in determining binding. The three-dimensional molecular structure of the binding site, the environment of the protein, the general location in the overall protein molecule, and allosteric effects are all factors that influence binding. Studies with toxicants, and even more extensive studies with drugs, provided an adequate elucidation of these factors. Binding appears to be too complex a phenomenon to be accurately described by any one set of equations.

There are many methods for analyzing binding, but equilibrium dialysis is the most extensively used. Again, the focus of these studies is to determine the percentage of toxicant bound, the number of binding sites (n), and the affinity constant (Ka). The examples presented here are greatly simplified to avoid the undue confusion engendered by a very complex subject.

Toxicant–protein complexes that utilize relatively weak bonds (energies of the order of hydrogen bonds or less) readily associate and dissociate at physiological temperatures, and the law of mass action applies to the thermodynamic equilibrium.

$$K_{\text{binding}} = \frac{[TP]}{[T][P]} = \frac{1}{K_{\text{diss}}}$$

where K_{binding} is the equilibrium constant for association, $[TP]$ is the molar concentration of toxicant–protein complex, $[T]$ is the molar concentration of free toxicant, and $[P]$ is the molar concentration of free protein. This equation does not describe the binding site(s) or the binding affinity. To incorporate these parameters and estimate the extent of binding, double-reciprocal plots $1/[TP]$ *versus* $1/[T]$ may be used to test the specificity of binding. The $1/[TP]$ term can also be interpreted as moles of albumin per moles of toxicant. The slope of the straight line equals 1/nKa, and the intercept of this line with the x-axis equals –Ka. Regression lines passing through the origin imply infinite binding, and the validity of calculating an affinity constant under these circumstances is questionable. Figure 5.10a illustrates one such case with four pesticides, and the insert illustrates the low-affinity, "unsaturable" nature of binding in this example.

The two classes of toxicant–protein interactions encountered may be defined as (1) specific, high affinity, low capacity; and (2) nonspecific, low affinity, high capacity. The term high affinity implies an affinity constant (K_{binding}) of the order of 10^8/M, whereas low affinity implies concentrations of 10^4/M. Nonspecific, low-affinity binding is probably most characteristic of nonpolar compounds, although most cases are not as extreme as that shown in Figure 5.10.

An alternative and well-accepted treatment for binding studies is the Scatchard equation especially in situations of high-affinity binding:

$$v = \frac{nk[T]}{1 + k[T]}$$

which is simplified for graphic estimates to

$$\frac{v}{[T]} = k(n - v)$$

where v is the moles of ligand (toxicant) bound per mole of protein, $[T]$ is the concentration of free toxicant, k is the intrinsic affinity constant, and n is the number of sites exhibiting such affinity. When v $[T]$ is plotted against v, a straight line is obtained if only one class of binding sites is evident. The slope is $-k$ and the intercept on the v axis becomes n (number of binding sites). If more than one class of sites

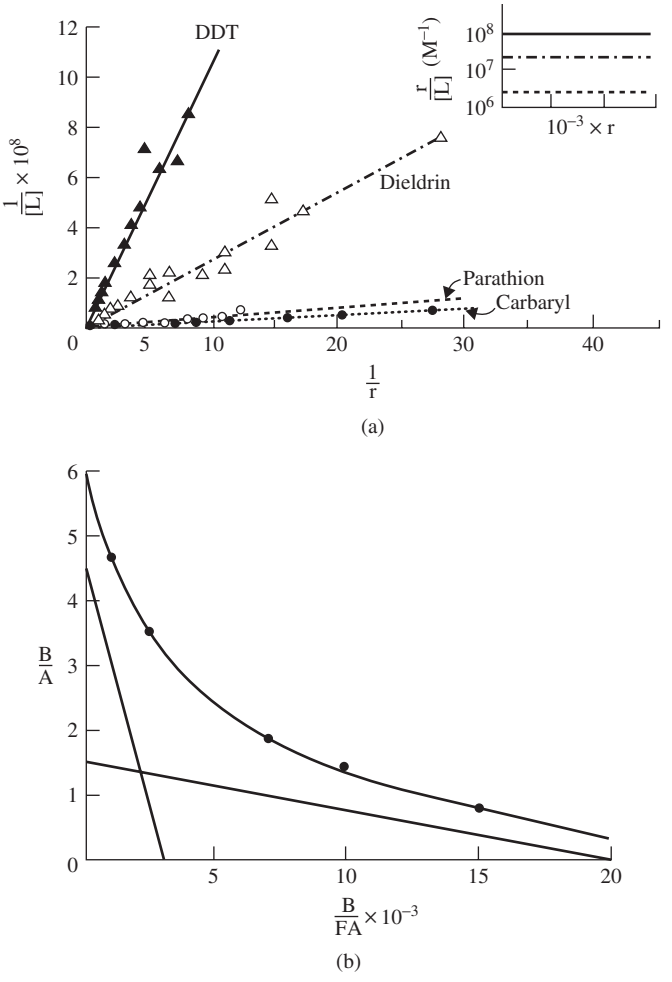


Figure 5.10 Binding of toxicants to blood proteins: (a) Double-reciprocal plot of binding of rat serum lipoprotein fraction with four insecticides. Insert illustrates magnitude of differences in slope with Scatchard plot. (b) Scatchard plot of binding of salicylate to human serum proteins. From (a) Skalsky and Guthrie, *Pest. Biochem. Physiol.* **7**:289, 1977 and (b) Moran and Walker, *Biochem. Pharmacol.* **17**:153, 1968.

occurs (probably the most common situation for toxicants), a curve is obtained from which the constants may be obtained. This is illustrated in Figure 5.10b, for which the data show not one but two species of binding sites: one with low capacity but high affinity and another with about three times the capacity but less affinity. Commonly used computer programs usually solve such data by determining one line for the specific binding and one line for nonspecific binding, the latter being an average of many possible solutions.

When hydrophobic binding of lipid toxicants occurs, as is the case for many environmental contaminants, binding is probably not limited to a single type of plasma protein. For example, the binding of the chlorinated hydrocarbon DDT is

strongest for lipoproteins and albumin, but other proteins account for a significant part of overall transport. Similar results have been observed for several compounds with a range of physiochemical properties.

The presence of another toxicant and/or drug that can bind at the same site can also increase the amount of free or unbound drug. This is an example of drug interaction, which can have serious toxicological or pharmacological consequences. *In general*, when bound concentrations are less than 90% of the total plasma concentrations, plasma protein binding has little clinical importance. Plasma protein binding *becomes important when it is >90%*. For example, if a toxicant is 99% bound to plasma proteins, then 1% is free, but if there is toxicant interaction (e.g., competitive binding) that results in 94% bound and 6% is now free. Note that because of this interaction, the amount of available toxicant to cause a toxicological response *has increased sixfold.* Such a scenario may result in severe acute toxicity. Extensive plasma protein binding can influence renal clearance if glomerular filtration is the major elimination process in the kidney, but not if it is by active secretion in the kidney. Binding can also affect drug clearance if the extraction ratio (ER) in the liver is low, but not if the ER is high for that toxicant. Plasma protein binding can vary between and within chemical classes, and it is also species specific. For example, humans tend to bind acidic drugs more extensively than do other species.

There are several other variables that can alter plasma protein concentrations. These include malnutrition, pregnancy, cancer, liver abscess, renal disease, and age that can reduce serum albumin. Furthermore, α_1-glycoprotein concentrations can increase with age, inflammation, infections, obesity, renal failure, and stress. Small changes in body temperature or changes in acid–base balance may alter chemical protein binding characteristics. Although termination of drug or toxicant effect is usually by biotransformation and excretion, it may also be associated with redistribution from its site of action into other tissues. The classical example of this is when highly lipid-soluble drugs or toxicants that act on the brain or cardiovascular system are administered by IV or by inhalation.

5.6.2 V_d

Usually, after a toxicant or drug is absorbed, it can be distributed into various physiologic fluid compartments. The total body water represents 57% of total body mass (0.57 L/kg). The plasma, interstitial fluid, extracellular fluid, and intracellular fluid represent about 5, 17, 22, and 35% body weight, respectively (Table 5.5). The extracellular fluid comprises the blood plasma, interstitial fluid, and lymph. Intracellular

TABLE 5.5 Examples of Several Compartment Fluid Volumes

Compartment	Volume of Distribution Liters/Kilogram Body Weight (Liters/70 kg Body Weight)
Plasma	0.05 (3.5 L)
Interstitial fluid	0.18 (12.6 L)
Extracellular fluid	0.23 (16.1 L)
Intracellular fluid	0.35 (24.5 L)
Total body water	0.55 (39 L)

fluid includes the sum of fluid contents of all cells in the body. There is also transcellular fluid which represents 2% body weight, and this includes cerebrospinal, intraocular, peritoneal, pleural, and synovial fluids, and digestive secretions. Fat is about 20% body weight, while the GIT contents in monogastrics make up 1% body weight, and in ruminants it can constitute 15% body weight.

It is sometimes useful to quantitate how well a drug or toxicant is distributed into these various fluid compartments, and in this context, the apparent V_d can be a useful parameter. The apparent V_d is defined as the volume of fluid required to contain the total amount, (A), of drug in the body at the same concentration as that present in plasma, C_p,

$$V_d = A/C_p$$

In general, the V_d for a drug is, to some extent, descriptive of its distribution pattern in the body. For example, drugs or toxicants with relatively small V_d values may be confined to the plasma as diffusion across the capillary wall is limited. There are other toxicants that have a slightly larger V_d (e.g., 0.23 L/kg), and these toxicants may be distributed in the extracellular compartment. This includes many polar compounds (e.g., tubocurarine, gentamicin; V_d = 0.2–0.4 L/kg). These toxicants cannot readily enter cells because of their low lipid solubility. If the V_d for some of these toxicants is in excess of the theoretical value, this may be due to limited degree of penetration into cells or from the extravascular compartment. Finally, there are many toxicants that are distributed throughout the body water ($V_d \geq 0.55$ L/kg), and may have V_d values much greater than that for total body water. This distribution is achieved by relatively lipid-soluble toxicants and drugs that readily cross cell membranes (e.g., ethanol, diazepam; V_d = 1–2 L/kg). Binding of the toxicant anywhere outside of the plasma compartment, as well as partitioning into body fat can increase V_d beyond the absolute value for total body water. In general, toxicants with a large V_d can even reach the brain, fetus, and other transcellular compartments. In general, toxicants with large V_d are a consequence of extensive tissue binding. The reader should be aware that we are talking about tissue binding, and not plasma protein binding where distribution is limited to plasma for obvious reasons.

The fraction of toxicant located in plasma is dependent on whether a toxicant binds to both plasma and tissue components. Plasma binding can be measured directly, but not tissue binding. It can, however, be inferred from the following relationship:

$$\text{Amount in Body} = \text{Amount in Plasma} + \text{Amount Outside Plasma}$$

$$V_d \times C = V_p \times C + V_{TW} \times C_{TW}$$

Where V_d = apparent volume of distribution; V_p = volume of plasma; V_{TW} = apparent volume of tissue; and C_{TW} = tissue concentration. If the above equation is divided by C, it now becomes:

$$V_d = V_p + V_{TW} \times \frac{C_{TW}}{C}$$

Recall, $fu = \mathrm{Cu}/C$ occurs with plasma. Then also fraction unbound in tissues, $fu_T = \mathrm{Cu_T}/\mathrm{C_{TW}}$.

Assuming at equilibrium that unbound concentration in tissue and plasma are equal, then the ratio of fu/fu_T replaces C_{TW}/C, and the V_{d} can be determined as follows:

$$V_{\mathrm{d}} = V_{\mathrm{p}} + V_{\mathrm{TW}} \times (fu/fu_T)$$

One can now predict what happens to V_{d} when fu or fu_T changes as a result of physiological or disease processes in the body that can change plasma and/or tissue protein concentrations. For example, V_{d} can increase with increased unbound toxicant in plasma or with a decrease in unbound toxicant tissue concentrations. The above equation explains why, because of both plasma and tissue binding, some V_{d} values rarely correspond to a real volume such as plasma volume, extracellular space, or total body water. Finally, interspecies differences in V_{d} values can be due to differences in body composition of body fat and protein, organ size, and blood flow as alluded to earlier in this section of this chapter. The reader should also be aware that in addition to V_{d}, there are other volumes of distribution that can be obtained from pharmacokinetic analysis of a given data set. These include the volume of distribution at steady state ($V_{\mathrm{d_{ss}}}$), volume of the central compartment (V_{c}), and the V_{d} that is operative over the elimination phase ($V_{\mathrm{d_{area}}}$). The reader is advised to consult other relevant text for a more detailed description of these parameters and when it is appropriate to use these parameters.

5.7 TOXICOKINETICS

The explanation of the pharmacokinetics or toxicokinetics involved in absorption, distribution, and elimination processes is a highly specialized branch of toxicology, and is beyond the scope of this chapter. However, our focus here is to introduce a few basic concepts that are related to the several transport rate processes that have been described earlier in this chapter. Toxicokinetics is an extension of pharmacokinetics in that these studies are conducted at higher doses than pharmacokinetic studies, and the principles of pharmacokinetics are applied to xenobiotics. In addition, these studies are essential to provide information on the fate of the xenobiotic following exposure by a define route. This information is essential if one is to adequately interpret the dose–response relationship in the risk assessment process. In recent years, these toxicokinetic data from laboratory animals are utilized in physiologically based pharmacokinetic (PBPK) models to help extrapolations to low-dose exposures in humans. The ultimate aim in all of these analyses is to provide an estimate of tissue concentrations at the target site associated with the toxicity.

Immediately on entering the body, a chemical begins changing location, concentration, or chemical identity. It may be transported independently by several components of the circulatory system, absorbed by various tissues, or stored; the chemical may effect an action, be detoxified, or be activated; the parent compound or its metabolite(s) may react with body constituents, be stored, or be eliminated—to name some of the more important actions. Each of these processes may be

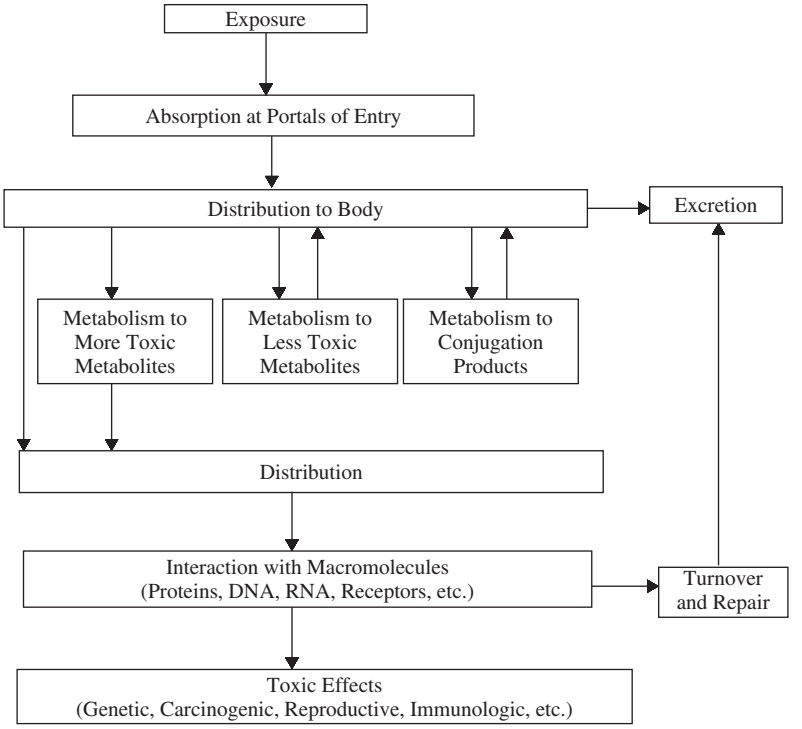


Figure 5.11 Sequence of events following exposure of an animal to exogenous chemicals.

described by rate constants similar to those described earlier in our discussion about first-order rate processes which are associated with toxicant absorption, distribution, and elimination that are occurring simultaneously. Thus, at no time is the situation stable but is constantly changing as indicated in Figure 5.11.

It should be noted however, that as the toxicant is being absorbed and distributed throughout the body, it is being simultaneously eliminated by various metabolism and/or excretion mechanisms which will be discussed in more detail in future chapters. However, one should mention here that an important pharmacokinetic parameter known as *clearance* (Cl) can be used to quantitatively assess elimination of a toxicant. Clearance is defined as the rate of toxicant excreted relative to its plasma concentration, C_p:

$$\text{Cl} = \text{Rate of Toxicant Excretion}/C_p$$

The rate of excretion is really the administered dose times the fractional elimination rate constant K_{el} described earlier. Therefore, we can express the above equation in terms of K_{el} and administered dose as well as V_d:

$$\text{Cl} = K_{el} \bullet \text{Dose}/C_p = K_{el} \bullet (V_d \bullet C_p)/C_p = K_{el} \bullet V_d$$

In physiological terms, we can also define clearance as the volume of blood cleared of the toxicant by an organ or body per unit time. Therefore, as the

equations above indicate, the body clearance of a toxicant is expressed in units of volume per unit time (e.g., liter/hour), and can be derived if we know the V_d of the toxicant and fractional rate constant. In many instances, this can only be derived by appropriate pharmacokinetic analysis of a given data set following blood or urine sample collection and appropriate chemical analyses to determine toxicant concentrations in either of these biological matrices.

Each of the processes discussed thus far, absorption, distribution, and elimination, can be described as a rate process. In general, these are assumed to be first-order processes in which the rate of transfer at any time is proportional to the amount of drug in the body at that time. Recall that the rate of transport (dC/dt) is proportional to toxicant concentration (C) or stated mathematically:

$$dC/dt = KC$$

where K is the rate constant (fraction per unit time). Many pharmacokinetic analyses of a chemical are based primarily on toxicant concentrations in blood or urine samples. It is often assumed in these analyses that the rate of change of toxicant concentration in blood reflects quantitatively the change in toxicant concentration throughout the body (first-order principles). Because of the elimination/clearance process which is also assumed to be a first-order rate process, the above rate equation now needs a negative sign. This is really a decaying process that is observed as a decline of toxicant concentration in blood or urine after IV administration. The IV route is preferred in these initial analyses because there is no absorption phase, but only chemical depletion phase. However, one cannot measure infinitesimal change of C or time, t; therefore there needs to be integration after rearrangement of the above equation:

$$-dC/C = kdt \text{ becomes } \int -dC/C = k\int dt$$

or expressed as:

$$C = C^0 e^{-kt}$$

where e is the base of the natural logarithm, and we can remove e by taking the ln of both sides:

$$\ln C^t = \ln C^0 - kt.$$

Please note that K is the slope of the straight line for a semilog plot of toxicant concentration versus time (Figure 5.12), and in the above equation, it is the elimination rate constant that is related to half-life of the toxicant described earlier in this chapter. The derived C^0 can now be used to calculate the V_d of the toxicant as follows:

$$V_d = \text{Dose}/C^0.$$

However, toxicokinetic data for many toxicants do not always provide a straight line when plotted as described above, and more complicated equations with more than one exponential term with rate constants may be necessary to mathematically describe the concentration-time profile. These numerous rate constants are indicative of chemical transport between various compartments in the body and not

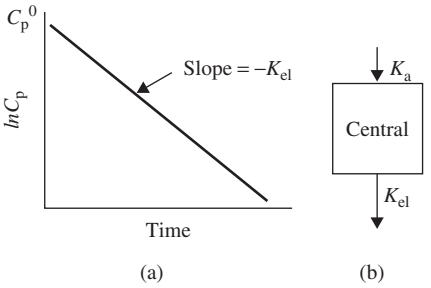


Figure 5.12 (a) Semilog plot of plasma concentration (C_p) versus time. C_p^o is the intercept on the *y*-axis, and K_{el} is the elimination rate constant. (b) Single compartment model with rate constants for absorption, K_a and for elimination, K_{el}.

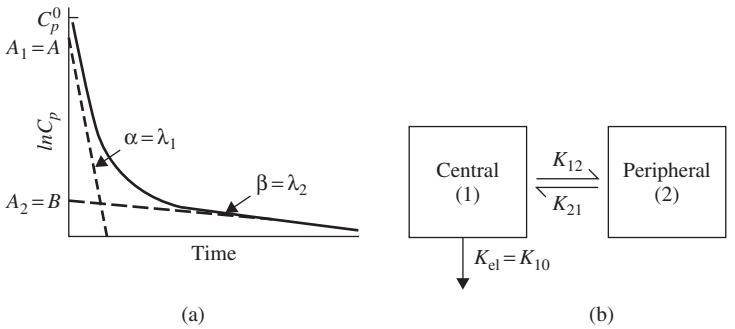


Figure 5.13 (a) Semilog plot of plasma concentration for (C_p) versus time representative of a two-compartment model. The curve can be broken down into an α or λ_1 distribution phase and β or λ_2 elimination phase. (b) Two-compartment model with transfer rate constants, K_{12} and K_{21}, and elimination rate constant, K_{el}.

a single central compartment as suggested in the simple equation and semilog plot described above in Figure 5.12. In some instances, the data may fit to a bi-exponential concentration-time profile Figure 5.13, and the equation to describe this model is:

$$C = Ae^{-\alpha t} + Be^{-\beta t}$$

In other instances, complex profiles may require a three- or multi-exponential concentration-time profile (Figure 5.14), and the equation to describe the former is:

$$C = Ae^{-\alpha t} + Be^{-\beta t} + Ce^{-\gamma t}$$

In the physiological sense, one can divide the body into "compartments" that may represent discrete parts of the whole—blood, liver, urine, and so on—or the mathematical model describing the process may be a composite representing the pooling of parts of tissues involved in distribution and bioactivation. Usually, pharmacokinetic compartments have no anatomical or physiological identity; they represent all locations within the body that have similar characteristics relative to the transport

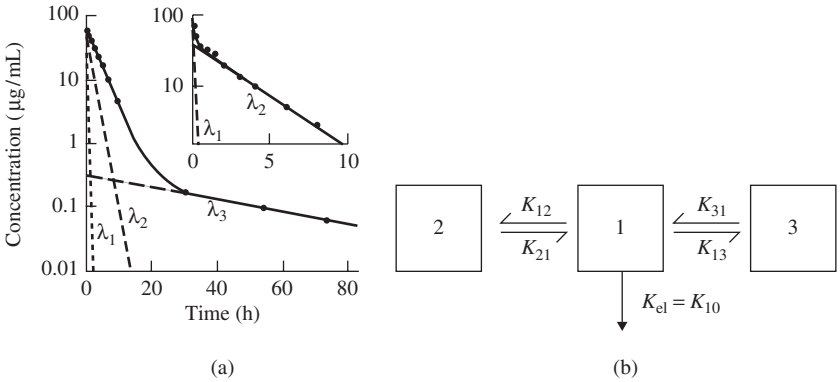


Figure 5.14 (a) Semilog plot of plasma concentration for (C_p) versus time representative of a three- or multicompartment model. The curve can be broken down into three phases, λ_1, λ_2, and λ_3. (b) Three-compartment model with transfer rate constants, K_{12}, K_{21}, K_{13}, K_{31}, and elimination rate constant, K_{el}. As these models can get more complicated; the α, β, and γ nomenclature may get replaced with λn as indicated in the profile.

rates of the particular toxicant. Simple first-order kinetics is usually accepted to describe individual rate processes for the toxicant after entry. The resolution of the model necessitates mathematical estimates (as a function of time) concerning the absorption, distribution, biotransformation, and excretion of the toxicant.

Drugs and toxicants with multi-exponential behavior depicted in Figure 5.14 require calculation of the various micro-constants. An alternative method involves using model-independent pharmacokinetic to arrive at relevant parameters. This would not be covered in any detail in this chapter, but very briefly it involves determination of AUC of the concentration-time profiles, and the emergence of microcomputers in recent years has greatly facilitated this approach.

In conclusion, pharmacokinetics is a study of the time course of absorption, distribution, and elimination of a chemical. We use pharmacokinetics as a tool to analyze plasma concentration time profiles after chemical exposure, and it is the derived rates and other parameters that reflect the underlying physiological processes that determine the fate of the chemical. There are numerous software packages available today to accomplish these analyses. The user should, however, be aware of the experimental conditions and time frame over which the data were collected and many of the assumptions embedded in the analyses. For example, many of the transport processes described in this chapter may not obey first-order kinetics and thus may be nonlinear especially at toxicological doses. The reader is advised to consult other text for more detailed descriptions of these nonlinear interactions and data analyses.

BIBLIOGRAPHY AND SUGGESTED READING

Bloom, D. and W. Fawcett. *Bloom and Fawcett: A Textbook of Histology*. Philadelphia: Saunders, 1975.

Bronaugh, R. and H. Maibach, eds. *Percutaneous Absorption*. New York: Marcel Dekker, 1989.

Gilman, A. G., T. W. Rall, A. S. Nies, eds. *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, 8th ed. Elmsford, NY: Pergamon Press, 1990.

Grandjean, P., ed. *Skin Penetration: Hazardous Chemicals at Work*. Philadelphia: Taylor & Francis, 1990.

Ham, A.W. and D. H. Cormack. *Histology*, 8th ed. Philadelphia: Lippincott, 1979.

Hodgson, E. and P. E. Levi, eds. *Introduction to Biochemical Toxicology*, 2nd ed. Norwalk, CT: Appleton & Lange, 1994, p. 12.

Krieger, R., ed. *Handbook of Pesticide Toxicology*, 2nd ed. San Diego, CA: Academic Press, 2001.

Maliwal, B. P. and F. E. Guthrie. Interactions of insecticides with human liopproteins. *Chem. Biol. Interact.* **35**:177–188, 1981.

Moran, C. J. and W. H. Walker. The binding of salicylate to human serum. *Biochem. Pharmacol.* **17**:153–156, 1968.

Rowland, M. and T. N. Tozer, eds. *Clinical Pharmacokinetics: Concepts and Applications*, 3rd ed. Philadelphia: Lea and Febiger, 1995.

Schottelius, B. A. and D. D. Schottelius. *Textbook of Physiology*. St. Louis, MO: Mosby, 1973.

Shargel, L. and A. B. C. Yu, eds. *Applied Biopharmaceutics and Pharmacokinetics*, 4th ed. Norwalk, CT: Appleton & Lange, 1999.

Singer, S. J. and G. L. Nicolson. The fluid mosaic model of the structure of cell membranes. *Science* **175**:720–731, 1972.

Skalsky, H. L. and F. E Guthrie. Affinities of parathion, DDT, dieldrin, and carbaryl for macromolecules in the blood of the rat and American cockroach and the competitive interaction of steroids. *Pest. Biochem. Physiol.* **7**:289–296, 1977.

SAMPLE QUESTIONS

1. **(a)** In carrier-mediated transport, chemical transport can reach saturation.
(b) In passive diffusion, energy is usually expended in chemical transport.
(c) In general, a chemical is more readily absorbed across human skin than mouse skin.
(d) The unionized form of a chemical is more readily absorbed than the ionized form.

2. **(a)** In zero-order transport, the amount moved per unit time is constant.
(b) In first-order transport, the fraction moved is dependent on dose.
(c) A chemical that binds extensively to plasma proteins will most likely have a high $V_{d.}$
(d) A chemical with high lipid solubility most likely has a high V_d.
(e) The half-life of any chemical can be increased by *increasing* the V_d and/or *decreasing* its clearance.

3. Explain how significant plasma protein binding can influence distribution of a toxicant in the body.

4. What are the differences between active and passive transport of toxicants?

5. Define the term partition coefficient. How is it related to Fick's law?

CHAPTER 6

Metabolism of Toxicants

ERNEST HODGSON and RANDY L. ROSE*

6.1 INTRODUCTION

Since the publication of the 3rd edition of this textbook, there have been few, if any, additions to the roster of xenobiotic-metabolizing enzymes (XMEs) or to the reactions they catalyze. However, important advances have been made in the molecular biology of XMEs and to their action in humans, the latter aspect being of particular importance in human health risk analysis.

One of the most important determinants of xenobiotic persistence in the body and subsequent toxicity to the organism is the extent to which they can be metabolized and excreted. Several families of metabolic enzymes, often with broad substrate specificity, are involved in xenobiotic metabolism. Some of the more important families of enzymes involved in xenobiotic metabolism include the cytochrome P450 monooxygenases (CYPs), flavin-containing monooxygenases (FMOs), alcohol and aldehyde dehydrogenases, amine oxidases, cyclooxygenases, reductases, hydrolases, and a variety of conjugating enzymes such as glucuronidases, sulfotransferases, methyltransferases, glutathione transferases, and acetyl transferases.

The majority of xenobiotic metabolism occurs in the liver, an organ devoted to the synthesis of many important biologically functional proteins, which also has the capacity to mediate chemical transformations of xenobiotics. Most xenobiotics that enter the body are lipophilic, a property that enables them to bind to lipid membranes and to be transported by lipoproteins in the blood. After entrance into the liver, as well as in other organs, xenobiotics may undergo one or two phases of metabolism. In Phase I, a polar reactive group is introduced into the molecule, rendering it a suitable substrate for Phase II enzymes. Enzymes typically involved in Phase I metabolism include the CYPs, FMOs, and hydrolases as will be discussed later. In Phase II, following the introduction of a polar group, conjugating enzymes typically add endogenous substituents, such as sugars, sulfates, or amino acids which result in substantially increasing the water solubility of the xenobiotic, making it

*deceased

A Textbook of Modern Toxicology, *Fourth Edition*. Edited by Ernest Hodgson

easily excreted. Although this process is generally a detoxication sequence, reactive intermediates may be formed that are much more toxic than the parent compound. It is, however, usually a sequence that increases water solubility and hence decreases the biological half-life ($t_{0.5}$) of the xenobiotic *in vivo*. The role of the transport proteins, known collectively as *transporters* (see Chapter 9) is often referred to as Phase III.

Phase I monooxygenations are more likely to form reactive intermediates than Phase II metabolism because the products are usually potent electrophiles capable of reacting with nucleophilic substituents on macromolecules, unless detoxified by some subsequent reaction. In the following discussion, examples of both detoxication and intoxication reactions are given, although greater emphasis on activation products is provided in Chapter 7.

6.2 PHASE I REACTIONS

Phase I reactions include microsomal monooxygenations, cytosolic and mitochondrial oxidations, co-oxidations in the prostaglandin synthetase reaction, reductions, hydrolyses, and epoxide hydration. All of these reactions, with the exception of reductions, introduce polar groups to the molecule that, in most cases, can be conjugated during Phase II metabolism. The major Phase I reactions are summarized in Table 6.1.

6.2.1 The Endoplasmic Reticulum, Microsomes, and Monooxygenations

Monooxygenations of xenobiotics are catalyzed either by the CYP-dependent monooxygenase system or by the FMOs. Both are located in the endoplasmic reticulum of the cell and have been studied in many tissues and organisms. This is particularly true of CYPs, probably the most studied of all enzymes.

Microsomes are derived from the endoplasmic reticulum as a result of tissue homogenization and are isolated by centrifugation of the postmitochondrial supernatant fraction, described below. The endoplasmic reticulum is an anastomosing network of lipoprotein membranes extending from the plasma membrane to the nucleus and mitochrondria, whereas the microsomal fraction derived from it consists of membranous vesicles contaminated with free ribosomes, glycogen granules, and fragments of other subcellular structures such as mitochondria and Golgi apparatus. The endoplasmic reticulum, and consequently the microsomes derived from it, consists of two types, rough and smooth, the former having an outer membrane studded with ribosomes, which the latter characteristically lack. Although both rough and smooth microsomes have all the components of the CYP-dependent monooxygenase system, the specific activity of the smooth type is usually higher.

The preparation of the postmitochondrial fraction (S9) microsomes and cytosolic fractions from tissue homogenates involves the use of two or three centrifugation steps. Following tissue extraction, careful mincing, and rinses of tissue for blood removal, the tissues are typically homogenized in buffer and centrifuged at $10{,}000 \times g$ for 20 min. The resulting supernatant, the S9 fraction, can be used in studies where both microsomal and cytosolic enzymes are desired. More often, however, the S9

TABLE 6.1 Summary of Some Important Oxidative and Reductive Reactions of Xenobiotics

Enzymes and Reactions	Examples
Cytochrome P450	
Epoxidation/hydroxylation	Aldrin, benzo(a)pyrene, aflatoxin, bromobenzene
N-, O-, S-dealkylation	Ethylmorphine, atrazine, *p*-nitroanisole, methylmercaptan
N-, S-, P-oxidation	Thiobenzamide, chlorpromazine, 2-acetylaminofluorene
Desulfuration	Parathion, carbon disulfide
Dehalogenation	Carbon tetrachloride, chloroform
Nitro reduction	Nitrobenzene
Azo reduction	*O*-aminoazotoluene
Flavin-containing monooxygenase	
N-, S-, P-oxidation	Nicotine, imipramine, thiourea, methimazole
Desulfuration	Fonofos
Prostaglandin synthetase co-oxidation	
Dehydrogenation	Acetaminophen, benzidine, epinephrine
N-dealkylation	Benzphetamine, dimethylaniline
Epoxidation/hydroxylation	Benzo(a)pyrene, 2-aminofluorene, phenylbutazone
Oxidation	FANFT (N(4-(5-nitro-2-furyl)-2-thiazolyl formamide)), ANFT (2-amino-4-(5-nitro-2-furyl) thiazole), bilirubin
Molybdenum hydroxylases	
Oxidation	Purines, pteridine, methotrexate, 6-deoxycyclovir
Reductions	Aromatic nitrocompounds, azo dyes, nitrosoamines
Alcohol dehydrogenase	
Oxidation	Methanol, ethanol, glycols, glycol ethers
Reduction	Aldehydes and ketones
Aldehyde dehydrogenase	
Oxidation	Aldehydes resulting from alcohol and glycol oxidations
Esterases and amidases	
Hydrolysis	Parathion, paraoxon, dimethoate
Epoxide hydrolase	
Hydrolysis	Benzo(a)pyrene epoxide, styrene oxide

fraction is centrifuged at 100,000 × *g* for 60 min to yield a microsomal pellet and a cytosolic supernatant. The pellet is typically resuspended in a volume of buffer, which will give 20–50 mg protein/mL and stored from –20 to –70°C. Often, the microsomal pellet is resuspended a second time and resedimented at 100,000 × *g* for 60 min to further remove contaminating hemoglobin and other proteins. As described above, enzymes within the microsomal fraction (or microsomes) include CYPs, FMOs, cyclooxygenases, and other membrane-bound enzymes including necessary coenzymes such as reduced nicotine adenine dinucleotide phosphate (NADPH)-cytochrome P450 reductase for CYP. Enzymes found in the cytosolic fraction (derived from the supernatant of the first 1,000,000 × *g* spin) include hydrolases and most of the conjugating enzymes such as glutathione transferases, glucuronidases, sulfotransferases, methyltransferases, and acetylases.

Monooxygenations, previously known as mixed-function oxidations, are those oxidations in which one atom of a molecule of oxygen is incorporated into the substrate while the other is reduced to water. Because the electrons involved in the reduction of CYPs or FMOs are derived from NADPH, the overall reaction can be written as follows (where RH is the substrate):

$$RH + O_2 + NADPH + H^+ \rightarrow NADP^+ + ROH + H_2O$$

6.2.2 The CYP-Dependent Monooxygenase System

The CYPs, the carbon monoxide-binding pigments of microsomes, are heme proteins of the b cytochrome type, containing protoporphyrin IX. Originally described as a single protein, over 7500 animal CYP isoforms in 781 gene families have been characterized across all taxa and genomic and protein sequences are known. As the list of CYPs is continually expanding, progress in this area can be readily accessed via the Internet at the website of the P450 Gene Superfamily Nomenclature Committee (http://drnelson.utmem.edu/nelsonhomepage.html) or at another excellent website (http://www.icgeb.trieste.it/p450).

A system of nomenclature based upon derived amino acid sequences was proposed in 1987 and entries are continuously updated (http://drnelson.utmem.edu/CytochromeP450.html). Degree of similarity in sequence classifies members to a CYP (cyp in the case of mice) numeric gene family, then letter subfamily such that individual isoforms have unique CYP number-letter-number annotations, for example, CYP1A1. Of the 110 animal CYP families, 18 are found in vertebrates.

In general, enzymes within a gene family share more than 40% amino acid sequence identity. Protein sequences within subfamilies have greater than 55% similarity in the case of mammalian genes, or 46% in the case of nonmammalian genes. So far, genes in the same subfamily have been found to lie on the same chromosome within the same gene cluster and are nonsegregating, suggesting a common origin through gene duplication events. Sequences showing less than 3% divergence are arbitrarily designated allelic variants unless other evidence exists to the contrary. Known sequences fit the classification scheme surprisingly well, with few exceptions found at the family, subfamily, or allelic variant levels, and in each case, additional information is available to justify the departure from the rules set out.

In some cases, a homologue of a particular CYP enzyme is found across species (e.g., CYP1A1). In other cases, the genes diverged subsequent to the divergence of the species and no exact analog is found in various species (e.g., the CYP2C subfamily). In this case, the genes are numbered in the order of discovery and the gene products from a particular subfamily may even have differing substrate specificity in different species (e.g., rodent vs. human).

The total number of functional CYP genes in any single mammalian species is thought to range from 60 to 200. Whereas some CYP isoforms are substrate specific, those involved in xenobiotic metabolism tend to be relatively nonspecific, although substrate preferences are usually evident. Although P450 is still appropriate as a prefix for the protein products, it is being rapidly replaced by the formal abbreviation, CYP. Unlike most cytochromes, the name is derived not from the absorption maximum of the reduced form in the visible region but from the unique wavelength

of the absorption maximum of the carbon monoxide derivative of the reduced form, namely 450 nm.

The role of CYP as the terminal oxidase in monooxygenase reactions is supported by considerable evidence. The initial proof was derived from the demonstration of the concomitant light reversibility of the CO complex of CYP and the inhibition, by CO, of the C-21 hydroxylation of 17 α-hydroxy-progesterone by adrenal gland microsomes. This was followed by a number of indirect, but nevertheless convincing proofs involving the effects on both CYP and monooxygenase activity of CO, inducing agents, and spectra resulting from ligand binding and the loss of activity on degradation of CYP to cytochrome P420. Direct proof was subsequently provided by the demonstration that monooxygenase systems, reconstituted from apparently homogenous purified CYP, NADPH-CYP reductase, and phosphatidylcholine, can catalyze many monooxygenase reactions.

CYPs, like other hemoproteins, have characteristic absorptions in the visible region. The addition of many organic, and some inorganic, ligands results in perturbations of these spectra. Although the detection and measurement of these spectra requires a high-resolution spectrophotometer, these perturbations, measured as optical difference spectra, have been of tremendous use in the characterization of CYPs, particularly in the decades preceding the molecular cloning and expression of specific CYP isoforms.

The most important difference spectra of oxidized CYP are type I, with an absorption maximum at 385–390 nm. Type I ligands are found in many different chemical classes and include drugs, environmental contaminants, pesticides, and so on. They appear to be generally unsuitable, on chemical grounds, as ligands for the heme iron and are believed to bind to a hydrophobic site in the protein that is close enough to the heme to allow both spectral perturbation and interaction with the activated oxygen. Although most type I ligands are substrates, it has not been possible to demonstrate a quantitative relationship between K_S (concentration required for half-maximal spectral development) and K_m (Michaelis constant). Type II ligands, however, interact directly with the heme iron of CYP, and are associated with organic compounds having nitrogen atoms with sp^2 or sp^3 nonbonded electrons that are sterically accessible. Such ligands are frequently inhibitors of CYP activity.

The two most important difference spectra of reduced CYP are the well-known CO spectrum, with its maximum at or about 450 nm, and the type III spectrum, with two pH-dependent peaks at approximately 430 nm and 455 nm. The CO spectrum forms the basis for the quantitative estimation of CYP. The best-known type III ligands for CYP are ethyl isocyanide and compounds such as the methylenedioxyphenyl synergists and SKF 525A, the last two forming stable type III complexes that appear to be related to the mechanism by which they inhibit monooxygenations.

In the catalytic cycle of CYP, reducing equivalents are transferred from NADPH to CYP by a flavoprotein enzyme known as NADPH-CYP reductase. The evidence that this enzyme is involved in CYP monooxygenations was originally derived from the observation that cytochrome c, which can function as an artificial electron acceptor for the enzyme, is an inhibitor of such monooxygenations. This reductase is an essential component in CYP-catalyzed enzyme systems reconstituted from purified components. Moreover, antibodies prepared from purified reductase are inhibitors

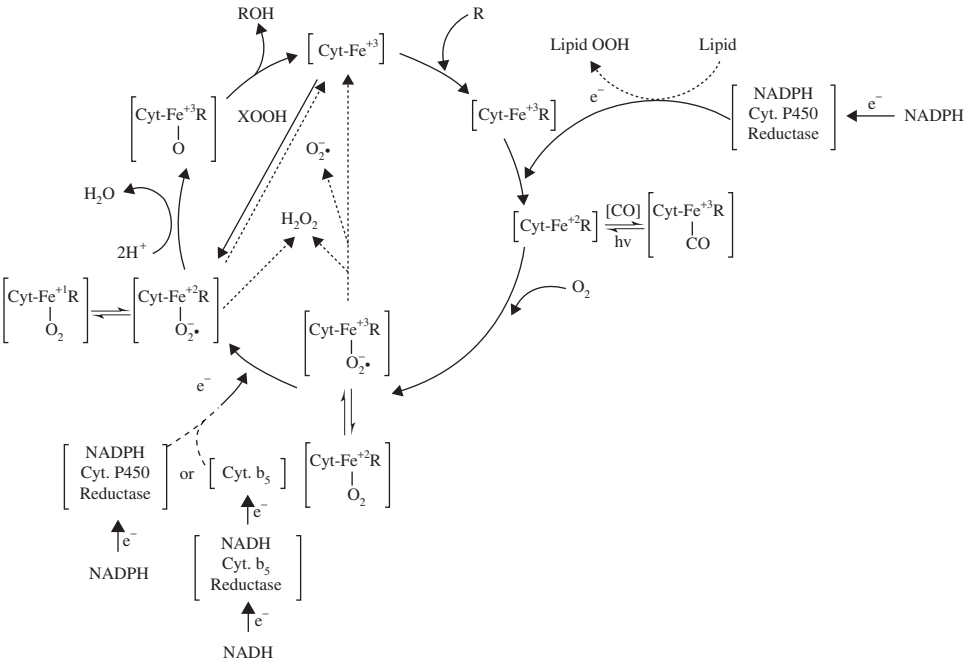


Figure 6.1 Generalized scheme showing the sequence of events for P450 monooxygenations.

of microsomal monooxygenase reactions. The reductase is a flavoprotein of approximately 80,000 Da that contain 2 mol each of flavin mononucleotide (FMN) and flavinadenine dinucleotied (FAD) per mole of enzyme. The only other component necessary for activity in the reconstituted system is a phospholipid, phosphatidylchloline. This is not involved directly in electron transfer but appears to be involved in the coupling of the reductase to the cytochrome and in the binding of the substrate to the cytochrome.

The generally recognized steps in the catalytic cycle are shown in Figure 6.1. The initial step consists of the binding of substrate to oxidized CYP followed by a one electron reduction catalyzed by NADPH-CYP reductase to form a reduced cytochrome–substrate complex. This complex can interact with CO to form the CO complex, which gives rise to the well-known difference spectrum with a peak at 450 nm and also inhibits monooxygenase activity. The next several steps are less well understood. They involve an initial interaction with molecular oxygen to form a ternary oxygenated complex. This ternary complex accepts a second electron, resulting in the further formation of one or more less understood complexes. One of these, however, is probably the equivalent of the peroxide anion derivative of the substrate-bound hemoprotein. Under some conditions, this complex may break down to yield hydrogen peroxide and the oxidized cytochrome–substrate complex. Normally, however, one atom of molecular oxygen is transferred to the substrate and the other is reduced to water, followed by dismutation reactions leading to the formation of the oxygenated product, water, and the oxidized cytochrome.

The possibility that the second electron is derived from NADH through cytochrome b_5 has been the subject of argument for some time and has yet to be completely resolved. Cytochrome b_5 is a widely distributed microsomal heme protein that is involved in metabolic reactions such as fatty acid desaturation that involve endogenous substrates. It is clear, however, that cytochrome b_5 is not essential for all CYP-dependent monooxygenations because many occur in systems reconstituted from NADPH, O_2, phosphatidylchloline, and highly purified CYP and NADPH-CYP reductase. Nevertheless, there is good evidence that many catalytic activities by isoforms including CYP3A4, CYP3A5, and CYP2E1 are stimulated by cytochrome b_5. In some cases, apocytochrome b_5 (devoid of heme) has also been found to be stimulatory, suggesting that an alternate role of cytochrome b_5 may be the result of conformational changes in the CYP/NADPH-CYP reductase systems. Thus, cytochrome b_5 may facilitate oxidative activity in the intact endoplasmic reticulum. The isolation of forms of CYP that bind avidly to cytochrome b_5 also tends to support this idea.

Distribution CYP In vertebrates, the liver is the richest source of CYP and is most active in the monooxygenation of xenobiotics. CYP and other components of the CYP-dependent monooxygenase system are also found in the skin, nasal mucosa, lung, and gastrointestinal tract, presumably reflecting the evolution of defense mechanisms at portals of entry. In addition to these organs, CYP has been demonstrated in the kidney, adrenal cortex and medulla, placenta, testes, ovaries, fetal and embryonic liver, corpus luteum, aorta, blood platelets, and the nervous system. In humans, CYP has been demonstrated in the fetal and adult liver, the placenta, kidney, testes, fetal and adult adrenal gland, skin, blood platelets, and lymphocytes.

Although CYPs are found in many tissues, the function of the particular subset of isoforms in a particular organ, tissue, or cell type does not appear to be the same in all cases. In the liver, CYPs oxidize a large number of xenobiotics as well as some endogenous steroids and bile pigments. The CYPs of the lung also appear to be concerned primarily with xenobiotic oxidations, although the range of substrates is more limited than that of the liver. The skin and small intestine also carry out xenobiotic oxidations, but their activities have been less well characterized. In normal pregnant females, the placental microsomes display little or no ability to oxidize foreign compounds, appearing to function as a steroid hormone metabolizing system. On induction of the CYP enzymes, such as occurs in pregnant women who smoke, CYP-catalyzed aryl hydrocarbon hydroxylase activity is readily apparent. The CYPs of the kidney are active in the ω-oxidation of fatty acids, such as lauric acid, but are relatively inactive in xenobiotic oxidation. Mitochondrial CYPs, such as those of the placenta and adrenal cortex, are active in the oxidation of steroid hormones rather than xenobiotics.

Distribution of CYPs within the cell has been studied primarily in the mammalian liver, where it is present in greatest quantity in the smooth endoplasmic reticulum and in smaller but appreciable amounts in the rough endoplasmic reticulum. The nuclear membrane has also been reported to contain CYP and to have detectable aryl hydrocarbon hydroxylase activity, a location that may be of importance in the metabolic activation of carcinogens.

Multiplicity of CYP, Purification, and Reconstitution of CYP Activity Even before appreciable purification of CYP had been accomplished, it was already apparent from indirect evidence that mammalian liver cells contained more than one CYP enzyme. Subsequent direct evidence on the multiplicity of CYPs included the separation and purification of CYP isozymes, distinguished from each other by chromatographic behavior, immunologic specificity, and/or substrate specificity after reconstitution and separation of distinct polypeptides by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), which could then be related to distinct CYPs present in the original microsomes.

Purification of CYP and its usual constituent isoforms was, for many years, an elusive goal; one, however, that was eventually resolved. However, the lengthy processes of CYP purification have now been largely replaced by the cloning and expression of transgenic isoforms.

Systems reconstituted from purified CYP, NADPH-CYP reductase, and phosphatidylchloline will, in the presence of NADPH and O_2, oxidize many xenobiotics, often at rates comparable to microsomes. Although systems reconstituted from this minimal number of components are enzymatically active, other microsomal components, such as cytochrome b_5, may facilitate activity either *in vivo* or *in vitro* or may even be essential for the oxidation of certain substrates.

One important finding from purification studies as well as cloning and expressing of individual isoforms is that the lack of substrate specificity of microsomes for monooxygenase activity is not an artifact caused by the presence of several specific cytochromes because it appears that many of the cytochromes isolated are still relatively nonspecific. The relative activity toward different substrates does, however, vary greatly from one CYP isoform to another even when both are relatively nonspecific. This lack of specificity is illustrated in Table 6.2, using human isoforms as examples.

Evolution of CYP Relationships between different CYP families and subfamilies are related to the rate and extent of CYP evolution.

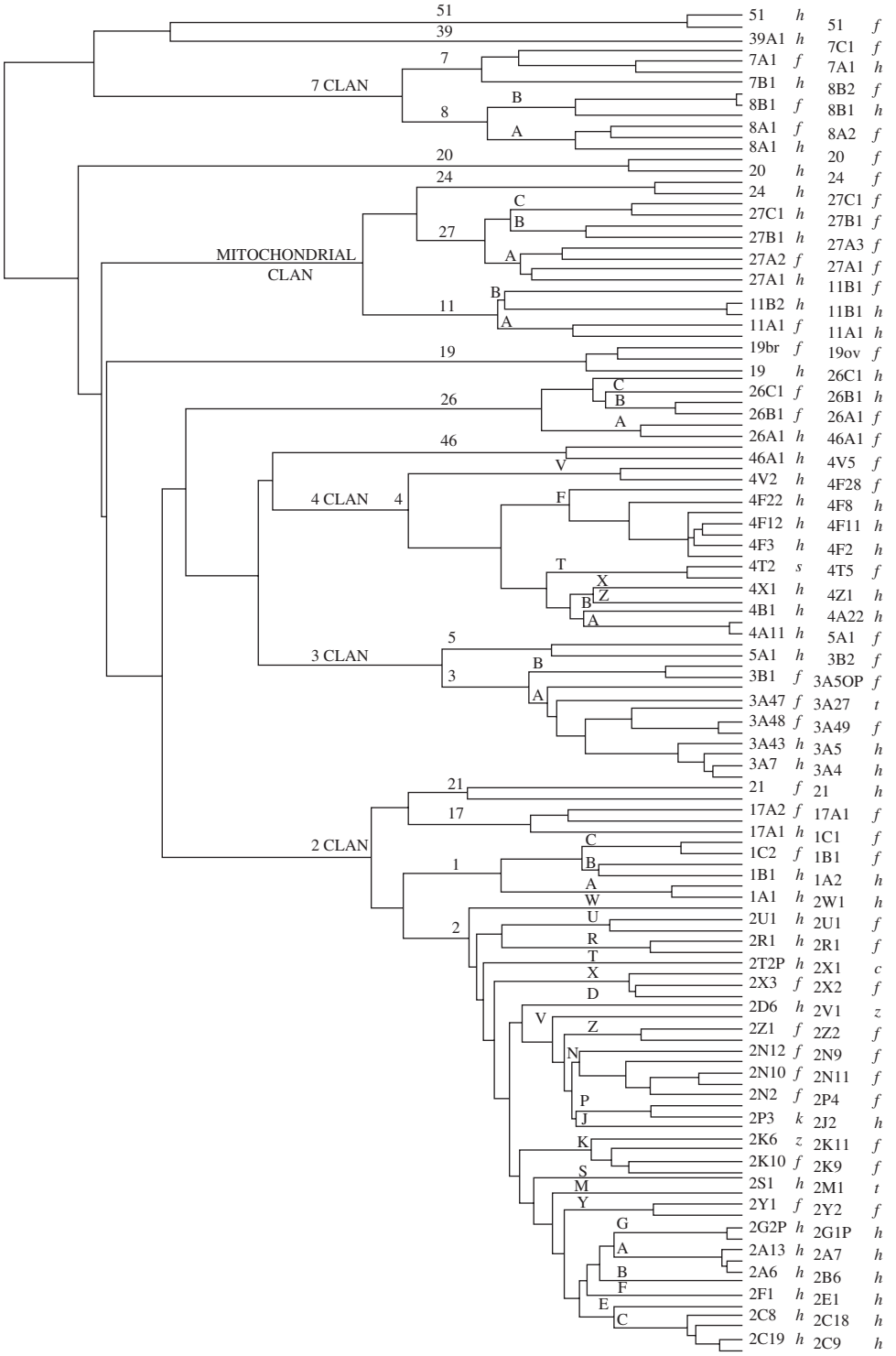
Figure 6.2 demonstrates some of the evolutionary relationships between P450 genes between some of the earliest vertebrates and humans. This dendrogram compares P450 genes from the puffer fish (fugu) and eight other fish species with human CYPs (including three pseudogenes). The unweighted pair group method arithmetic averaging (UPGMA) phylogenetic tree demonstrates the presence of five CYP clans (clusters of CYPs that are consistently grouped together) and delineates the 18 known human CYPs. This data set demonstrates that the defining characteristics of vertebrate CYPs have not changed much in 420 million years. Of these 18 human CYPs, only 1 family was missing in fugu (CYP39), indicating that the mammalian diversity of CYPs likely predates the tetrapod-ray finned fish divergence. The fish genome also has new CYP1C, 3B, and 7C subfamilies that are not seen in mammals.

CYP Families with Xenobiotic Metabolizing Potential Although mammals are known to have 18 CYP families, only three families are primarily responsible for most xenobiotic metabolism. These families, families 1–3, are considered to be more recently derived from the "ancestral" CYP families. The remaining families are less promiscuous in their metabolizing abilities and are often responsible for specific metabolic steps. For example, members of the CYP4 family are responsible for the

TABLE 6.2 Some Important Human Cytochrome P450 Isozymes and Selected Substrates

P450	Drugs	Carcinogens/Toxicants/Endogenous Substrates	Diagnostic Substrates *In Vivo* [*In Vitro*]
1A1	Verlukast (very few drugs)	Benzo(a)pyrene, dimethylbenz(a) anthracene	[Ethoxyresorufin, benzo(a)pyrene]
1A2	Phenacetin, theophylline, acetaminophen, warfarin, caffeine, cimetidine	Aromatic amines, arylhydrocarbons, NNK[a], aflatoxin, estradiol	Caffeine, [acetanilide, methoxyresorufin, ethoxyresorufin]
2A6	Coumarin, nicotine	Aflatoxin, diethylnitrosamine, NNK[a]	Coumarin
2B6	Cyclophosphamide, ifosfamide, nicotine	6 Aminochrysene, aflatoxin, NNK[a]	[7-ethoxy-4-trifluoro-methyl coumarin]
2C8	Taxol, tolbutamide, carbamazepine	—	[Chloromethylfluorescein diethly ether]
2C9	Tienilic acid, tolbutamide, warfarin, phenytoin, tetrahydrocannabinol, hexobarbital, diclofenac	—	[Diclofenac (4′-OH)]
2C19	*S*-mephenytoin, diazepam, phenytoin, omeprazole, indomethacin, imipramine, propanolol, proguanil	—	[*S*-mephenytoin (4′-OH)]
2D6	Debrisoquine, sparteine, bufuralol, propanolol, thioridazine, quinidine, phenytoin, fluoxetine	NNK[a]	Dextromethorphan, [bufuralol (4′-OH)]
2E1	Chlorzoxazone, isoniazid, acetaminophen, halothane, enflurane, methoxyflurane	Dimethylnitrosamine, benzene, halogenated alkanes (e.g., CCl_4), acylonitrile, alcohols, aniline, styrene, vinyl chloride	Chlorzoxazone (6-OH), [*p*-nitrophenol]
3A4	Nifedipine, ethylmorphine, warfarin, quinidine, taxol, ketoconazole, verapamil, erythromycin, diazepam	Aflatoxin, 1-nitropyrene, benzo(a) pyrene 7,8-diol, 6 aminochrysene, estradiol, progesterone, testosterone, other steroids, bile acids	Erythromycin, nifedipine [testosterone (6-β)]
4A9/11	(Very few drugs)	Fatty acids, prostaglandins, thromboxane, prostacyclin	[Lauric acid]

[a] NNK, 4(methylnitrosamino)-1-(3-pyridyl)-1-butanone, a tobacco-smoke specific nitrosamine.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7A1 f
7B1 h
8B1 f
8A1 f
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27C1 h
27B1 h
27A2 f
27A1 h
11B2 h
11A1 f
19br f
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26C1 f
26B1 f
26A1 h
46A1 h
4V2 h
4F22 h
4F12 h
4F3 h
4T2 s
4X1 h
4B1 h
4A11 h
5A1 h
3B1 f
3A47 f
3A48 f
3A43 h
3A7 h
21 f
17A2 f
17A1 h
1C2 f
1B1 h
1A1 h
2U1 h
2R1 h
2T2P h
2X3 f
2D6 h
2Z1 f
2N12 f
2N10 f
2N2 f
2P3 k
2K6 z
2K10 f
2S1 h
2Y1 f
2G2P h
2A13 h
2A6 h
2F1 h
2C8 h
2C19 h
51 f
7C1 f
7A1 h
8B2 f
8B1 h
8A2 f
20 f
24 f
27C1 f
27B1 f
27A3 f
27A1 f
11B1 f
11B1 h
11A1 h
19ov f
26C1 h
26B1 h
26A1 f
46A1 f
4V5 f
4F28 f
4F8 h
4F11 h
4F2 h
4T5 f
4Z1 h
4A22 h
5A1 f
3B2 f
3A5OP f
3A27 t
3A49 f
3A5 h
3A4 h
21 h
17A1 f
1C1 f
1B1 f
1A2 h
2W1 h
2U1 f
2R1 f
2X1 c
2X2 f
2V1 z
2Z2 f
2N9 f
2N11 f
2P4 f
2J2 h
2K11 f
2K9 f
2M1 t
2Y2 f
2G1P h
2A7 h
2B6 h
2E1 h
2C18 h
2C9 h

end-chain hydroxylation of long chain fatty acids. The remaining mammalian CYP families are involved in biosynthesis of steroid hormones. In fact, some of the nomenclature for some of these families is actually derived from the various positions in the steroid nucleus where the metabolism takes place. For example, CYP7 mediates hydroxylation of cholesterol at the 7 α-position, while CYP 17 and 21 catalyze the 17α and 21-hydroxylations of progesterone, respectively. CYP 19 is responsible for the aromatization of androgens to estrogen by the initial step of hydroxylation at the 19-position. Many of the CYPs responsible for steroidogenesis are found in the adrenal cortex, while those involved in xenobiotic metabolism are found predominantly in tissues that are more likely to be involved in exposure such as liver, kidneys, lungs, and olfactory tissues.

To simplify discussion of important CYP family members, the following discussion concentrates upon human CYP family members. However, since there is a great deal of homology among family members, many of the points of discussion are generally applicable to CYP families belonging to several species.

The CYP1 family contains three known human members, CYP1A1, CYP1A2, and CYP1B1. CYP1A1 and CYP1A2 are found in all classes of the animal kingdom. CYP2E1 is the only other CYP that retains the same gene designation in many different species.

CYP1A1 and CYP1A2 possess distinct but overlapping substrate specificities: CYP1A1 preferring neutral polycyclic aromatic hydrocarbons (PAHs), while the latter prefers polyaromatic and heterocyclic amines and amides. Because of the preference of this family for molecules with highly planar molecular structures, CYP1 family members are closely associated with metabolic activation of many procarcinogens and mutagens including benzo(a)pyrene, aflatoxin B1, dimethylbenzanthracene, β-naphthylamine, 4-aminobiphenyl, 2-acetylaminofluorene, and benzidine. Figure 6.3 illustrates a typical reaction sequence that leads to the formation of epoxide and epoxide diols that are often implicated in the formation of carcinogenic metabolites formed by these enzymes.

Many of the planar PAH compounds induce their own metabolism by inducing transcription of the aryl hydrocarbon receptor (Ah receptor). Although expression of CYP1A1 and 1A2 is often coordinately induced, there are clear differences in regulation, not only with respect to substrate specificity but also in their biological expression. For example, CYP1A1 does not appear to be expressed in human liver unless induced, whereas CYP1A2 is endogenously expressed in the liver. CYP1A1, however, is present in many extrahepatic tissues including the lung, where there is a possible association between CYP-mediated activation of benzo(a) pyrene and other related chemicals present in cigarette smoke and lung cancer in man.

Figure 6.2 UPGMA tree of 54 puffer fish (fugu), 60 human, and 8 other fish P450s. Species are indicated by f, h, z, c, k, s, and t for fugu, human, zebrafish, catfish, killifish, sea bass, and trout, respectively. Reprinted from: Nelson, D. R. Comparison of P450s from human and fugu: 420 million years of vertebrate P450 evolution. *Arch. Biochem. Biophys.* **409**:18–24, 2003; with permission from Academic Press.

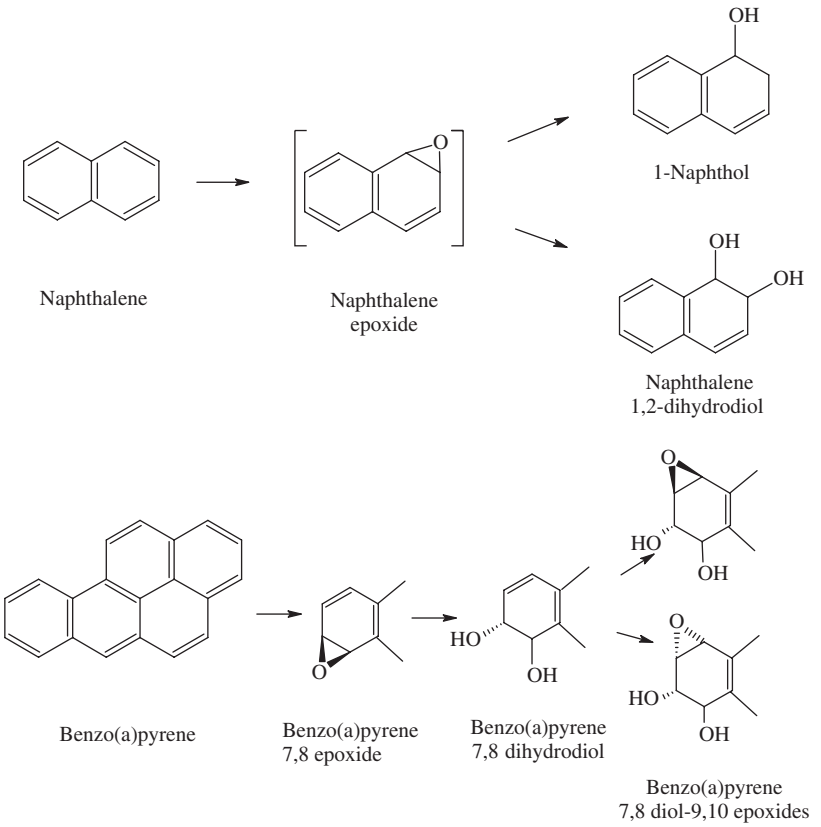


Figure 6.3 Examples of epoxidation reactions.

The CYP2 family consists of 10 subfamilies, five of which are present in mammalian liver. Some of the more important isoforms found in humans within this family are CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP2E1. The enzyme CYP2A6 is expressed primarily in liver tissue, where it represents 1–10% of total CYP content. CYP2A6 is responsible for the 7-hydroxylation of the naturally occurring plant compound coumarin, and its activity is often phenotyped by monitoring this particular metabolic pathway. Other drugs metabolized by CYP2A6 include nicotine, 2-acetylaminofluorine, methoxyflurane, halothane, valproic acid, and disulfiram. Precarcinogens likely activated by CYP2A6 include aflatoxin B1, 1,3 butadiene, 2,6-dichlorobenzonitrile, and a number of nitrosamines. Because CYP2A6 is responsible for up to 80% of the human metabolism of nicotine, a number of studies have been conducted to determine whether individuals with CYP2A6 polymorphisms have reduced risk of lung cancers. Although theoretically, individuals lacking CYP2A6 would be expected to smoke less and be less likely to activate carcinogens found in tobacco smoke, studies have not conclusively demonstrated any clear associations between CYP2A6 polymorphisms and risk of lung cancer.

Like CYP2A6, the human isoform CYP2B6 has recently gained greater recognition for its role in metabolism of many clinical drugs. Some common pharmaceutical

substrates for CYP2B6 include cyclophosphamide, nevirapine, *S*-mephobarbital, artemisinin, bupropion, propofol, ifosfamide, ketamine, selegiline, and methadone. CYP2B6 has also been demonstrated to have a role in the activation of the organophosphorus insecticide, chlorpyrifos, and in the degradation of the commonly used insecticide repellant, diethyl toluamide (DEET). Historically, it was thought that CYP2B6 is found in only a small proportion of livers (<25%), but more recent data using antibodies prepared from human proteins have demonstrated that most liver samples have detectable levels of CYP2B6, although greater than 20-fold differences in levels of protein have been observed. Recently, it has become apparent that CYP2B6 is important in the monooxidation of occupational chemicals, particularly agrochemicals. Since it is highly inducible, individual variation in concentration may be important in risk analysis and xenobiotic interactions.

In contrast with CYP2A6 and CYP2B6, members of the CYP2C family constitute a fairly large percentage of CYP in human liver (ca. 20%) and are responsible for the metabolism of several drugs. All four members of the subfamily in humans exhibit genetic polymorphisms, many of which have important clinical consequences in affected individuals. Genetic polymorphisms in CYP2C19 were shown to be responsible for one of the earliest described polymorphic effects, that involving mephenytoin metabolism. This particular polymorphism significantly reduces the metabolism of mephenytoin resulting in the classification of those individuals possessing this trait as poor metabolizers (PM). Among Caucasians, PMs represent only 3–5% of the populations, while in Asian and Polynesian populations 12–23% and 38–79% of the populations are represented, respectively. At least seven different mutations in this allele have been described, some of which negatively affect catalytic activity while others prevent expression of the protein. Other important drugs affected by these CYP2C19 polymorphisms include the antiulcer drug omeprazole, other important proton pump inhibitors, barbiturates, certain tricyclic antidepressants such as imipramine, and the antimalarial drug proguanil. Other important members of the CYP2C family in humans include CYP2C8, CYP2C9, and CYP2C18. Substrates metabolized exclusively by CYP2C8 include retinol, retinoic acid, taxol, and arachidonic acid. CYP2C9, the principal CYP2C in human liver, metabolizes several important drugs including the diabetic agent tolbutamide, the anticonvulsant phenytoin, the anticoagulant warfarin, and a number of anti-inflammatory drugs including ibuprofen, diclofenac, and others. Both CYP2C9 and CYP2C8, which are responsible for metabolism of the anticancer drug paclitaxel, have been demonstrated to be polymorphic.

CYP2E1 is the only member of the CYP2E family in most mammals with the exception of rabbits. Substrates for this family tend to be of small molecular weight and include ethanol, carbon tetrachloride, benzene, and acetaminophen. In contrast with many other inducible CYP families, CYP2E1 is regulated by a combination of increased transcription levels and increased message and protein stabilization.

Undoubtedly, the largest amount of CYP in human liver is that of the CYP3 family. CYP3A4 is the most abundant CYP in the human liver, on average accounting for approximately 30% of the total amount, and is known to metabolize many important drugs, including cyclosporin A, nifedipine, rapamycin, ethinyl \estradiol, quinidine, digitonin, lidocaine, erythromycin, midazolam, triazolam, lovastatin, and tamoxifen. Other important oxidations ascribed to the CYP3 family

include many steroid hormones, macrolide antibiotics, alkaloids, benzodiazepines, dihydropyridines, organophosphorus insecticides and other insecticides, warfarin, polycyclic hydrocarbon-derived dihydrodiols, and aflatoxin B_1. Many chemicals are also capable of inducing CYPs of this family including phenobarbital, rifampicin, and dexamethasone. Because of potential difficulties arising from CYP induction, drugs metabolized by this family must be closely examined for the possibility of harmful drug–drug interactions.

CYP Reactions Although microsomal monooxygenase reactions are basically similar in the role played by molecular oxygen and in the supply of electrons, the many CYP isoforms can attack a large variety of xenobiotic substrates, with both substrates and products falling into many different chemical classes. In the following sections, therefore, enzyme activities are classified on the basis of the overall chemical reaction catalyzed; one should bear in mind, however, that not only do these classes often overlap, but often a substrate may also undergo more than one reaction. See Table 6.1 for a listing of important oxidation and reduction reactions of CYPs.

Epoxidation and Aromatic Hydroxylation Epoxidation is an extremely important microsomal reaction because not only can stable and environmentally persistent epoxides be formed (see aliphatic epoxidations below), but highly reactive intermediates of aromatic hydroxylations, such as arene oxides, can also be produced. These highly reactive intermediates are known to be involved in chemical carcinogesis as well as chemically induced cellular and tissue necrosis.

The oxidation of naphthalene was one of the earliest examples of an epoxide as an intermediate in aromatic hydroxylation. As shown in Figure 6.3, the epoxide can rearrange nonenzymatically to yield predominantly 1-naphthol, or interact with the enzyme epoxide hydrolase to yield the dihydrodiol, or interact with glutathione S-transferase (GST) to yield the glutathione conjugate, which is ultimately metabolized to a mercapturic acid.

More recent studies of the *in vitro* human metabolism of naphthalene have shown that the primary naphthalene metabolites from pooled human liver microsomes are *trans*-1,2-dihydro-1,2-naphthalenediol (dihydrodiol), 1-naphthol, and 2-naphthol. CYP1A2 was identified as the most efficient isoform for producing dihydrodiol and 1-naphthol while CYP3A4 was the most effective for 2-naphthol production. Further metabolism of the primary metabolites of naphthalene was also studied to identify secondary metabolites, and reactive metabolites such as 1,4-naphthoquinone were observed, and CYP1A2 and 2D6*1 were identified as the most active isoforms for the production of 1,4-naphthoquinone.

These aromatic epoxidation reactions are also of importance in the metabolism of other xenobiotics that contain an aromatic nucleus, such as the insecticide carbaryl and the carcinogen benzo(a)pyrene.

The ultimate carcinogens arising from the metabolic activation of benzo(a) pyrene are stereoisomers of benzo(a)pyrene 7,8-diol-9,10-epoxide (Figure 6.3). These metabolites arise by prior formation of the 7,8 epoxide, which gives rise to the 7,8-dihydrodiol through the action of epoxide hydrolase. This is further metabolized by the CYP to the 7,8-diol-9,10-epoxides, which are both potent mutagens and unsuitable substrates for the further action of epoxide hydrolase. Stereochemistry

is important in the final product. Of the four possible isomers of the diol epoxide, the (+)-benzo(a)pyrene diol epoxide-2 is the most active carcinogen.

Aliphatic Hydroxylation Simple aliphatic molecules such as *n*-butane, *n*-pentane, *n*-hexane, and so on, as well as alicyclic compounds such as cyclohexane, are known to be oxidized to alcohols. However, alkyl side chains of aromatic compounds are more readily oxidized, often at more than one position, and provide good examples of this type of oxidation. For example, the *n*-propyl side chain of *n*-propyl benzene can be oxidized at any one of three carbons to yield 3-phenylpropan-1-ol ($C_6H_5CH_2CH_2CH_2OH$) by ω-oxidation, benzylmethylcarbinol ($C_6H_5CH_2CHOHCH_3$) by ω-1 oxidation, and ethyl-phenylcarbinol ($C_6H_5CHOHCH_2CH_3$) by α-oxidation. Further oxidation of these alcohols is also possible.

Aliphatic Epoxidation Many aliphatic and alicyclic compounds containing unsaturated carbon atoms are thought to be metabolized to epoxide intermediates (Figure 6.4). In the case of aldrin, the product, dieldrin, is an extremely stable epoxide and represents the principle residue found in animals exposed to aldrin. Epoxide formation in the case of aflatoxin is believed to be the final step in formation of the ultimate carcinogenic species and is, therefore, an activation reaction.

Dealkylation: O-, N-, and S-Dealkylation Probably the best-known example of O-dealkylation is the demethylation of *p*-nitroanisole. Due to the ease with which the product, *p*-nitrophenol, can be measured, it is a frequently used substrate for the demonstration of CYP activity. The reaction likely proceeds through formation of an unstable methylol intermediate (Figure 6.5).

The O-dealkylation of organophosphorus triesters differs from that of *p*-nitroanisole in that it involves the dealkylation of an ester rather than an ether. The reaction was first described for the insecticide chlorfenvinphos and is known to occur with a wide variety of vinyl, phenyl, phenylvinyl, and naphthyl phosphate and thionophosphate triesters (Figure 6.5).

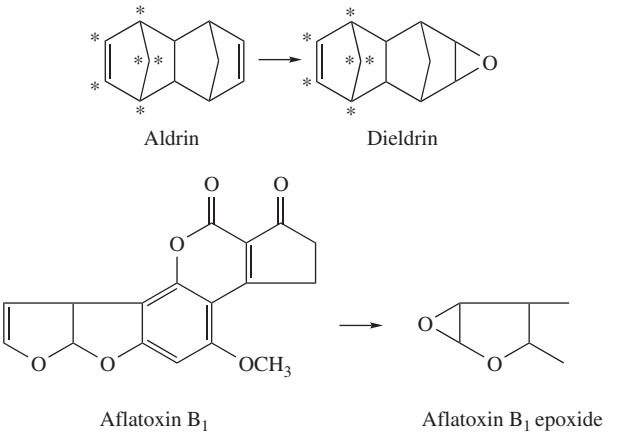


Figure 6.4 Examples of aliphatic epoxidation.

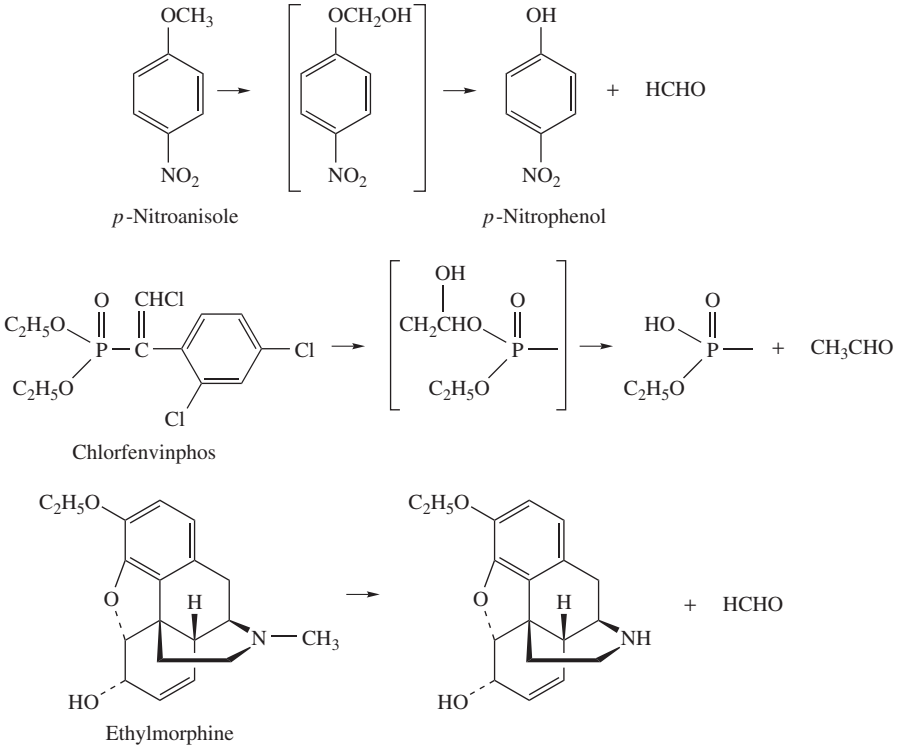


Figure 6.5 Examples of dealkylation.

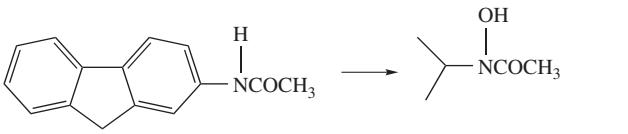
N-dealkylation is a common reaction in the metabolism of drugs, insecticides, and other xenobiotics. The drug ethylmorphine is a useful model compound for this reaction. In this case, the methyl group is oxidized to formaldehyde, which can be readily detected by the Nash reaction.

S-dealkylation is believed to occur with a number of thioethers, including methylmercaptan and 6-methylthiopurine, although with newer knowledge of the specificity of the FMO, it is possible that the initial attack is through sulfoxidation mediated by FMO rather than CYP.

N-Oxidation N-oxidation can occur in a number of ways, including hydroxylamine formation, oxime formation, and N-oxide formation, although the latter is primarily dependent on the FMO enzyme. Hydroxylamine formation occurs with a number of amines such as aniline and many of its substituted derivatives. In the case of 2-acetylaminofluorene, the product is a potent carcinogen, and thus the reaction is an activation reaction (Figure 6.6).

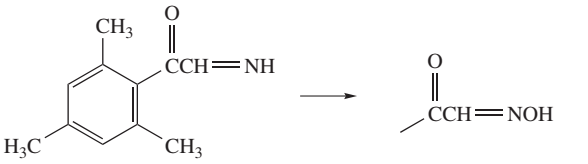
Oximes can be formed by the N-hydroxylation of imines and primary amines. Imines have been suggested as intermediates in the formation of oximes from primary amines (Figure 6.6).

Oxidative Deamination Oxidative deamination of amphetamine occurs in the rabbit liver but not to any extent in the liver of either the dog or the rat, which tend



2-Acetylaminofluorene — *N*-Hydroxy-2-acetylaminofluorene

(a) Hydroxylamine formation



Trimethylacetophenone imine — Trimethylacetophenone oxime

(b) Oxime formation

Figure 6.6 Examples of N-oxidation.

to hydroxylate the aromatic ring. A close examination of the reaction indicates that it is probably not an attack on the nitrogen but rather on the adjacent carbon atom, giving rise to a carbinol amine, which eliminates ammonia, producing a ketone.

$$R_2CHNH_2 \xrightarrow{+O} R_2C(OH)NH_2 \xrightarrow{-NH_3} R_2C{=}O$$

The carbinol, by another reaction sequence, can also give rise to an oxime, which can be hydrolyzed to yield the ketone, which is thus formed by two different routes.

$$R_2C(OH)NH_2 \xrightarrow{-H_2O} R_2C{=}NH \xrightarrow{+O} R_2CNOH \xrightarrow{+H_2O} R_2C{=}O$$

S-Oxidation Thioethers in general are oxidized by microsomal monooxygenases to sulfoxides, some of which are further oxidized to sulfones. This reaction is very common among insecticides of several different chemical classes, including carbamates, organophosphorus compounds, and chlorinated hydrocarbons. Recent work suggests that members of the CYP2C family are involved in sulfoxidation of several organophosphorus compounds including phorate, coumaphos, and demeton. The carbamate methiocarb is oxidized to a series of sulfoxides and sulfones, and among the chlorinated hydrocarbons, endosulfan is oxidized to endosulfan sulfate and methiochlor to a series of sulfoxides and sulfones, eventually yielding the bis-sulfone. Drugs, including chlorpromazine, and solvents, such as dimethyl sulfoxide, are also subject to S-oxidation. The fact that FMOs are versatile sulfur oxidation enzymes capable of carrying out many of the previously mentioned reactions raises important questions as to the relative role of this enzyme versus that of CYP.

P-Oxidation P-oxidation, a little known reaction, involves the conversion of trisubstituted phosphines to phosphine oxides, for example, diphenylmethylphosphine to diphenylmethylphosphine oxide. Although this reaction is described as a typical CYP-dependent monooxygenation, it also is now known to be catalyzed by the FMO.

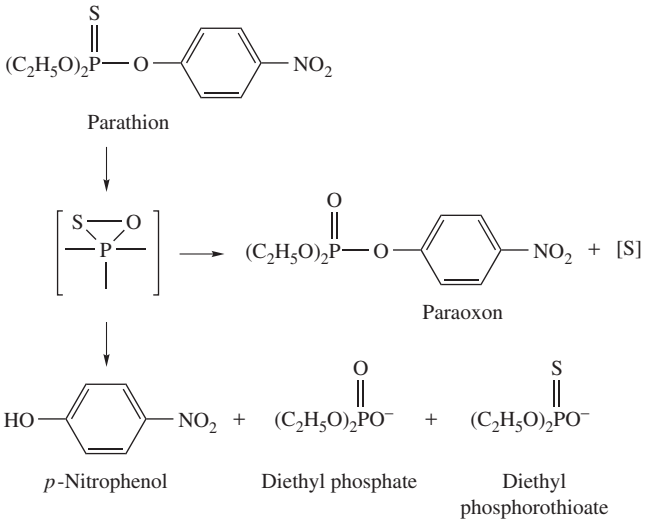


Figure 6.7 Desulfuration and oxidative dearylation.

Desulfuration and Ester Cleavage The phosphorothionates $[(R^1O)_2P(S)OR^2]$ and phosphorodithioates $[(R^1O)_2P(S)SR^2]$ owe their insecticidal activity and their mammalian toxicity to an oxidative reaction in which the P=S group is converted to P=O, thereby converting the compounds from chemicals relatively inactive toward cholinesterase into potent inhibitors (see Chapter 15 for a discussion of the mechanism of cholinesterase inhibition). This reaction has been described for many organophosphorus compounds. Much of the splitting of the phosphorus ester bonds in organophosphorus insecticides, formerly believed to be due to hydrolysis, is now known to be due to oxidative dearylation. This is a typical CYP-dependent monooxygenation, requiring NADPH and O_2 and being inhibited by CO. Current evidence supports the hypothesis that this reaction and oxidative desulfuration involve a common intermediate of the "phosphooxithirane" type (Figure 6.7). Some organophosphorus insecticides, all phosphonates such as fonofos, are activated by the FMO as well as the CYP.

Methylenedioxy (Benzodioxole) Ring Cleavage Methylenedioxyphenyl compounds, such as safrole or the insecticide synergist, piperonyl butoxide, many of which are effective inhibitors of CYP monooxygenations, are themselves metabolized to catechols. The most probable mechanism appears to be an attack on the methylene carbon, followed by elimination of water to yield a carbene. The highly reactive carbene either reacts with the heme iron to form a CYP-inhibitory complex or breaks down to yield the catechol (Figure 6.8).

6.2.3 The FMO

Tertiary amines such as trimethylamine and dimethylamine had long been known to be metabolized to N-oxides by a microsomal amine oxidase that was not dependent on CYP. This enzyme, now known as the microsomal FMO, is also dependent

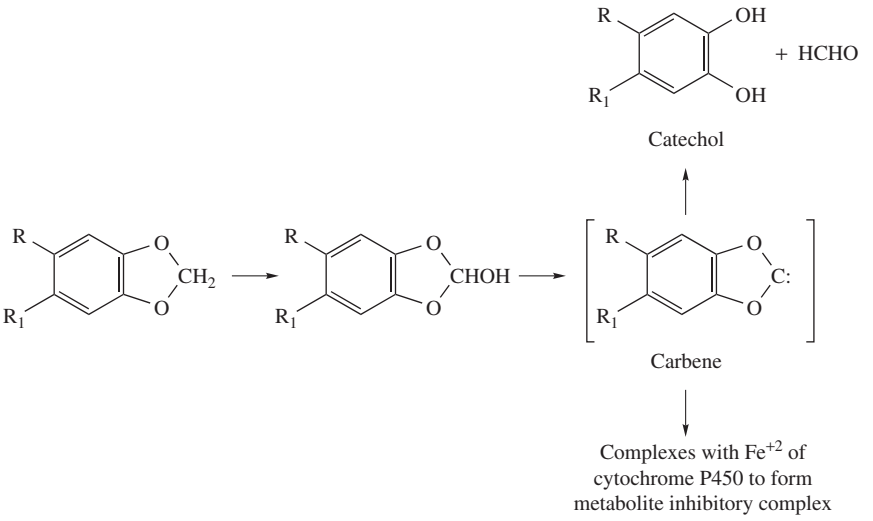


Figure 6.8 Monooxygenation of methylenedioxyphenyl compounds.

on NADPH and O_2, and has been purified to homogeneity from a number of species. Isolation and characterization of the enzyme from liver and lung samples provided evidence of clearly distinct physicochemical properties and substrate specificities suggesting the presence of at least two different isoforms. Subsequent studies have verified the presence of multiple forms of the enzyme.

At least six different isoforms have been described by amino acid or cDNA sequencing, and are classified as FMO1 to FMO6. These isoforms share approximately 50–60% amino acid identity across species lines. The identity of orthologs is greater than 82%. Although each isoform has been characterized in humans, several are essentially nonfunctional in adults. For example, FMO1, expressed in the embryo, disappears relatively quickly after birth. FMO2 in most Caucasians and Asians contains a premature stop codon, preventing the expression of functional protein. Functional FMO2 is found in 26% of the African-American population and is, perhaps, also found in the Hispanic population. FMO3, the predominant human FMO, is poorly expressed in neonatal humans but is expressed in most individuals by 1 year of age. Gender-independent expression of FMO3 (contrasting with what is observed in other mammals) continues to increase through childhood, reaching maximal levels of expression at adulthood. Several polymorphic forms of FMO3 are responsible for the disease, trimethylaminuria, also known as "fish odor syndrome," characterized by the inability of some individuals to convert the malodorous trimethylamine, either from the diet or from metabolism, to its odorless N-oxide. Although the FMO4 transcript is found in several species, the protein has yet to be successfully expressed in any species. Although FMO5 is expressed in humans at low levels, the poor catalytic activity of FMO5 for most classical FMO substrates suggests that it has minimal participation in xenobiotic oxidation. No data is yet available on the role and abundance of the most recently discovered FMO, FMO6.

Substrates containing soft nucleopohiles (e.g., nitrogen, sulfur, phosphorus, and selenium) are good candidates for FMO oxidation (Figure 6.9). A short list of

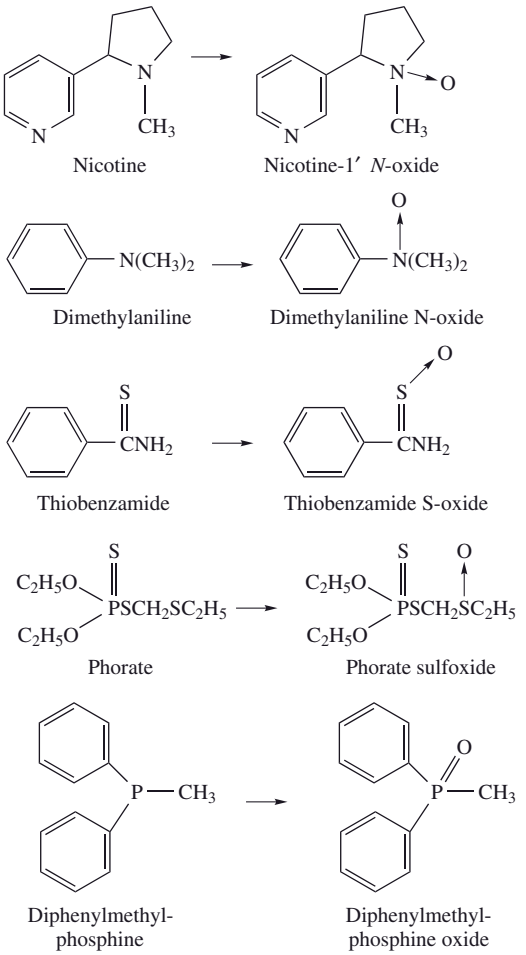


Figure 6.9 Examples of oxidations catalyzed by the flavin-containing monooxygenase (FMO).

known substrates include drugs such as dimethylaniline, imipramine, thiobenzamide, chlorpromazine, promethazine, cimetidine, and tamoxifen; pesticides such as phorate, fonofos, and methiocarb; environmental agents including the carcinogen 2-aminofluorine, and the neurotoxicants nicotine and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Although there is no known physiologically relevant substrate for FMO, a few dietary and/or endogenous substrates have been identified, including trimethylamine, cysteamine, methionine, and several cysteine-S-conjugates. In most cases, metabolism by FMO results in detoxication products, although there are several examples of substrates that are bioactivated by FMO oxidation; particularly in the case of substrates involving sulfur oxidation.

Most FMO substrates are also substrates for CYP. Since both enzymes are microsomal and require NADPH and oxygen, it is difficult to distinguish which enzyme is responsible for oxidation without the use of techniques involving specific inactivation or inhibition or one or the other of these enzymes while simultaneously

examining the metabolic contribution of the other. Since FMOs are generally heat labile, heating the microsomal preparation to 50°C for 1 min inactivates the FMOs while having minimal effects of CYPs. Alternatively, the contribution of FMO can be assessed by use of a general CYP inhibitor such as *N*-benzylimidazole or by an inhibitory antibody to NADPH-CYP reductase, a necessary CYP coenzyme. Typically, results of these tests are sought in combination so that the best estimates of CYP and FMO contribution can be obtained.

Toxicologically, it is of interest that the FMO enzyme is responsible for the oxidation of nicotine to nicotine-1′-N-oxide, whereas the oxidation of nicotine to cotinine is catalyzed by two enzymes acting in sequence: CYP followed by a soluble aldehyde dehydrogenase. Thus, nicotine is metabolized by two different routes, the relative contributions of which may vary with both the extrinsic and intrinsic factors outlined in Chapter 8.

6.2.4 Nonmicrosomal Oxidations

In addition to the microsomal monooxygenases, other enzymes are involved in the oxidation of xenobiotics. These enzymes are located in the mitochondria or in the soluble cytoplasm of the cell.

Alcohol Dehydrogenase Alcohol dehydrogenases catalyze the conversion of alcohols to aldehydes or ketones:

$$RCH_2OH + NAD^+ \rightarrow RCHO + NADH + H^+$$

This reaction should not be confused with the monooxygenation of ethanol by CYP that occurs in the microsomes. The alcohol dehydrogenase reaction is reversible, with the carbonyl compounds being reduced to alcohols.

Alcohol dehydrogenase is found in the soluble fraction of the liver, kidney, and lung and is probably the most important enzyme involved in the metabolism of foreign alcohols. Alcohol dehydrogenase is a dimer, the subunits of which can occur in several forms that are under genetic control, thus giving rise to a large number of variants of the enzyme. In mammals, six classes of enzymes have been described. Alcohol dehydrogenase can use either NAD or NADP as a coenzyme, but the reaction proceeds at a much slower rate with NADP. In the intact organism, the reaction proceeds in the direction of alcohol consumption, because aldehydes are further oxidized to acids. Because aldehydes are toxic and are not readily excreted because of their lipophilicity, alcohol oxidation may be considered an activation reaction, the further oxidation to an acid being a detoxication step.

Although short chain aliphatic alcohols, particularly ethanol, are the most studied substrates for alcohol dehydrogenase, larger molecules, such as phenoxybenzyl alcohol, the hydrolysis product of permethrin, have Km values up to two orders of magnitude lower than that for ethanol.

Primary alcohols are oxidized to aldehydes, *n*-butanol being the substrate oxidized at the highest rate. Although secondary alcohols are oxidized to ketones, the rate is less than for primary alcohols, and tertiary alcohols are not readily oxidized. Alcohol dehydrogenase is inhibited by a number of heterocyclic compounds such as pyrazole, imidazole, and their derivatives.

Aldehyde Dehydrogense Aldehydes are generated from a variety of endogenous and exogenous substrates. Endogenous aldehydes may be formed during metabolism of amino acids, carbohydrates, lipids, biogenic amines, vitamins, and steroids. Metabolism of many drugs and environmental agents produce aldehydes. Aldehydes are highly reactive electrophilic compounds and may react with thiol and amino groups to produce a variety of effects. Some aldehydes produce therapeutic effects, but more often effects are cytotoxic, genotoxic, mutagenic, or carcinogenic. Aldehyde dehydrogenases are important in helping to alleviate some of the toxic effects of aldehyde generation. This enzyme catalyzes the formation of acids from aliphatic and aromatic aldehydes; the acids are then available as substrates for conjugating enzymes:

$$\mathrm{RCHO} + \mathrm{NAD}^{+} \rightarrow \mathrm{RCOOH} + \mathrm{NADH} + \mathrm{H}^{+}$$

The aldehyde dehydrogenase gene superfamily is large, with more than 330 aldehyde dehydrogenase genes in prokaryote and eukaryotic species. The eukaryotic aldehyde dehydrogenase gene superfamily consists of 20 gene families, 9 of which contain 16 human genes and 3 pseudogenes. The importance of some of these genes in detoxication pathways is underscored by the fact that identified polymorphisms are associated with several metabolic diseases.

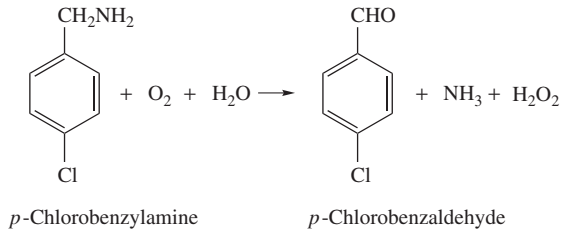
One especially interesting polymorphism is that which occurs at the aldehyde dehydrogenase 2 locus. When inherited as the homozygous trait, this aldehyde dehydrogenase polymorphism results in a 20-fold greater generation of acetaldehyde from ethanol, resulting in the flushing syndrome characteristic of many Asian individuals after ethanol consumption. Alcoholics are not likely to be found among individuals expressing this particular polymorphism.

Other enzymes in the soluble fraction of liver that oxidize aldehydes are aldehyde oxidase and xanthine oxidase, both flavoproteins that contain molybdenum; however, their primary role seems to be the oxidation of endogenous aldehydes formed as a result of deamination reactions.

Amine Oxidases The most important function of amine oxidases appears to be the oxidation of amines formed during normal processes. Two types of amine oxidases are concerned with oxidative deamination of both endogenous and exogenous amines. Typical substrates are shown in Figure 6.10.

Monoamine Oxidases The monomine oxidases are a family of flavoproteins found in the mitochondria of a wide variety of tissues: liver, kidney, brain, intestine, and blood platelets. They are a group of similar enzymes with overlapping specificities and inhibition. Although the enzyme in the central nervous system is concerned primarily with neurotransmitter turnover, that in the liver will deaminate primary, secondary, and tertiary aliphatic amines, reaction rates with the primary amines being faster. Electron-withdrawing substitutions on an aromatic ring increase the reaction rate, whereas compounds with a methyl group on the α-carbon such as amphetamine and ephedrine are not metabolized.

Diamine Oxidases Diamine oxidases are enzymes that also oxidize amines to aldehydes. The preferred substates are aliphatic diamines in which the chain length



(a) Monoamine oxidase

$$H_2N(CH_2)_5NH_2 + O_2 + H_2O \longrightarrow H_2N(CH_2)_4CHO + NH_3 + H_2O_2$$

Cadaverine

(b) Diamine oxidase

Figure 6.10 Examples of oxidations catalyzed by amine oxidases.

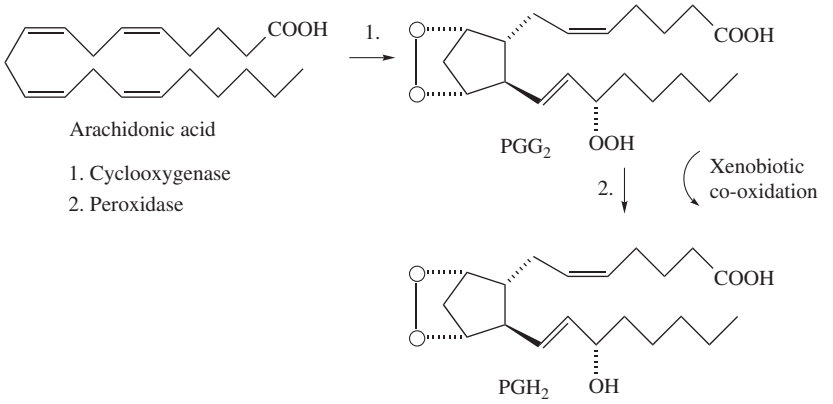


Figure 6.11 Co-oxidation during prostaglandin biosynthesis.

is four (putrescine) or five (cadaverine) carbon atoms. Diamines with carbon chains longer than nine will not serve as substrates but can be oxidized by monoamine oxidases. Secondary and tertiary amines are not metabolized. Diamine oxidases are typically soluble pyridoxal phosphate-containing proteins that also contain copper. They have been found in a number of tissues, including liver, intestine, kidney, and placenta.

6.2.5 Co-oxidation by Cyclooxygenase (COX)

During the biosynthesis of prostaglandins, a polyunsaturated fatty acid, such as arachidonic acid, is first oxygenated to yield a hydroperoxy endoperoxide, prostaglandin G2. This is then further metabolized to prostaglandin H2, both reactions being catalyzed by the same enzyme, COX, also known as prostaglandin synthase (Figure 6.11). This enzyme is located in the microsomal membrane and is found in greatest levels in respiratory tissues such as the lung. It is also common in the kidney

and seminal vesicle. It is a glycoprotein with a subunit molecular mass of about 70,000 Da, containing one heme per subunit. During the second step of the previous sequence (peroxidase), many xenobiotics can be co-oxidized, and investigations of the mechanism have shown that the reactions are hydroperoxide-dependent reactions catalyzed by a peroxidase that uses prostaglandin G as a substrate. In at least some of these cases, the identity of this peroxidase has been established as a prostaglandin synthase. Many of the reactions are similar or identical to those catalyzed by other peroxidases and also by microsomal monooxygenases; they include both detoxication and activation reactions. This mechanism is important in xenobiotic metabolism, particularly in tissues that are low in CYP and/or the FMO but high in prostaglandin synthase.

The COX enzyme is known to exist as two distinct isoforms. COX-1 is a constitutively expressed housekeeping enzyme found in nearly all tissues and mediates physiological responses. COX-2 is an inducible form expressed primarily by cells involved in the inflammatory response. Several tissues low in CYP expression are rich in COX, which is believed to have significance in the carcinogenic effects of aromatic amines in these organs.

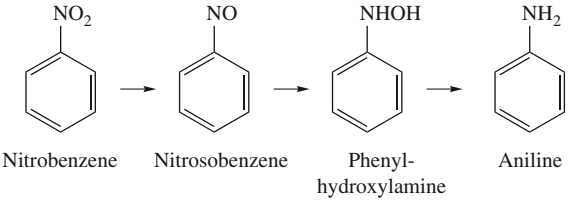
During co-oxidation, some substrates are activated to become more toxic than they were originally. In some cases, substrate oxidation results in the production of free radicals, which may initiate lipid peroxidation or bind to cellular proteins or DNA. Another activation pathway involves the formation of a peroxyl radical from subsequent metabolism of prostaglandin G2. This reactive molecule can epoxidize many substates including polycyclic aromatic hydrocarbons, generally resulting in increasing toxicity of the respective substrates.

To differentiate between xenobiotic oxidations by COX and CYP, *in vitro* microsomal incubations of the xenobiotic may be performed in the presence of either arachidonic acid (COX catalyzed), or in the presence of NADPH (CYP catalyzed). In the presence of arachidonic acid while in the absence of NADPH, substrates co-oxidized by COX will be formed while those requiring CYP will not. Specific inhibitors of PG synthase (indomethacin) and CYP (metyrapone or SKF 525A) have also been used.

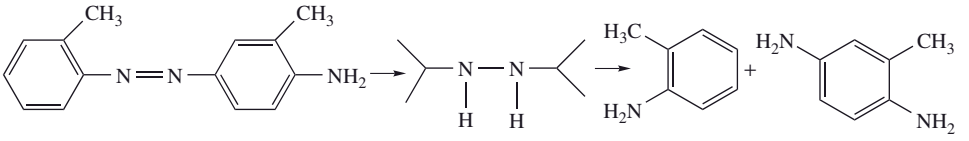
6.2.6 Reduction Reactions

A number of functional groups, such as nitro, diazo, carbonyl, disulfide sulfoxide, alkene, pentavalent arsenic, and so on, are susceptible to reduction, although in many cases it is difficult to tell whether the reaction proceeds enzymatically or nonenzymatically by the action of such biologic reducing agents as reduced flavins or reduced pyridine nucleotides. In some cases, such as the reduction of the double bond in cinnamic acid ($C_6H_5CH{=}CHCOOH$), the reaction has been attributed to the intestinal microflora. Examples of reduction reactions are shown in Figure 6.12.

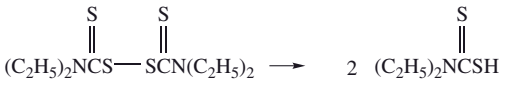
Nitro Reduction Aromatic amines are susceptible to reduction by both bacterial and mammalian nitroreductase systems. Convincing evidence has been presented that this reaction sequence is catalyzed by CYP. It is inhibited by oxygen, although NADPH is still consumed. Earlier workers had suggested a flavoprotein reductase was involved, and it is not clear if this is incorrect or if both mechanisms occur. It



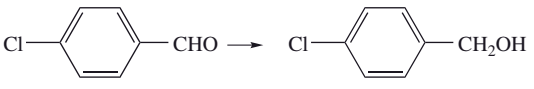
(a) Nitro reduction



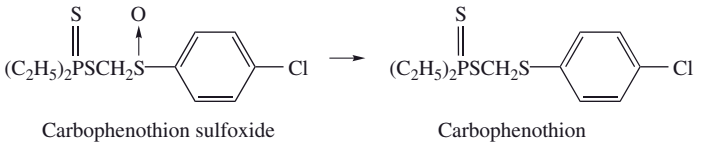
(b) Azo reduction



(c) Disulfide reduction



(d) Aldehyde reduction



(e) Sulfoxide reduction

Figure 6.12 Examples of metabolic reduction reactions.

is true, however, that high concentration of FAD or FMN will catalyze the nonenzymatic reduction of nitro groups.

Azo Reduction Requirements for azo reduction are similar to those for nitroreduction, namely, anaerobic conditions and NADPH. They are also inhibited by CO, and presumably they involve CYP. The ability of mammalian cells to reduce azo bonds is rather poor, and intestinal microflora may play a role.

Disulfide Reduction Some disulfides, such as the drug disulfiram (Antabuse), are reduced to their sulfhydryl constituents. Many of these reactions are three-step

sequences, the last reaction of which is catalyzed by glutathione reductase, using glutathione (GSH) as a cofactor.

$$\mathrm{RSSR + GSH \rightarrow RSSG + RSH}$$
$$\mathrm{RSSG + GSH \rightarrow GSSG + RSH}$$
$$\mathrm{GSSG + NADPH + H^{+} \rightarrow 2GSH + NADP^{+}}$$

Ketone and Aldehyde Reduction In addition to the reduction of aldehyde and ketones through the reverse reaction of alcohol dehydrogenase, a family of aldehyde reductases also reduces these compounds. These reductases are NADPH-dependent, cytoplasmic enzymes of low molecular weight, and have been found in liver, brain, kidney, and other tissues.

Sulfoxide Reduction The reduction of sulfoxides has been reported to occur in mammalian tissues. Soluble thioredoxin-dependent enzymes in the liver are responsible in some cases. It has been suggested that oxidation in the endoplasmic reticulum followed by reduction in the cytoplasm may be a form of recycling that could extend the *in vivo* half-life of certain toxicants.

6.2.7 Hydrolysis

Enzymes with carboxylesterase and amidases activity are widely distributed in the body, occurring in many tissues and in both microsomal and soluble fractions. They catalyze the following general reactions:

$\mathrm{RC(O)OR' + H_2O \rightarrow RCOOH + HOR'}$	Carboxylester hydrolysis
$\mathrm{RC(O)NR'R'' + H_2O \rightarrow RCOOH + HNR'R''}$	Carboxyamide hydrolysis
$\mathrm{RC(O)SR' + H_2O \rightarrow RCOOH + HSR'}$	Carboxythioester hydrolysis

Although carboxylesterases and amidases were thought to be different, no purified carboxylesterase has been found that does not have amidase activity toward the corresponding amide. Similarly, enzymes purified on the basis of their amidase activity have been found to have esterase activity. Thus, these two activities are now regarded as different manifestations of the same activity, specificity depending on the nature of R, R′, and R″ groups and, to a lesser extent, on the atom (O, S, or N) adjacent to the carboxyl group.

In view of the large number of esterases in many tissues and subcellular fractons, as well as the large number of substrates hydrolyzed by them, it is difficult to derive a meaningful classification scheme. The division into A-, B-, and C-esterases on the basis of their behavior toward such phosphate triesters as paraoxon, first devised by Aldridge, is still of some value, although not entirely satisfactory.

A-esterases, also referred to as arylesterases, are distinguished by their ability to hydrolyze esters derived from aromatic compounds. Organophosphates, such as the paraoxon, the active metabolite of the insecticide, parathion, are often used to characterize this group. B-esterases, the largest and most important group, are inhibited by organophosphates. All the B-esterases have a serine residue in their

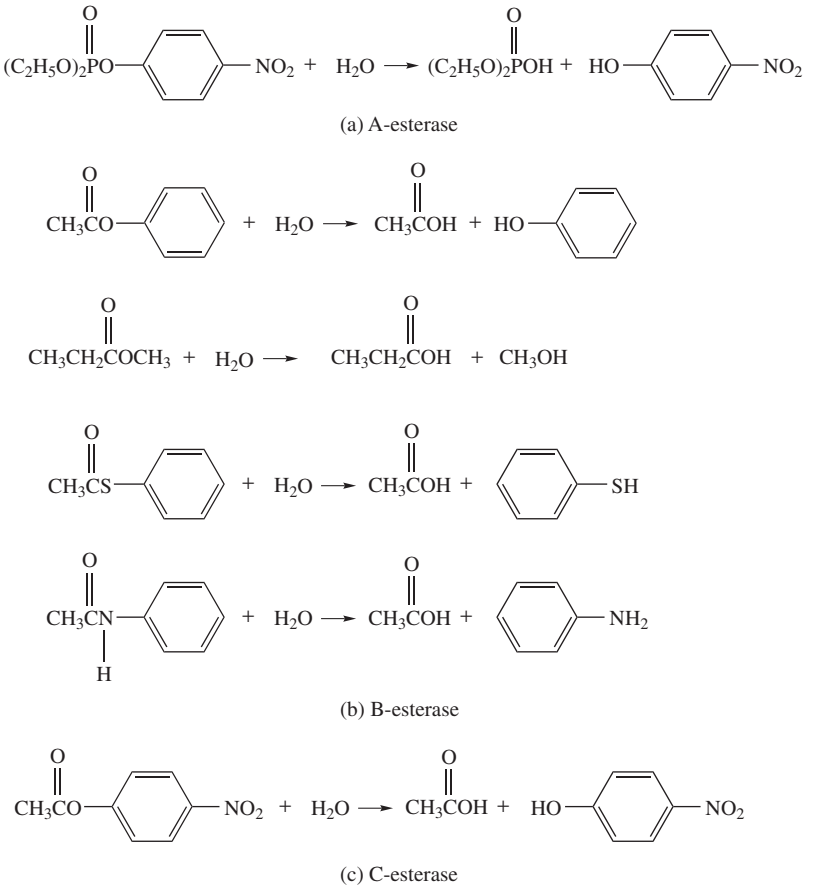


Figure 6.13 Examples of esterase/amidase reactions involving xenobiotics.

active site that is phosphorylated by these inhibitors. This group includes a number of different enzymes and their isozymes, many of which have quite different substrate specificities. For example, the group contains carboxylesterase, amidases, cholinesterases, monoacylglycerol lipases, and arylamidases. Many of these enzymes hydrolyze physiological (endogenous) substrates as well as xenobiotics. Several examples of their activity toward xenobiotic substrates are shown in Figure 6.13. C-esterases, or acetylesterases, are defined as those esterases that prefer acetyl esters as substrates, and for which paraoxon serves as neither substrate nor inhibitor.

Two esterases that have received considerable attention in recent years are the carboxylesterase that hydrolyzes pyrethroid insecticides and PON1 (paraoxonase 1). In the first case, the esterase that hydrolyzes pyrethroids into acid and alcohol moieties has been of particular interest, particularly the human forms, hCE1 and hCE2. While the levels of each are only slightly variable in human liver microsomes, the level of hCE1 is some 46 times higher than of hCE2. While pyrethroids such as bioresmethrin are substrates for hCE1 but not hCE2, procaine is a substrate for hCE2 but not hCE1. It has also been shown that these carboxylases have

endogenous substrates such as cholesterol esters and triglycerides and are important in cardiovascular function and health. Cholesteryl ester hydrolase and CE1 appear to be the same enzyme.

Paraxonase, originally named for its ability to hydrolyze paraoxon, has been shown to have broad substrate specificity as well as a role in the risk of vascular disease in humans. The form known as PON1 has been characterized with regard to its ability to hydrolyze paraoxon, diazoxon, chlorpyrifos oxon, and the chemical warfare agents, sarin and soman, all substrates. PON1 is a plasma enzyme associated with high-density lipoproteins that is also found in the liver. PON1 is polymorphic, and the GLn/Arg substitution at position 192 affects the catalytic activity. However, in addition to the variable catalytic activity determined by this polymorphism, the levels of PON1 vary as much as 15-fold between individuals. As a result, PON1 status cannot be assessed by genotyping alone; the determination of the rates of hydrolysis of different substrates (e.g., diazoxon vs. paraxon) relative to each other is also essential.

6.2.8 Epoxide Hydration

Epoxide rings of alkene and arene compounds are hydrated by enzymes known as epoxide hydrolases, the animal enzyme forming the corresponding *trans*-diols, although bacterial hydrolases are known that form *cis*-diols. Although in general, the hydration of the oxirane ring results in detoxication of the very reactive epoxide, in some cases, such as benzo(a)pyrene, the hydration of an epoxide is the first step in an activation sequence that ultimately yields highly toxic *trans*-dihydrodiol intermediates. In others, reactive epoxides are detoxified by both glutathione transferase and epoxide hydrolase. The reaction probably involves a nucleophilic attack by -OH on the oxirane carbon. The most studied epoxide hydrolase is microsomal, and the enzyme has been purified from hepatic microsomes of several species. Although less well-known, soluble epoxide hydrolases with different substrate specificities have also been described. Examples of epoxide hydrolase reactions are shown in Figure 6.14.

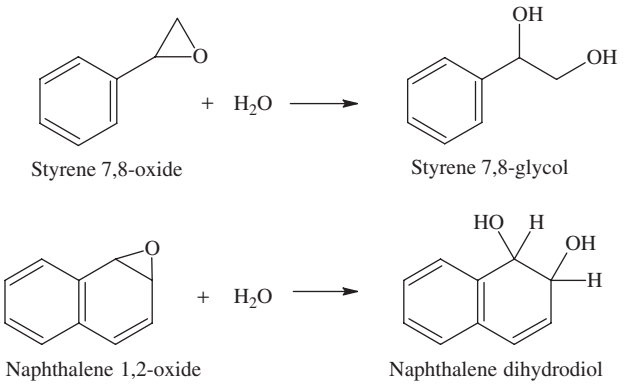


Figure 6.14 Examples of epoxide hydrolase reactions.

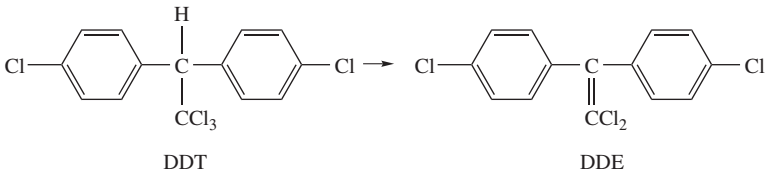


Figure 6.15 DDT-dehydrochlorinase.

6.2.9 DDT Dehydrochlorinase

DDT (1,1,1-trichloro-2,2-bis(4-chlorophenylethane))-dehydrochlorinase is an enzyme that occurs in both mammals and insects and has been studied most intensively in DDT-resistant houseflies. It catalyzes the dehydrochlorination of DDT to DDE (1,1-dichloro-bis(4-chlorophenyl) ethane) and occurs in the soluble fraction of tissue homogenates. Although the reaction requires GSH, it apparently serves in a catalytic role because it does not appear to be consumed during the reaction. The Km for DDT is 5×10^{-7} mol/L with optimum activity at pH 7.4. The monomeric form of the enzyme has a molecular mass of about 36,000 Da, but the enzyme normally exists as a tetramer. In addition to catalyzing the dehydrochlorination of DDT to DDE and DDD (2,2-bis(*p*-chlorophenyl)-1,1 -dichloroethane) to TDE (2,2-bis(*p*-chlorophenyl)-1-chlorothylen), DDT dehydrochlorinase also catalyzes the dehydrohalogenation of a number of other DDT analogs. In all cases, the p,p configuration is required, o,p and other analogs are not utilized as substrates. The reaction is illustrated in Figure 6.15.

6.3 PHASE II REACTIONS

Products of Phase I metabolism and other xenobiotics containing functional groups such as hydroxyl, amino, carboxyl, epoxide, or halogen can undergo conjugation reactions with endogenous metabolites, these conjugations being collectively termed Phase II reactions. The endogenous metabolites in question include sugars, amino acids, GSH, sulfate, and so on. Conjugation products, with rare exceptions, are more polar, less toxic, and more readily excreted than are their parent compounds.

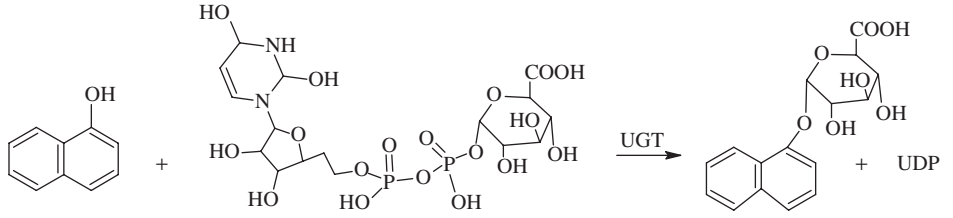
Conjugation reactions usually involve activation by some high-energy intermediate and have been classified into two general types: type I, in which an activated conjugating agent combines with the substrate to yield the conjugated product, and type II, in which the substrate is activated and then combines with an amino acid to yield a conjugated product. The formation of sulfates and glycosides are examples of type I, whereas type II consists primarily of amino acid conjugation.

6.3.1 Glucuronide Conjugation

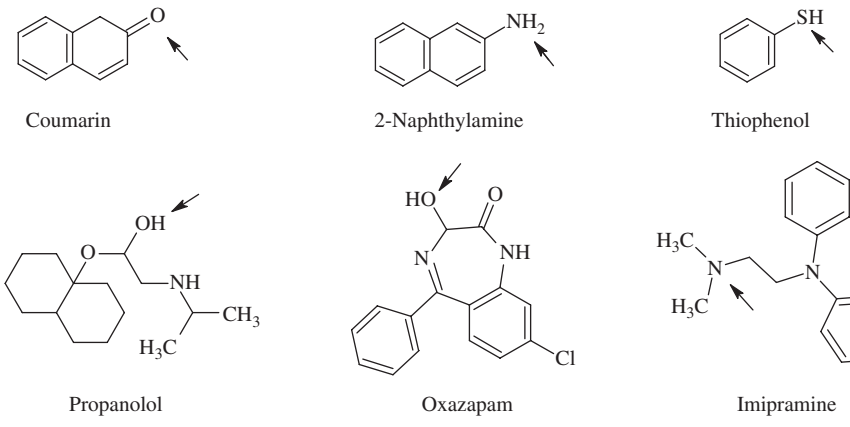
The glucuronidation reaction is one of the major pathways for elimination of many lipophilic xenobiotics and endobiotics from the body. The mechanism for this conjugation involves the reaction of one of many possible functional groups (R-OH, Ar-OH, R-NH2, AR-NH2, R-COOH, Ar-COOH) with the sugar derivative, uridine

5′-diphosphoglucuronic acid (UDPGA). Homogeneous glucuronosyl transferase has been isolated as a single polypeptide chain of about 59,000 Da, apparently containing carbohydrate, the activity of which appears to be dependent on reconstitution with microsomal lipid. There appears to be an absolute requirement for UDPGA; related UDP-sugars will not suffice. This enzyme, as it exists in the microsomal membrane, does not exhibit its maximal capacity for conjugation; activation by some means (e.g., detergents) is required. The reaction involves a nucleophilic displacement (SN^2 reaction) of the functional group of the substrate with Walden inversion. UDPGS is in the α-configuration whereas, due to the inversion, the glucuronide formed is in the β-configuration. The enzyme involved, the UDP glucuronosyl transferase (UGT), is found in the microsomal fraction of liver, kidney, intestine, and other tissues. Examples of various types of glucuronides are shown in Figure 6.16.

Glucuronide conjugation generally results in the formation of products that are less biologically and chemically reactive. This, combined with their greater polarity and greater susceptibility to excretion, contributes greatly to the detoxication of most xenobiotics. However, there are examples of glucuronide conjugation resulting in greater toxicity. Perhaps the best-known example involves the bioactivation



(a) Reaction sequence



(b) Substrate examples

Figure 6.16 Reaction sequences of uridine diphospho glucuronosyl transferase and chemical structures of compounds that form glurcuronides. Arrows indicate the position on each molecule where glucuronidation occurs.

of *N*-hydroxy-2-acetylaminofluorine. This substrate, unlike 2-acetylaminofluorine, is unable to bind to DNA without metabolic activation. However, following glucuronide conjugation by linkage of the oxygen through the *N*-hydroxy group, this substrate becomes equipotent as a hepatocarcinogen with 2-acetylaminofluorine based on its ability to bind to DNA. Another relatively large class of xenobiotics that are often activated by glucuronide conjugation are the acyl glucuronides of carboxylic acids. Useful therapeutic drugs within this class include nonsteroidal anti-inflammatory drugs (NSAIDS), hypolipidemic drugs (clofibrate), and anticonvulsants (valproic acid). The various syndromes associated with the clinical use of some of these drugs (including cytotoxic, carcinogenic, and various immunologic effects) are thought to be the result of the ability of the glucuronide conjugates to react with nucleophilic macromolecules (protein and DNA).

A wide variety of reactions are mediated by glucuronosyltransferases. *O*-glucuronides, *N*-glucuronides, and *S*-glucuronides have all been identified. At this time, over 35 different UGT gene products have been described from several different species. These are responsible for the biotransformation of greater than 350 different substrates. Evidence from molecular cloning suggests that the UGTs belong to one of two large superfamilies, sharing less than 50% amino acid identity. Nomenclature of these genes is similar to that of the CYP superfamily. The UGT1 gene family consists of a number of UGTs that arise from alternate splicing of multiple first exons and share common exons 2–5. Members of the UGT2 family catalyze the glucuronidation of a wide variety of substrates including steroids, bile acids, and opioids.

There are nine known human isozymes within the UGT1 family and six within the UGT2 family. Polymorphic forms of some of these enzymes are associated with diseases and significant adverse effects to some drugs.

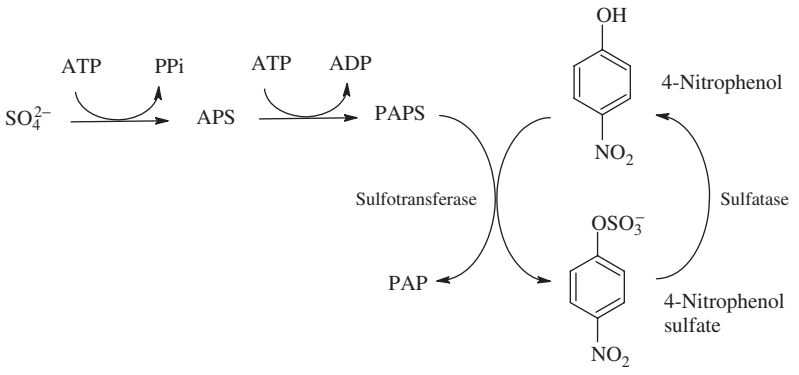
Jaundice, a condition resulting from the failure of either transport or conjugation of bilirubin, becomes clinically evident when serum bilirubin levels exceed 35 μM/L. Although the human UGT1A locus encompasses nine functional transferase genes, only one isoform, UGT1A1, is involved in inherited diseases of bilirubin metabolism. All three inheritable hyperbilirubinemias are the result of either mutant UGT1A1 alleles or UGT1A1 promoter polymorphisms. To date, 33 mutant UGT1A1 alleles have been identified. For the disease to be clinically manifest, one must either be homozygous for the mutant allele or have multiple heterozygous mutant alleles.

6.3.2 Glucoside Conjugation

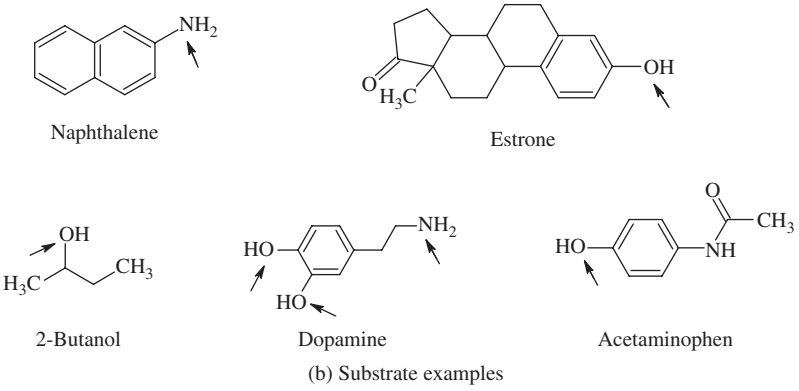
Although rare in vertebrates, glucosides formed from xenobiotics are common in insects and plants. Formed from UDP-glucose, they appear to fall into the same classes as the glucuronides.

6.3.3 Sulfate Conjugation

Sulfation and sulfate conjugate hydrolysis, catalyzed by various members of the sulfotransferases (SULT) and sulfatase enzyme superfamilies, play important roles in the metabolism and disposition of many xenobiotics and endogenous substrates. Reactions of the sulfotransferase enzyme with various xenobiotics including



(a) Reaction sequence



(b) Substrate examples

Figure 6.17 Reaction sequence of sulfotransferases and chemical structures of compounds that form sulfates. Arrows indicate positions on each molecule where sulfotransferases may attack.

alcohols, arylamines, and phenols result in the production of water-soluble sulfate esters, which often are readily eliminated from the organism. Although generally these reactions are important in detoxication, they have also been shown to be involved in carcinogen activation, prodrug processing, cellular signaling pathways, and the regulation of several potent endogenous chemicals including thyroid hormones, steroids, and catechols. The overall sulfation pathway shown in Figure 6.17 consists of two enzyme systems: the SULTs, which catalyze the sulfation reaction, and the sulfatases, which catalyze the hydrolysis of sulfate esters formed by the action of the SULTs.

Sulfation is expensive in energy terms for the cell, since two molecules of ATP are necessary for the synthesis of one molecule of 3′-phosphoadenosine 5′-phosphosulfate (PAPS). Both enzymes involved in the synthesis of PAPS, ATP sulfurylase and adenosine phosphosulfate (APS) kinase, reside within a single bifunctional cytosolic protein of approximately 56 kDa, where substrate channeling of APS from ATP sulfurylase to APS kinase occurs. Several group VI anions other than sulfate can also serve as substrates, although the resultant anhydrides are unstable. Because

this instability would lead to the overall consumption of ATP, these other anions can exert a toxic effect by depleting the cell of ATP.

In humans, there are five well-characterized SULT genes, each possessing widely different amino acid sequences and with widely different substrate specificities. Based on amino acid sequence identity as well as substrate preference, these can be separated into two families, phenol SULTs (P-PST, SULT1A2, M-PST, and EST) and hydroxysteroid sulfotransferase (HST). Phenol SULTs from rat liver have been separated into four distinct forms, each of which catalyzes the sulfation of various phenols and catecholamines. They differ, however, in pH optimum, relative substrate specificity, and immunologic properties. The molecules of all of them are in the range of 61,000–64,000 Da.

HST also appears to exist in several forms. This reaction is now known to be important, not only as a detoxication mechanism, but also in the synthesis and possibly the transport of steroids. HST will react with hydroxysterols and primary and secondary alcohols but not with hydroxyl groups in the aromatic rings of steroids.

6.3.4 Methyltransferases

A large number of both endogenous and exogenous compounds can be methylated by several *N*-, *O*-, and *S*-methyltransferases. The most common methyl donor is *S*-adenosylmethionine (SAM), formed from methionine and ATP. Even though these reactions may involve a decrease in water solubility, they are generally detoxication reactions. Examples of biologic methylation reactions are seen in Figure 6.18.

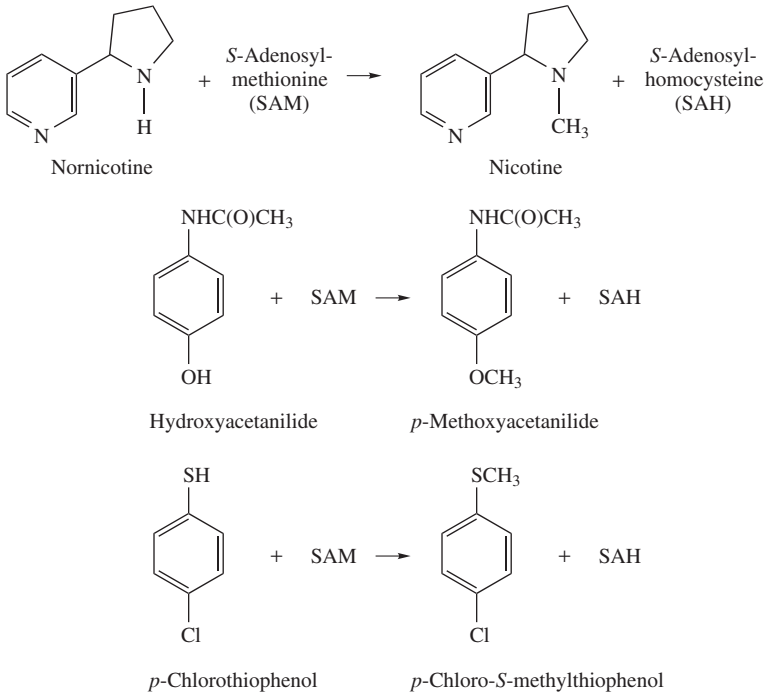


Figure 6.18 Examples of methyltransferase reactions.

N-*Methylation* Several enzymes are known that catalyze N-methylation reactions. They include histamine *N*-methyltransferase, a highly specific enzyme that occurs in the soluble fraction of the cell, phenylethanolamine *N*-methyltransferase, which catalyzes the methylation of noradrenaline to adrenaline as well as the methylation of other phenylethanolamine derivatives. A third *N*-methyltransferase is the indoethylamine *N*-methyltansferase, or nonspecific *N*-methyltransferase. This enzyme occurs in various tissues. It methylates endogenous compounds such as serotonin and tyrptamine and exogenous compounds such as nornicotine and norcodeine. The relationship between this enzyme and phenylethanolamine *N*-methyltransferase is not yet clear.

O-*Methylation* Catechol *O*-methyltransferase occurs in the soluble fraction of several tissues and has been purified from rat liver. The purified form has a molecular weight 23,000 Da, requires *S*-adenosylmethionine and Mg^+, and catalyzes the methylation of epinephrine, norepinephrine, and other catechol derivatives. There is evidence that this enzyme exists in multiple forms.

A microsomal *O*-methyltransferase that methylates a number of alkyl-, methoxy-, and halophenols has been described from rabbit liver and lungs. These methylations are inhibited by SKF-525, *N*-ethylmaleimide and *p*-chloromercuribenzoate. A hydroxyindole *O*-methyltransferase, which methylates *N*-acetylserotonin to melatonin and, to a lesser extent, other 5-hydroxyindoles and 5,6-dihydroxyindoles, has been described from the pineal gland of mammals, birds, reptiles, amphibians, and fish.

S-*Methylation* Thiol groups of some foreign compounds are also methylated, the reaction being catalyzed by the enzyme, thiol *S*-methyltransferase. This enzyme is microsomal and, as with most methyltransferases, utilizes *S*-adenosylmethionine. It has been purified from rat liver and is a monomer of about 28,000 Da. A wide variety of substrates are methylated, including thioacetanilide, mercaptoethanol, and diphenyl sulfide. This enzyme may also be important in the detoxication of hydrogen sulfide, which is methylated in two steps, first to the highly toxic methanethiol and then to dimethyl sulfide.

Methylthiolation, or the transfer of a methylthio (CH_3S–) group to a foreign compound may occur through the action of another recently discovered enzyme, cysteine conjugate β-lyase. This enzyme acts on cysteine conjugates of foreign compounds as follows:

$$RSCH_2CH(NH_2)COOH \rightarrow RSH + NH_3 + CH_3C(O)COOH$$

The thiol group can then be methylated to yield the methylthio derivative of the original xenobiotic.

Biomethylation of Elements The biomethylation of elements is carried out principally by microorganisms and is important in environmental toxicology, particularly in the case of heavy metals, because the methylated compounds are absorbed through the membranes of the gut, the blood–brain barrier, and the placenta more readily than are the inorganic forms. For example, inorganic mercury can be methylated first to monomethylmercury and subsequently, to dimethylmercury.

$$Hg^{2+} \rightarrow CH_3HG^{+} \rightarrow (CH_3)_2\,Hg$$

The enzymes involved are reported to use either *S*-adenosylmethionine or vitamin B_{12} derivatives as methyl donors and, in addition to mercury, the metals, lead, tin, and thallium as well as the metalloids, arsenic, selenium, tellurium, and sulfur, are methylated. Even the unreactive metals, gold and platinum, are reported as substrates for these reactions.

6.3.5 GSTs and Mercapturic Acid Formation

Although mercapturic acids, the *N*-acetylcysteine conjugates of xenobiotics, have been known since the early part of the twentieth century, only since the early 1960s has the source of the cysteine moiety (GSH) and the enzymes required for the formation of these acids been identified and characterized. The overall pathway is shown in Figure 6.19.

The initial reaction is the conjugation of xenobiotics having electrophilic substituents with GSH, a reaction catalyzed by one of the various forms of GST. This is followed by transfer of the glutamate by γ-glutamyltanspeptidase, by loss of glycine through cysteinyl glycinase, and finally by acetylation of the cysteine amino group. The overall sequence, particularly the initial reaction is extremely important in toxicology because, by removing reactive electrophiles, vital nucleophilic groups in macromolecules such as proteins and nucleic acids are protected. The mercapturic acids formed can be excreted either in the bile or in the urine.

The GSTs, the family of enzymes that catalyzes the initial step, are widely distributed, being found in essentially all groups of living organisms. Although the best-known examples have been described from the soluble fraction of mammalian liver, these enzymes have also been described in microsomes. All forms appear to be highly specific with respect to GSH but nonspecific with respect to xenobiotic

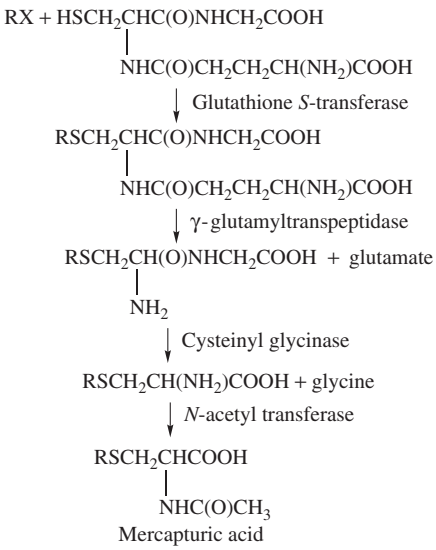


Figure 6.19 Glutathione transferase reaction and formation of mercapturic acids.

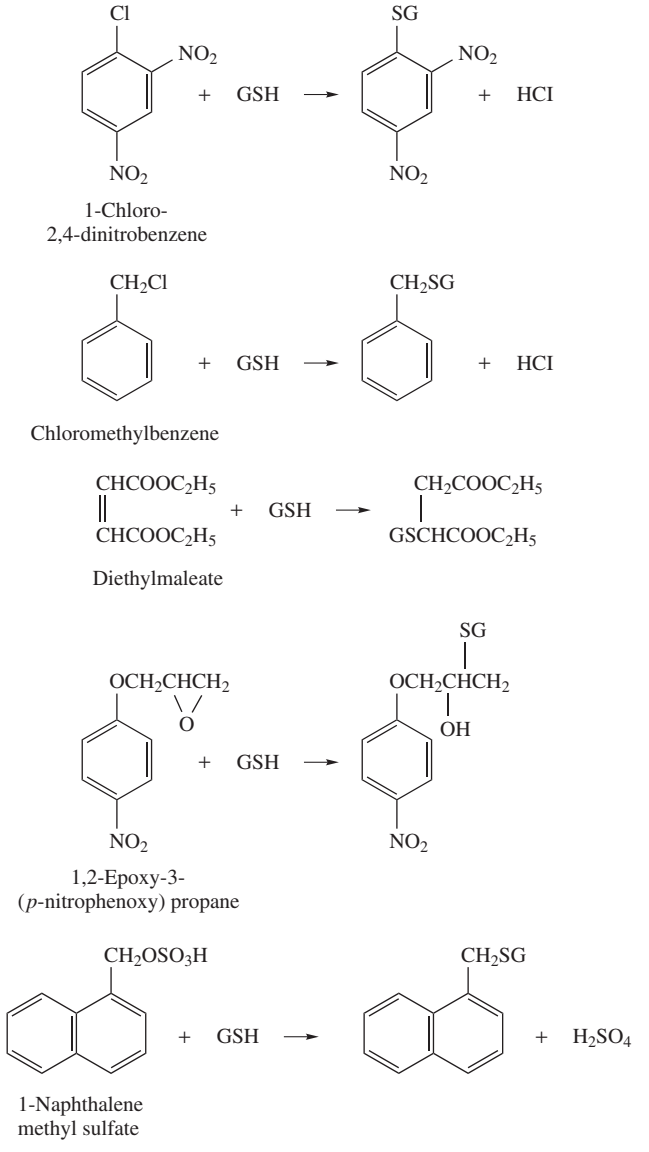


Figure 6.20 Examples of glutathione transferases reactions.

substrates, although the relative rates for different substrates can vary widely from one form to another. The types of reactions catalyzed include the following: alkyltransferase, aryltransferase, aralkyltransferase, alkenetransferase, and epoxidetransferase. Examples are shown in Figure 6.20.

Multiple forms of GST have been demonstrated in the liver of many mammalian species; multiple forms also occur in insects. Most GSTs are soluble dimeric proteins with molecular weights ranging between 45,000–50,000 Da. All forms appear to be nonspecific with respect to the reaction types described, although the kinetic constants for particular substrates vary from one form to another. They are usually

named from their chromatographic behavior. At least two are membrane bound glutathione transferases, one of which is involved in metabolism of xenobiotics and is designated the microsomal GST. The cytosolic GSTs are divided into six families (historically called classes): the α (alpha), κ (kappa), μ (mu), π (pi), σ (sigma), and θ (theta) families. A new system of nomenclature proposes the term GST for the enzyme, preceded by the use of a small roman letter for the species (m for mouse, h for humans, etc.) followed by a capital roman letter for the family (A for α, K for κ, etc.). Subunits are to be designated by Arabic numbers, with the two subunits represented with a hyphen between them. For example, hGSTM1-2 designates a heterodimer of the human family mu which possesses subunits one and two.

Glutathione conjugation dramatically increases the water solubility of the metabolites compared to the parent compounds. The metabolites are released from the cell by an active transport system belonging to the multidrug resistance protein (mdr). Prior to excretion, the metabolites are usually processed by multiple enzymes to release the substrate conjugated to a mercapturic acid (Figure 6.19). The enzymes involved in this process are γ-glutamyltranspeptidase, cysteinyl glycinase, and *N*-acetyltransferase.

γ-Glutamyltranspeptidase is a membrane-bound glycoprotein that has been purified from both the liver and kidney of several species. Molecular weights for the kidney enzyme are in the range of 68,000–90,000 Da, and the enzyme appears to consist of two unequal subunits; the different forms appear to differ in the degree of sialylation. This enzyme, which exhibits wide specificity toward γ-glutamyl peptides and has a number of acceptor amino acids, catalyzes two types of reactions:

Hydrolysis	γ-Glu-R + H_2O	Glu + HR
Transpeptidation	γ-Glu-R + Acceptor	γ-Glu-Acceptor + HR
	γ-Glu-r + γ-Glu-R	γ-Glu γ-Glu-R + HR

Aminopeptidases that catalyze the hydrolysis of cysteinyl peptides are known. The membrane-bound aminopeptidases are glycoproteins, usually with molecular weights of about 100,000 Da. They appear to be metalloproteins, one of the better known being a zinc-containing enzyme. Other enzymes, such as the leucine aminopeptidase, are cytosolic, but, at least in this case, are also zinc containing. The substrate specificity of these enzymes varies, but most are relatively nonspecific.

Little is known of the *N*-acetyltransferase(s) responsible for the acetylation of the S-substituted cysteine. It is found in the microsomes of the kidney and the liver, however, and is specific for acetyl CoA as the actyl donor. It is distinguished from other *N*-acetyltransferases by its substrate specificity and subcellular location.

6.3.6 Cysteine Conjugate β-Lyase

This enzyme uses cysteine conjugates as substrates, releasing the thiol of the xenobiotic, pyruvic acid, and ammonia; with subsequent methylation giving rise to the methylthio derivative, the enzyme from the cytosolic fraction of rat liver is pyridoxal phosphate requiring protein of about 175,000 Da. Cysteine conjugates of aromatic compounds are the best substrates, and it is necessary for the cysteine amino and carboxyl groups to be unsubstituted for enzyme activity.

6.3.7 Acylation

Acylation reactions are of two general types, the first involving an activated conjugation agent, coenzyme A (CoA), and the second involving activation of the foreign compounds and subsequent acylation of an amino acid. This type of conjugation is commonly undergone by exogenous carboxylic acids and amides and, although the products are often less water soluble than the parent compound, they are usually less toxic. Examples of acylation reactions are shown in Figure 6.21.

Acetylation Acetylated derivatives of foreign exogenous amines are acetylated by *N*-acetyltransferase, the acetyl donor being CoA. This enzyme is cytosolic, has been purified from rat liver, and is known to occur in several other organs. Evidence exists for the existence of multiple forms of this enzyme. Although endogenous amino, hydroxy, and thiol compounds are acetylated *in vivo*, the acetylation of exogenous hydroxy and thiol groups is presently unknown.

Acetylation of foreign compounds is influenced by both development and genetics. Newborn mammals generally have a low level of the transferase whereas, due to the different genes involved, fast and slow acetylators have been identified in both rabbit and human populations. Slow acetylators are more susceptible to the effects of compounds detoxified by acetylation.

N, O-*acyltransferase* The *N*-acyltransferase enzyme is believed to be involved in the carcinogenicity of arylamines. These compounds are first N-oxidized, and then, in species capable of their N-acetylation, acetylated to arylhydroxamic acids. The effect of *N*-, *O*-transacetylation is shown in Figure 6.22. The *N*-acyl group of

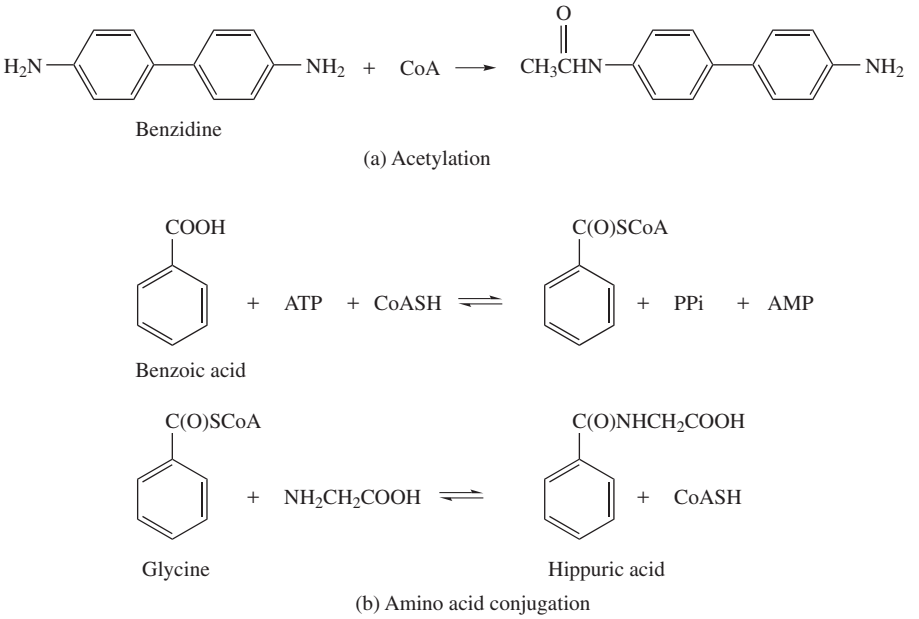


Figure 6.21 Examples of acylation reactions.

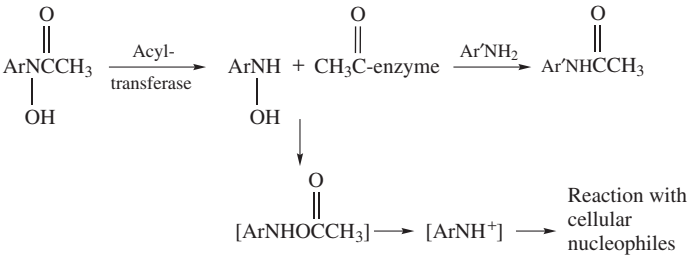


Figure 6.22 *N-, O*-acyltransferase reactions of arylhydroxamic acid. Ar, aryl group.

the hydroxamic acid is first removed and is then transferred, either to an amine to yield a stable amide, or to the oxygen of the hydroxylamine to yield a reactive *N*-acyloxyarylamine. These compounds are highly reactive in the formation of adducts with both proteins and nucleic acids, and *N, O*-acyltransferase, added to the medium in the Ames test, increases the mutagenicity of compounds such as *N*-hydroxy-2-acetylaminofluorene. In spite of its great instability, this enzyme has been purified from the cytosolic fraction of the rat liver.

Amino Acid Conjugation In the second type of acylation reaction, exogenous carboxylic acids are activated to form S-CoA derivatives in a reaction involving ATP and CoA. These CoA derivatives then acylate the amino group of a variety of amino acids. Glycine and glutamate appear to be the most common acceptor of amino acids in mammals. In other organisms, other amino acids are involved. These include ornithine in reptiles and birds and taurine in fish.

The activating enzyme occurs in the mitochondria and belongs to a class of enzymes known as the ATP-dependent acid: CoA ligases but has also been known as acyl CoA synthetase and acid-activating enzyme. It appears to be identical to the intermediate chain length fatty acyl–CoA-synthetase.

Two acyl-CoA:amino acid *N*-acyltransferases have been purified from liver mitochondria of cattle, Rhesus monkeys, and humans. One is a benzoyltransferase CoA that utilizes benzyl-CoA, isovaleryl-CoA, and tiglyl-CoA, but not phenylacetyl CoA, malonyl-CoA, or indolacetyl-CoA. The other is a phenylacetyl transferase that utilizes phenylacetyl-CoA and indolacetyl-CoA but is inactive toward benzoyl-CoA. Neither is specific for glycine, as had been supposed from studies using less defined systems; both also utilize asparagine and glutamine, although at lesser rates than glycine.

Bile acids are also conjugated by a similar sequence of reactions involving a microsomal bile acid: CoA ligase and a soluble bile acid *N*-acyltransferase. The latter has been extensively purified, and differences in acceptor amino acids, of which taurine is the most common, have been related to the evolutionary history of the species.

Deacetylation Deacetylation occurs in a number of species, but there is a large difference between species, strains, and individuals in the extent to which the reaction occurs. Because acetylation and deacetylation are catalyzed by different enzymes, the levels of which vary independently in different species, the importance

of deacetylation as a xenobiotic-metabolizing mechanism also varies between species. This can be seen in a comparison of the rabbit and the dog. The rabbit, which has high acetyltransferase activity and low deacetylase, excretes significant amounts of acetylated amines. The dog, in which the opposite situation obtains, does not.

A typical substrate for the aromatic deacetylases of the liver and kidney is acetanilide, which is deacylated to yield aniline.

6.3.8 Phosphate Conjugation

Phosphorylation of xenobiotics is not a widely distributed conjugation reaction, insects being the only major group of animals in which it is found. The enzyme from the gut of cockroaches utilizes ATP, requires MG^{+}, and is active in the phosphorylation of 1-naphthol and *p*-nitrophenol.

BIBLIOGRAPHY AND SUGGESTED READING

Benedetti, M. S. Biotransformation of xenobiotics by amine oxidases. *Fundam. Clin. Pharmacol.* **15**:75–84, 2001.

Cashman, J. R. and J. Zhang. Human flavin-containing monooxygenases. *Annu. Rev. Pharmacol. Toxicol.* **46**:65–100, 2006.

Coughtrie, M. W. H., S. Sharp, K. Maxwell, et al. Biology and function of the reversible sulfation pathway catalyzed by human sulfotransferases and sulfatases. *Chem. Biol. Interact.* **109**:3–27, 1998.

Duffel, M. W., A. D. Marshall, P. McPhie, et al. Enzymatic aspects of the phenol (aryl) sulfotransferases. *Drug Metab. Rev.* **33**:369–395, 2001.

Guengerich, F. P. Cytochrome P450 and chemical toxicology. *Chem. Res. Toxicol.* **21**:70–83, 2008.

Hayes, J. D. and D. J. Pulford. The glutathione S-transferase supergene family: Regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. *Crit. Rev. Biochem. Mol. Biol.* **30**:445–600, 1995.

Hodgson, E., P. K. Das, T. M. Cho, et al. Phase 1—Metabolism of toxicants and metabolic interactions. In *Molecular and Biochemical Toxicology*, 4th ed., ed. R. C. Smart and E. Hodgson. Hoboken, NJ: John Wiley and Sons, 2008.

Koukouritaki, S. B., P. Simpson, C. K. Yeung, et al. Human hepatic flavin-containing monooxygenases 1 (FMO1) and 3 (FMO3) developmental expression. *Pediatric. Res.* **51**:236–243, 2002.

Lawton, M. P., J. R. Cashman, T. Cresteil, et al. A nomenclature for the mammalian flavin-containing monooxygenase gene family based on amino acid sequence identities. *Arch. Biochem. Biophys.* **308**:254–257, 1994.

LeBlanc, G. A. Phase II—Conjugation of toxicants. In *Molecular and Biochemical Toxicology*, 4th ed., ed. R. C. Smart and E. Hodgson. Hoboken, NJ: John Wiley and Sons, 2008.

Nebert, D. W., D. R. Nelson, M. J. Coon, et al. Superfamily—update on new sequences, gene-mapping, and recommended nomenclature. *DNA Cell Biol.* **10**:1–4, 1991.

Nelson, D. R. Comparison of P450s from human and fugu: 420 million years of vertebrate P450 evolution. *Arch. Biochem. Biophys.* **409**:18–24, 2003.

Ortiz de Montellano, P. R., ed. *Cytochrome P450: Structure, Mechanism and Biochemistry*. New York: Springer, 2004.

Richter, R., G. P. Jarvik, and C. E. Furlong. Paraoxonase 1 (PON1) status and substrate hydrolysis. *Toxicol. Appl. Pharmacol.* **235**:1–9, 2009.

Ritter, J. K. Roles of glucuronidation and UDP-glucuronosyltransferases in xenobiotic bioactivation reactions. *Chem. Biol. Interact.* **129**:171–193, 2001.

Ross, M. K. and J. A. Crow. Human carboxylases and their role in xenobiotic and endobiotic metabolism. *J. Biochem. Mol. Toxicol.* **21**:187–196, 2007.

Smart, R. C. and E. Hodgson, eds. *Molecular and Biochemical Toxicology*, 4th ed. Hoboken, NJ: John Wiley and Sons, 2008.

Tukey, R. H. and C. P. Strassburg. Human UDP-glucuronosyltransferases: Metabolism, expression, and disease. *Annu. Rev. Pharmacol. Toxicol.* **40**:581–616, 2000.

Vasiliou, V., A. Pappa, and D. R. Petersen. Role of aldehyde dehydrogenases in endogenous and xenobiotic metabolism. *Chem. Biol. Interact.* **129**:1–19, 2000.

Zeldin, D. C. and J. M. Seubert. Structure, mechanism and regulation of cytochromes P450. In *Molecular and Biochemical Toxicology*, 4th ed., ed. R. C. Smart and E. Hodgson. Hoboken, NJ: John Wiley and Sons, 2008.

SAMPLE QUESTIONS

1. Compare and contrast the following terms:
   a. Cytochrome P450 (CYP) and flavin-containing monooxygenase (FMO);
   b. Induction and activation of xenobiotic metabolizing enzymes (XMEs);
   c. Phase I and Phase II xenobiotic metabolism.

2. Give examples of the following:
   a. Epoxidation;
   b. O-Dealkylation;
   c. Desulfuration and ester cleavage.

3. Name the cofactors involved in the following xenobiotic conjugations:
   a. Mercapturic acid formation;
   b. Glucuronic acid formation;
   c. Sulfation;
   d. Methylation.

4. Outline the combined action of alcohol dehydrogenase and aldehyde dehydrogenase on ethanol.

5. Describe how the action of cysteine conjugate β-lyase can lead to methylthiolation of a xenobiotic.

CHAPTER 7

Reactive Metabolites

ERNEST HODGSON and RANDY L. ROSE*

7.1 INTRODUCTION

Many xenobiotics that are relatively inert undergo metabolism to highly reactive intermediates. These metabolites may interact with cellular constituents in numerous ways, such as binding covalently to macromolecules and/or stimulating lipid peroxidation to produce toxic effects, or they may be detoxified and the products excreted. This biotransformation of relatively inert chemicals to highly reactive intermediary metabolites is commonly referred to as metabolic activation or bioactivation and is often the initial event in chemically induced toxicities. Some toxicants are direct acting and require no activation, whereas other chemicals may be activated nonenzymatically. The focus of this chapter, however, relates to toxicants requiring metabolic activation and to the processes involved in activation.

In the 1940s and 1950s, the pioneering studies of James and Elizabeth Miller provided early evidence for *in vivo* conversion of chemical carcinogens to reactive metabolites. They found that reactive metabolites of the aminoazo dye *N,N*-dimethyl-4-aminoazobenzene, a hepatocarcinogen in rats, would bind covalently to proteins and nucleic acids. The term, metabolic activation, was coined by the Millers to describe this process. Moreover, they demonstrated that covalent binding of these chemicals was an essential part of the carcinogenic process.

The overall scheme of metabolism for potentially toxic xenobiotics is outlined in Figure 7.1. As illustrated by this diagram, xenobiotic metabolism can produce not only nontoxic metabolites, which are more polar and more readily excreted (detoxication), but also highly reactive metabolites, which can interact with vital intracellular macromolecules, resulting in toxicity. In addition, reactive metabolites can be detoxified—for example, by interaction with glutathione or by epoxide hydration. In general, reactive metabolites are electrophiles (molecules containing positive centers). Those electrophiles not detoxified can in turn can react with cellular nucleophiles (molecules containing negative centers) such as proteins and nucleic acids. Other reactive metabolites may be free radicals or act as radical generators

*deceased

A Textbook of Modern Toxicology, *Fourth Edition*. Edited by Ernest Hodgson

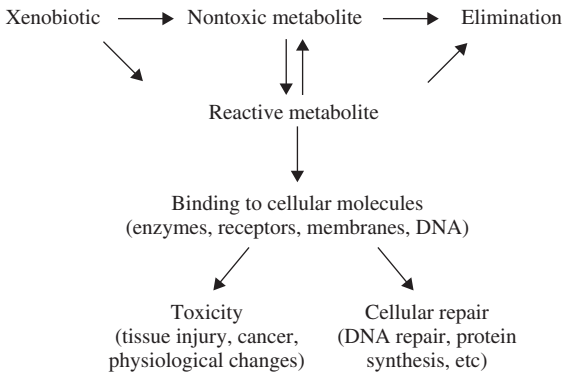


Figure 7.1 The relationship between metabolism, activation, detoxication, and toxicity of a chemical.

that interact with oxygen to produce reactive oxygen species that are capable of causing damage to membranes, DNA, and other macromolecules.

Although a chemical can be metabolized by several routes, the activation pathway is often a minor route with the remainder of the pathways resulting in detoxication. Activation, however, may become a more dominant pathway in certain situations, thus leading to toxicity. Several examples illustrating these situations are discussed later in this chapter. Some important terms that are often used when discussing activation include parent compound, sometimes referred to as procarcinogen in the case of a carcinogen or prodrug for pharmaceutical compounds; proximate toxic metabolite or proximate carcinogen for one or more of the intermediates; and ultimate toxic metabolite or ultimate carcinogen for the reactive species that binds to macromolecules such as DNA and protein.

7.2 ACTIVATION ENZYMES

Whereas most, if not all, of the enzymes involved in xenobiotic metabolism can form reactive metabolites (examples are summarized in Table 7.1 and are described in more detail in Sections 7.7.1–7.7.10), the enzyme systems most frequently involved in the activation of xenobiotics are those that catalyze oxidation reactions. The cytochrome P450 (CYPs) monooxygenases are by far the most important enzymes involved in the oxidation of xenobiotics. This is because of the abundance of CYPs (especially in the liver), the numerous CYP isoforms, and the ability of CYPs to be induced by xenobiotics.

Although CYPs are most abundant in the liver, they are also present in other tissues including the skin, kidney, intestine, lung, placenta, and nasal mucosa. Because CYP exists as multiple isoforms with different substrate specificities, the presence or absence of a particular CYP isozyme may contribute to tissue-specific toxicities. Many drugs and other xenobiotics are known to induce one or more of the CYP isoforms, resulting in an increase, decrease, or an alteration in the metabolic pathway of chemicals metabolized by the CYP isoforms involved. Specific examples of these types of interactions are given later in this section.

TABLE 7.1 Some Examples of Activation Enzymes, Substrates, and Reactive Intermediates

Enzyme	Reaction	Substrate	Reactive Intermediate
Alcohol dehydrogenase	Oxidation	Methanol	Formaldehyde
Aldehyde dehydrogenase	Oxidation	Formaldehyde	Formic acid
CYP	Oxidation	Acetaminophen	*N*-acetylbenzoquinoneimine
CYP	Epoxidation	Aflatoxinb1	2,3-epoxide
CYP	N-hydroxylation	Acetylaminofluorene	*N*-hydroxyacetylaminofluorene
Sulfotransferase	Sulfate conjugation	*N*-hydroxyacetylaminofluorene	Sulfate conjugate
CYP	Epoxidation	Benzo(a)pyrene	Benzo(a)pyrene 7,8-epoxide
Epoxide hydrolase	Hydrolysis	Benzo(a)pyrene , 7,8-epoxide	Benzo(a)pyrene 7,8-dihydrodiol
CYP	Epoxidation	Benzo(a)pyrene 7,8-dihydrodiol	Benzo(a)pyrene 7,8-diol-9,10-epoxide
CYP	Oxidative dechlorination	Carbon tetrachloride	Trichloromethyl radical
CYP	Oxidative desulfuration	Chlorpyrifos	Chlorpyrifos oxon Reactive sulfur
CYP	Methylenedioxy ring cleavage	Piperonyl butoxide	Carbene derivative on methylene carbon
CYP	Epoxidation	Vinyl chloride	Epoxide
B-glucosidase (gut flora)	Hydrolysis	Cycasin	Methylazoxymethanol

In addition to activations catalyzed by CYPs and flavin-containing monooxygenases (FMOs), Phase II conjugations, co-oxidation by cyclooxygenase (COX) during prostaglandin biosynthesis, and metabolism by intestinal microflora may also lead to the formation of reactive toxic products. With some chemicals, only one enzymatic reaction is involved, whereas with other compounds, several reactions, often involving multiple pathways, are necessary for the production of the ultimate reactive metabolite.

7.3 NATURE AND STABILITY OF REACTIVE METABOLITES

Reactive metabolites include such diverse groups as epoxides, quinones, free radicals, reactive oxygen species, and unstable conjugates. Figure 7.2 gives some examples of activation reactions, the reactive metabolites formed, and the enzymes catalyzing their bioactivation.

As a result of their high reactivity, reactive metabolites are often considered to be short-lived. This is not always true, however, because reactive intermediates can be transported from one tissue to another, where they may exert their deleterious effects. Thus, reactive intermediates can be seen as occurring in several stability categories, or better as a continuum consisting of a continuous range of stabilities

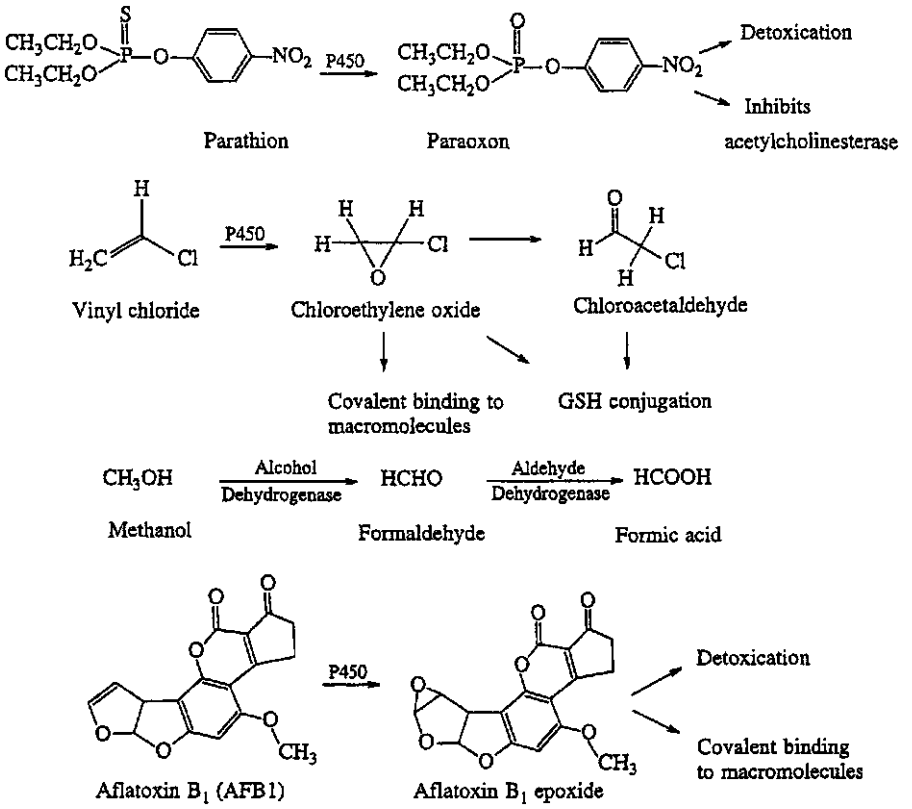


Figure 7.2 Examples of some activation reactions.

that reflect their half-life under physiological conditions and how far they may be transported from the site of activation.

Some of these metabolites are ultrashort-lived and bind primarily to the enzyme involved in their formation. This category includes intermediates that form enzyme-bound complexes with the active site of the enzyme, hence the parent substrate is often referred to as a mechanism-based inhibitor or a "suicide substrate." A number of compounds are known to react in this manner with CYP, and such compounds are often used experimentally as CYP inhibitors (see discussion of piperonyl butoxide, Section 7.7.1 and of chlorpyrifos, Section 7.7.2). Other compounds, although not true suicide substrates, produce reactive metabolites that bind primarily to the activating enzyme or adjacent proteins altering the function of the protein.

Short-lived metabolites (as opposed to ultrashort lived) remain in the cell or travel only to nearby cells. In this case, covalent binding is restricted to the cell of origin and to adjacent cells. Many xenobiotics fall into this group and give rise to localized tissue damage occurring close to the sites of activation. For example, in the lung, the Clara cells contain high concentrations of CYP and several lung toxicants that require activation often result in damage primarily to Clara cells.

Longer-lived metabolites may be transported to other cells and tissues so that although the site of activation may be the liver, the target site may be in a distant organ. Reactive intermediates may also be transported to other tissues, not in their original form but as conjugates, which then release the reactive intermediate under the specific conditions of the target tissue. For example, carcinogenic aromatic amines are metabolized in the liver to the N-hydroxylated derivatives that, following glucuronide conjugation, are transported to the bladder, where the N-hydroxy derivative is released under the acidic conditions of urine.

7.4 FATE OF REACTIVE METABOLITES

While the production of reactive metabolites is the initial process in the sequence of events leading to toxicity, this sequence is not inevitable since the reactive metabolites are also subject to detoxication. Thus, a variety of reactions may occur depending on the nature of the reactive species and the physiology of the organism.

7.4.1 Binding to Cellular Macromolecules

As mentioned previously, most reactive metabolites are electrophiles that can bind covalently to nucleophilic sites on cellular macromolecules such as proteins, polypeptides, RNA, and DNA. This covalent binding is considered to be the initiating event for many toxic processes such as mutagenesis, carcinogenesis, and cellular necrosis, and is discussed in greater detail in chapters dealing with modes of toxic action.

7.4.2 Lipid Peroxidation

Radicals such as $CCl_3\bullet$, produced during the oxidation of carbon tetrachloride, may induce lipid peroxidation and subsequent destruction of lipid membranes (Figure 7.3). Because of the critical nature of various cellular membranes (nuclear, mitochondrial, lysosomal, etc.), lipid peroxidation can be a pivotal event in cellular necrosis.

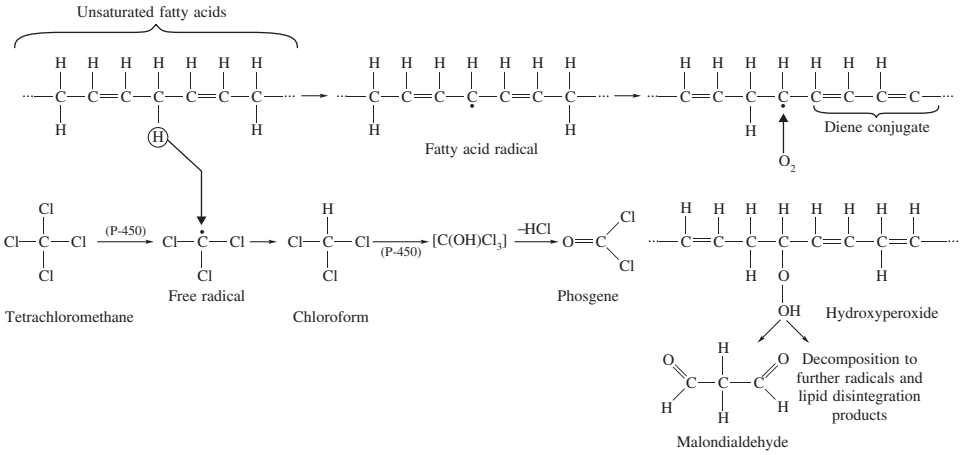


Figure 7.3 Metabolism of tetrachloromethane. Upon metabolic activation a CCl_3 radical is formed. This radical extracts protons from unsaturated fatty acids to form a free fatty acid radical. This leads to diene conjugates. At the same time, O_2 forms a hydroperoxide with the C radical. Upon its decomposition, malondialdehyde and other disintegration products are formed. In contrast, the CCl_3 radical is converted to chloroform, which undergoes further oxidative metabolism. Reprinted from: Bolt, H. M. and J. T. Borlak. Halogenated hydrocarbons. *Toxicol.* 645–657, 1999; with permission from Elsevier.

7.4.3 Trapping and Removal: Role of Glutathione

Once reactive metabolites are formed, mechanisms within the cell may bring about their rapid removal or inactivation. Toxicity then depends primarily on the balance between the rate of metabolite formation and the rate of removal. With some compounds, reduced glutathione plays an important protective role by trapping electrophilic metabolites and preventing their binding to hepatic proteins and enzymes. Although conjugation reactions occasionally result in bioactivation of a compound, the acetyl-, glutathione-, glucuronyl-, or sulfo-transferases usually result in the formation of a nontoxic, water-soluble metabolite that is easily excreted. Thus, availability of the conjugating chemical is an important factor in determining the fate of the reactive intermediates.

7.4.4 Trapping and Removal: Role of Epoxide Hydration

Reactive epoxides, in addition to being conjugated by glutathione S-transferase, may also be hydrolyzed by epoxide hydrolases to yield less toxic diols. Examples include styrene 7,8-oxide and naphthalene 1,2-oxide (see Chapter 6, Figure 6.14).

7.5 FACTORS AFFECTING TOXICITY OF REACTIVE METABOLITES

A number of factors can influence the balance between the rate of formation of reactive metabolites and the rate of removal, thereby affecting toxicity. The major factors discussed in this chapter are summarized in the following subsections.

A more in-depth discussion of other factors affecting metabolism and toxicity are presented in Chapter 8.

7.5.1 Levels of Activating Enzymes

Specific isozymes of CYPs are often important in determining metabolic activation of a foreign compound. As mentioned previously, many xenobiotics induce specific CYP isoforms. Frequently, the CYP isoforms induced are those involved in the metabolism of the inducing agent. Thus, a carcinogen or other toxicant has the potential for inducing its own activation. In addition, there are species and gender differences in enzyme levels as well as specific differences in the expression of particular isozymes.

7.5.2 Levels of Conjugating Enzymes

Levels of conjugating enzymes, such as glutathione transferases, are also known to be influenced by gender and species differences as well as by drugs and other environmental factors. All of these factors will, in turn, affect the detoxication process.

7.5.3 Levels of Cofactors or Conjugating Chemicals

Treatment of animals with *N*-acetylcysteine, a precursor of glutathione, protects animals against acetaminophen-induced hepatic necrosis, possibly by reducing covalent binding to tissue macromolecules. However, depletion of glutathione potentiates covalent binding and hepatotoxicity.

7.6 REACTIVE OXYGEN SPECIES

Although molecular oxygen normally exists in a relatively unreactive triplet state ($^3O_2$), reactive oxygen species are formed *in vivo*, either during, or as a consequence of, aerobic metabolism. These reactive species include superoxide anion, hydrogen peroxide, singlet oxygen, nitric oxide and the highly reactive hydroxyl radical. There is a great deal of evidence that these reactive oxygen species are linked to a number of toxic end points, and this phemomenon is known as oxidative stress.

Oxygen is first converted to the oxidizing agent superoxide anion ($O_2^{\bullet -}$) by cellular nicotine adenine dinucleophosphate (NADPH) oxidase systems or by xanthine oxidase and, subsequently, hydrogen peroxide (H_2O_2) is formed by the further oxidation of $O_2^{\bullet -}$ by the enzyme, superoxide dismutase:

$$2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$$

H_2O_2 can then be converted to the highly toxic hydroxyl radical (•OH) via the iron-catalyzed Fenton reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {\bullet}OH + OH^-$$

In addition, in the presence of $O_2^{\bullet-}$ and a divalent metal, H_2O_2 can produce •OH via the iron-catalyzed Haber–Weiss reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} \bullet OH + OH^-$$
$$Fe^{3+} + O_2^{\bullet-} \rightarrow Fe^{2+} + O_2$$
$$O_2^{\bullet-} + H_2O_2 \rightarrow \bullet OH + OH^-$$

Further details of the deleterious effects of these reactive oxygen species can be found in the various chapters dealing with modes of toxic action.

7.7 EXAMPLES OF ACTIVATING REACTIONS

The following examples have been selected to illustrate the various concepts of activation and detoxication discussed in the previous sections. They are also summarized in Table 7.1.

7.7.1 Piperonyl Butoxide

Methylenedioxyphenyl compounds, such as the insecticide synergist, piperonyl butoxide, are effective inhibitors of CYP monooxygenations, and are themselves metabolized to catechols. The most probable mechanism for inhibition and metabolism to a catechol appears to be oxidation at the methylene carbon, followed by elimination of water to yield a carbene. The highly reactive carbene either reacts with the heme iron to form a CYP-inhibitory complex or breaks down to yield the catechol (Chapter 6, Figure 6.8).

7.7.2 Chlorpyrifos

Chlorpyrifos is one of several organophosphorus insecticides of economic importance. Like all of the organophosphorus cholinesterase inhibitors containing the P=S moiety, chlorpyrifos must be metabolized to the reactive oxon (the P=O derivative). Oxon toxicity is the result of excessive stimulation of cholinergic nerves, which is dependent upon their ability to inhibit acetylcholinesterases (Figure 7.2). The activation reaction, which is CYP-catalyzed, is known as oxidative desulfuration. However, oxons are not the only activated products of oxidative desulfuration, as *in vitro* studies of rat and human liver have demonstrated that CYP isoforms are inactivated by the electrophilic sulfur atom released during oxidation of chlorpyrifos to chlorpyrifos oxon as well as other organophosphorus insecticides such the oxidation of parathion to paraoxon. The specific isoforms responsible for the metabolic activation are the ones destroyed in the process. For example, preincubations of NADPH-supplemented human liver microsomes with either chlorpyrifos or parathion resulted in the inhibition of some isoform-specific reactions including testosterone and estradiol oxidation (CYP3A4). These losses of metabolic activity are also associated with the loss of CYP content as measured by the CO-difference spectra. Thus, chlorpyrifos acts as a suicide substrate, in that its metabolism results in the destruction of the particular isoforms involved in its metabolism. This becomes

particularly important because the principal CYP involved in chlorpyrifos metabolism is CYP3A4, which is the dominant CYP in human liver; accounting for between 30% and 50% of the total liver CYP. Because of this enzyme's importance in drug and steroid hormone metabolism, the strong potential for inhibition by organophosphorus compounds may have serious consequences for individuals undergoing drug therapy.

7.7.3 Vinyl Chloride

Another example of a suicide inhibitor is vinyl chloride. The first step in the biotransformation of vinyl chloride involves the CYP-mediated oxidation of the double bond leading to the formation of an epoxide, or oxirane, which is highly reactive and can easily bind to proteins and nucleic acids. Following activation by CYP, reactive metabolites such as those formed by vinyl chloride bind covalently to the pyrrole nitrogens present in the heme moiety, resulting in destruction of the heme and loss of CYP activity. The interaction of the oxirane structure with nucleic acids results in mutations and cancer. The first indications that vinyl chloride was a human carcinogen involved individuals who cleaned reactor vessels in polymerization plants who were exposed to high concentrations of vinyl chloride and developed angiosarcomas of the liver as a result of their exposure (Figure 7.2).

7.7.4 Methanol

Ingestion of methanol, particularly during the prohibition era, resulted in significant illness and mortality. Where epidemics of methanol poisoning have been reported, one-third of the exposed population recovered with no ill effects, one-third had severe visual loss or blindness, and one-third had died. Methanol itself is not responsible for the toxic effects but is rapidly metabolized in humans by alcohol dehydrogenase to formaldehyde that is subsequently metabolized by aldehyde dehydrogenase to form the highly toxic formic acid (Figure 7.2). The aldehyde dehydrogenase is so efficient in its metabolism of formaldehyde that it is actually difficult to detect formaldehyde in postmortem tissues. Accumulation of formic acid in the tissues results first in blindness through edema of the retina, and eventually to death as a result of acidosis. Successful treatment of acidosis by treatment with base was often still unsuccessful in preventing mortality due to subsequent effects on the central nervous system. Treatment generally consists of hemodialysis to remove the methanol, but where this option is not available, administration of ethanol effectively competes with methanol for the alcohol dehydrogenase pathway, a competition that is therapeutic inasmuch as acetaldehyde is much less toxic than formaldehyde.

7.7.5 Aflatoxin B_1

Aflatoxin B_1 (AFB1) is one of the mycotoxins produced by *Aspergillus flavus* and *A. parasiticus* and is a well-known hepatotoxicant and hepatocarcinogen. It is generally accepted that the activated form of AFB1 that binds covalently to DNA is the 2,3-epoxide (Figure 7.2). AFB1-induced hepatotoxicity and carcinogenicity is known to vary among species of livestock and laboratory animals. The selective toxicity of AFB1 appears to be dependent on quantitative differences in formation

of the 2,3-epoxide, which is related to the particular enzyme complement of the organism. Because the epoxides of foreign compounds are frequently further metabolized by epoxide hydrolases or are nonenzymatically converted to the corresponding dihydrodiols, existence of the dihydrodiol is considered as evidence for prior formation of the epoxide. Because epoxide formation is catalyzed by CYP enzymes, the amount of AFB1-dihydrodiol produced by microsomes is reflective of the CYP isozyme complement involved in AFB1 metabolism. It has been shown that in rat microsomes in which specific CYP isozymes have been induced by phenobarbital (PB), dihydrodiol formation is considerably higher than that in control microsomes.

7.7.6 Carbon Tetrachloride (Tetrachloromethane)

Carbon tetrachloride has long been known to cause fatty acid accumulation and hepatic necrosis. Extraction of a chlorine atom from carbon tetrachloride by CYP results in the formation of a trichloromethyl radical which extracts protons from esterified desaturated fatty acids resulting in the production of chloroform (Figure 7.3). Chloroform also undergoes subsequent metabolism by CYP leading to the production of phosgene which covalently binds to sulfhydryl-containing enzymes and proteins, leading to toxicity. Differences between hepatic and renal effects of carbon tetrachloride and chloroform toxicity suggest that each tissue produces its own toxic metabolites from these chemicals.

In the case of hepatic toxicity due to carbon tetrachloride, the extraction of protons from fatty acids by the trichloromethyl radical results in the formation of highly unstable lipid radicals that undergo a series of transformations, including rearrangement of double bonds to produce conjugated dienes (Figure 7.3). Lipid radicals also readily react with oxygen, with the subsequent process, termed lipid peroxidation, producing damage to the membranes and enzymes. The resulting lipid peroxyl radicals decompose to aldehydes, the most abundant being malondialdehyde and 4-hydroxy-2,3-nonenal (Figure 7.3).

Since desaturated fatty acids are highly susceptible to free radical attack, neighboring fatty acids are readily affected and the initial metabolic transformation results in a cascade of detrimental effects on the tissue. The initial production of the trichloromethyl radical from carbon tetrachloride also results in irreversible covalent binding to CYP, resulting in its inactivation. In cases of carbon tetrachloride poisoning, preliminary sublethal doses actually become protective to an organism in the event of further poisoning since the metabolic activating enzymes are effectively inhibited by the first dose.

7.7.7 Acetylaminofluorene

In the case of the hepatocarcinogen, 2-acetylaminofluorene (2-AAF), two activation steps are necessary to form the reactive metabolites (Figure 7.4). The initial reaction, N-hydroxylation, is a CYP-dependent Phase I reaction, whereas the second reaction, resulting in the formation of the unstable sulfate ester, is a Phase II conjugation reaction that results in the formation of the reactive intermediate. Another Phase II reaction, glucuronide conjugation, is a detoxication step, resulting in a readily excreted conjugation product.

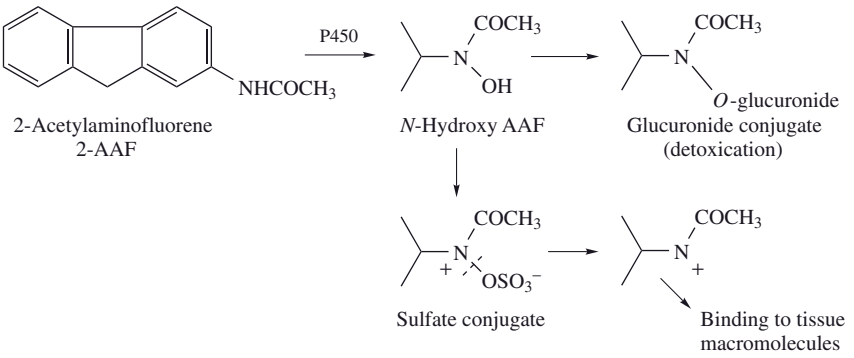


Figure 7.4 Bioactivation of 2-acetylaminofluorene.

In some animal species, 2-AAF is known to be carcinogenic, whereas in other species, it is noncarcinogenic. The species- and sex-specific carcinogenic potential of 2-AAF is correlated with the ability of the organism to sequentially produce the N-hydroxylated metabolite followed by the sulfate ester. Therefore, in an animal such as the guinea pig, which does not produce the N-hydroxylated metabolite, 2-AAF is not carcinogenic. In contrast, both male and female rats produce the N-hydroxylated metabolite, but only male rats have high rates of tumor formation. This is because male rats have up to 10-fold greater expression of sulfotransferase 1C1 than female rats, which has been implicated in the sulfate conjugation of 2-AAF resulting in higher production of the carcinogenic metabolite.

7.7.8 Benzo(a)pyrene

The polycyclic aromatic hydrocarbons are a group of chemicals consisting of two or more condensed aromatic rings that are formed primarily from incomplete combustion of organic materials including wood, coal, mineral oil, motor vehicle exhaust, and cigarette smoke. Early studies of cancer in the 1920s involving the fractionation of coal tar identified the carcinogenic potency of pure polycyclic aromatic hydrocarbons, including dibenz(a,h)anthracene and benzo(a)pyrene. Although several hundred different polycyclic aromatic hydrocarbons are known, environmental monitoring usually only detects a few compounds, one of the most important of which is benzo(a)pyrene. Benzo(a)pyrene is also one of the most prevalent polycyclic aromatic hydrocarbons found in cigarette smoke.

Extensive studies of metabolism of benzo(a)pyrene have identified at least 15 Phase I metabolites. The majority of these are the result of CYP1A1 and epoxide hydrolase reactions. Many of these metabolites are further metabolized by Phase II enzymes to produce numerous different metabolites. Studies examining the carcinogenicity of this compound have identified the 7,8-oxide and 7,8-dihydrodiol as proximate carcinogens and the 7,8-diol-9,10 epoxide as a strong mutagen and ultimate carcinogen. Because of the stereoselective metabolizing abilities of CYP isoforms, the reactive 7,8-diol-9,10-epoxide can appear as four different isomers (Figure 7.5). Interestingly, only one of these isomers(+)-benzo(a)pyrene 7,8-diol-9,10 epoxide-2 has significant carcinogenic potential. Comparative studies with

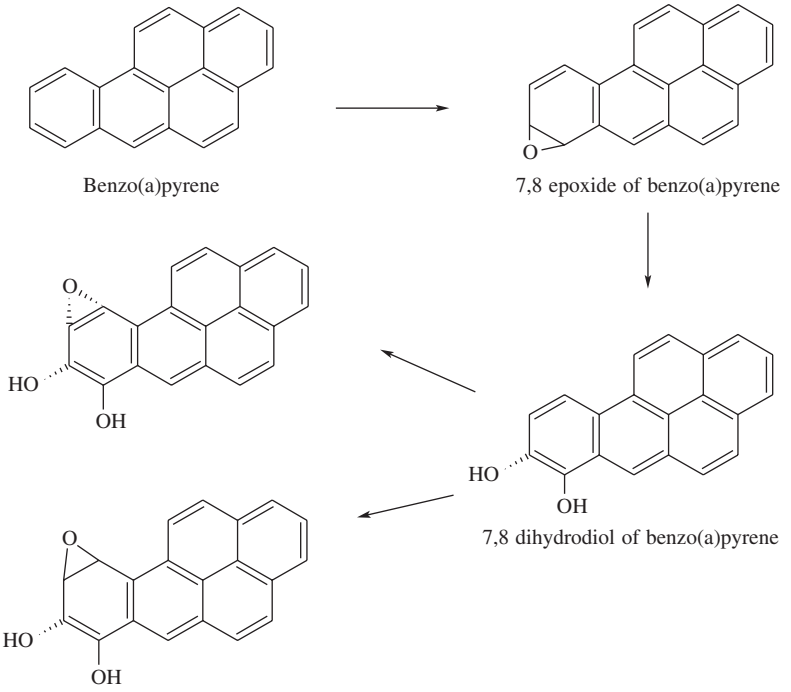


Figure 7.5 Selected stages of biotransformation of benzo(a)pyrene. The diol epoxide can exist in four diastereoisomeric forms of which the key carcinogenic metabolite is (+)-benzo(a) pyrene 7,8-diol-9,10-epoxide.

several other polycyclic aromatic hydrocarbons have demonstrated that only those substances that are epoxidized in the bay region of the ring system possess carcinogenic properties.

7.7.9 Acetaminophen

A good example of the importance of tissue availability of the conjugating chemical is found with acetaminophen. At normal therapeutic doses, acetaminophen is safe, but can be hepatotoxic at high doses. The major portion of acetaminophen is conjugated with either sulfate or glucuronic acid to form water-soluble, readily excreted metabolites, and only small amounts of the reactive intermediate, believed to be a quinoneimine (*N*-acetylbenzoquinoneimine), are formed by the CYP isoforms, principally by CYP2E1 (Figure 7.6).

When therapeutic doses of acetaminophen are ingested, the small amount of reactive intermediate formed is efficiently deactivated by conjugation with glutathione. When large doses are ingested, however, the sulfate and glucuronide cofactors (phosphoadenosine phosphosulfate and uridinediphosphoglucuronic acid) become depleted, resulting in more of the acetaminophen being metabolized to the reactive intermediate.

As long as glutathione (GSH) is available, most of the reactive intermediate can be detoxified. When the concentration of GSH in the liver also becomes depleted,

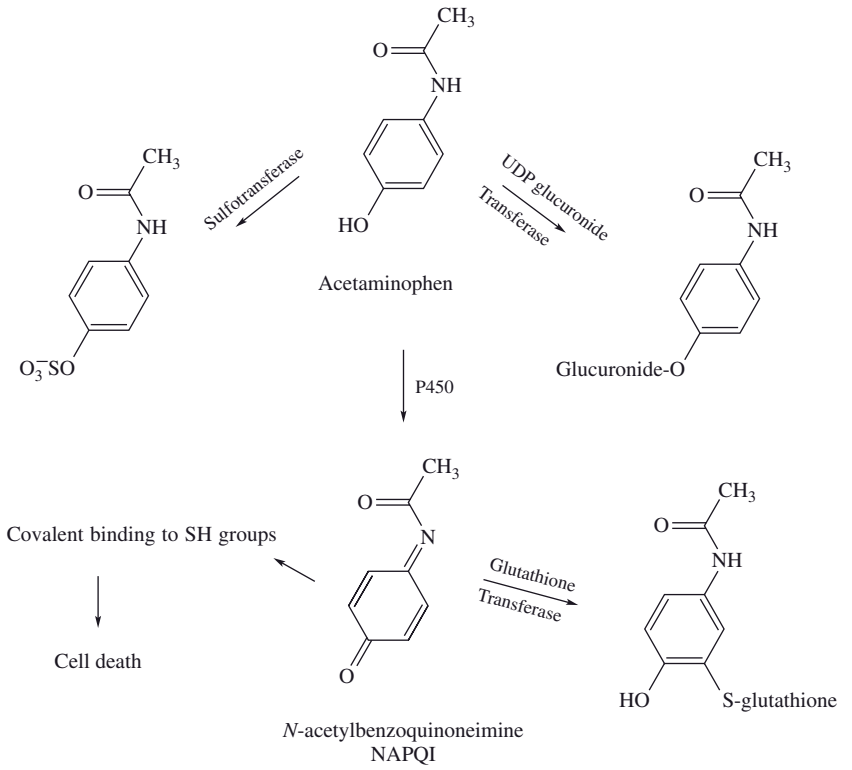


Figure 7.6 Metabolism of acetaminophen and formation of reactive metabolites.

however, covalent binding to sulfhydryl (-SH) groups of various cellular proteins increases, resulting in hepatic necrosis. If sufficiently large amounts of acetaminophen are ingested, as in drug overdoses and suicide attempts, extensive liver damage and death may result.

A further complication may result from the fact that ethanol is an inducer of CYP2E1. Thus, prior consumption of ethanol increases the isoform responsible for the generation of the reactive metabolite, *N*-acetylbenzoquinoneimine.

7.7.10 Cycasin

When flour from the cycad nut, which is used extensively among residents of South Pacific Islands, is fed to rats, it leads to cancers of the liver, kidney, and digestive tract. The active compound in cycasin, is the β-glucoside of methylazoxymethanol (Figure 7.7). If this compound is injected intraperitoneally rather than given orally, or if the compound is fed to germ-free rats, no tumors occur. Intestinal microflora possess the necessary enzyme, β-glucosidase, to form the active compound methylazoxymethanol, which is then absorbed into the body. The parent compound, cycasin, is carcinogenic only if administered orally because β-glucosidases are not present in mammalian tissues but are present in the gut. However, it can be

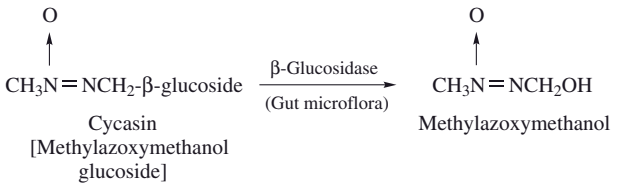


Figure 7.7 Bioactivation of cycasin by intestinal microflora to the carcinogen methylazoxymethanol.

demonstrated that the metabolite, methylazoxymethanol, will lead to tumors in both normal and germ-free animals regardless of the route of administration.

7.8 SUMMARY AND CONCLUSIONS

The current procedures for assessing safety and carcinogenic potential of chemicals using whole animal studies are expensive as well as becoming less socially acceptable. Moreover, the scientific validity of such tests for human risk assessment is also being questioned. Currently, a battery of short-term mutagenicity tests are used extensively as early predictors of mutagenicity and possible carcinogenicity.

Most of these systems use test organisms—for example, bacteria, that lack suitable enzyme systems to bioactivate chemicals, and therefore, an exogenous activating system is used. Usually, the post-mitochondrial fraction from rat liver, containing both Phase I and Phase II enzymes, is used as the activating system. The critical question is to what extent does this rat system represent the true *in vivo* situation, especially in humans? If not this system, then what is the better alternative? As some of the examples in this chapter illustrate, a chemical that is toxic or carcinogenic in one species or gender may be inactive in another, and this phenomenon is often related to the complement of enzymes, either activation or detoxication, expressed in the exposed organism.

Another factor to consider is the ability of many foreign compounds to selectively induce the CYP enzymes involved in their metabolism, especially if this induction results in the activation of the compound. With molecular techniques now available, considerable progress is being made in defining the enzyme and isozyme complements of human and laboratory species and understanding their mechanisms of control. Another area of active research is the use of *in vitro* expression systems to study the oxidation of foreign chemicals (e.g., bacteria containing genes for specific human CYP isozymes).

In summary, in studies of chemical toxicity, pathways and rates of metabolism as well as effects resulting from toxicokinetic factors and receptor affinities are critical in the choice of the animal species and experimental design. Therefore, it is important that the animal species chosen as a model for humans in safety evaluations metabolize the test chemical by the same routes as humans and, furthermore, that quantitative differences are considered in the interpretation of animal toxicity data. Risk assessment methods involving the extrapolation of toxic or carcinogenic potential of a chemical from one species to another must consider the metabolic and toxicokinetic characteristics of both species.

BIBLIOGRAPHY AND SUGGESTED READING

Anders, M. W., W. Dekant, and S. Vamvakas. Glutathione-dependent toxicity. *Xenobiotica* **22**:1135–1145, 1992.

Bolt, H. M. and J. T. Borlak. Halogenated hydrocarbons. In *Toxicology*, ed. H. Marquardt. San Diego, CA: Academic, 1999.

Bonner, J. C. Respiratory toxicology. In *Molecular and Biochemical Toxicology*, 4th ed., eds. R. C. Smart and E. Hodgson. Hoboken, NJ: Wiley, 2008.

Coughtrie, M. W. H., S. Sharp, K. Maxwell, et al. Biology and function of the reversible sulfation pathway catalysed by human sulfotransferases and sulfatases. *Chem. Biol. Interact.* **109**:3–27, 1998.

Gonzalez, F. J. Role of cytochromes P450 in chemical toxicity and oxidative stress: Studies with CYP2E1. *Mutat. Res.* **569**:101–110, 2005.

Gonzalez, F. J. and H. V. Gelboin. Role of human cytochromes P450 in the metabolic activation of chemical carcinogens and toxins. *Drug Metab. Rev.* **26**:165–183, 1994.

Guengerich, F. P. Bioactivation and detoxication of toxic and carcinogenic chemicals. *Drug Metab. Dispos.* **21**:1–6, 1993.

Guengerich, F. P. Metabolic activation of carcinogens. *Pharmacol. Ther.* **54**:17–61, 1992.

MacKenzie, E. L. Reactive oxygen species/reactive metabolites and toxicity. In *Molecular and Biochemical Toxicology*, 4th ed., eds. R. C. Smart and E. Hodgson. Hoboken, NJ: Wiley, 2008.

Omiecinski, C. J., R. P. Remmel, and V. P. Hosagrahara, Concise review of the cytochrome P450s and their roles in toxicology. *Toxicol. Sci.* **48**:151–156, 1999.

Park, B., N. Kitteringham, J. Maggs, et al. The role of metabolic activation in drug-induced hepatoxicity. *Annu. Rev. Pharmacol. Toxicol.* **45**:177–202, 2005.

Rinaldi, R., E. Eliasson, S. Swedmark, et al. Reactive intermediates and the dynamics of glutathione transferases. *Drug Metab. Dispos.* **30**:1053–1058, 2002.

Ritter, J. K. Roles of glucuronidation and UDP-glucuronosyltransferases in xenobiotic bioactivation reactions. *Chem. Biol. Interact.* **129**:171–193, 2000.

Vasiliou, V., A. Pappa, and D. R. Petersen. Role of aldehyde dehydrogenases in endogenous and xenobiotic metabolism. *Chem. Biol. Interact.* **129**:1–19, 2000.

SAMPLE QUESTIONS

1. Which xenobiotic-metabolizing enzymes are involved in the production of reactive metabolites? Which are most important?

2. Name the reactive metabolite(s) formed from piperonyl butoxide; chlorpyrifos; methanol; carbon tetrachloride; cycasin.

3. What are the principal ways in which reactive metabolites exert their toxic effects?

4. What are the principal ways that reactive metabolites may be detoxified before exerting their toxic effects?

5. Outline the process, starting with molecular oxygen, by which reactive oxygen species can be formed *in vivo*.

CHAPTER 8

Chemical and Physiological Effects on Xenobiotic Metabolism

ANDREW D. WALLACE and ERNEST HODGSON

8.1 INTRODUCTION

Xenobiotic metabolism can be modified by many factors both extrinsic and intrinsic to the normal functioning of the organism, and it is probable that many changes in toxicity are due to changes in metabolism of the toxicant, because most sequences of events that lead to overt toxicity involve either activation or detoxication of the toxicant in question. However, due to the difficulty of relating single events, measured *in vitro*, to the complex and interrelated effects that occur *in vivo*, the chain of cause and effect is not always entirely clear. Nevertheless, the relationship between *in vitro* and *in vivo* studies is important and is one of the themes of this chapter. It is important to note that the chemical, nutritional, physiological, and other effects noted herein have been described primarily from experiments carried out on experimental animals. While these studies indicate that similar effects may occur in humans or other animals, they do not indicate that they must occur, or that they occur at the same magnitude in all species, if they occur at all.

8.2 NUTRITIONAL EFFECTS

Many nutritional effects on xenobiotic metabolism have been noted, but the information is scattered and often appears contradictory. This section (8.2) is concerned only with the effects of nutritional constituents of the diet; the effects of other xenobiotics in the diet are discussed under chemical effects (Section 8.5).

8.2.1 Protein

Low-protein diets generally decrease monooxygenase activity in rat liver microsomes, and gender and substrate differences may be seen in the effect. For example, aminopyrine N-demethylation, hexobarbital hydroxylation, and aniline

A Textbook of Modern Toxicology, *Fourth Edition*. Edited by Ernest Hodgson

hydroxylation are all decreased, but the effect on the first two is greater in males than in females. In the third case, aniline hydroxylation, the reduction in males is equal to that in females. Tissue differences may also be seen. These changes are presumably related to the reductions in the levels of cytochrome P450 (CYP) and Nicotinamide adenine dinucleotide phosphate (NADPH)-CYP reductase that are also noted. One might speculate that the gender and other variations are due to differential effects on specific CYP isoforms. Even though enzyme levels are reduced by low-protein diets, they can still be induced to some extent by compounds such as phenobarbital. Such changes may also be reflected in changes in toxicity. Changes in the level of azoreductase activity in rat liver brought about by a low-protein diet are reflected in an increased severity in the carcinogenic effect of dimethylaminoazobenzene. The liver carcinogen dimethylnitrosamine, which must be activated metabolically, is almost without effect in protein-deficient rats.

Strychnine, which is detoxified by microsomal monooxygenase action, is more toxic to animals on low-protein diets, whereas octamethylpyrophosphoramide, carbon tetrachloride, and heptachlor, which are activated by CYP monooxygenases, are less toxic. Phase II reactions may also be affected by dietary protein levels. Chloramphenicol glucuronidation is reduced in protein-deficient guinea pigs, although no effect is seen on sulfotransferase activity in protein-deficient rats.

8.2.2 Carbohydrates

High dietary carbohydrate levels in the rat tend to have much the same effect as low dietary protein, decreasing such activities as aminopyrine N-demethylase, pentobarbital hydroxylation, and *p*-nitrobenzoic acid reduction along with a concomitant decrease in the enzymes of the CYP-dependent monooxygenase system. Because rats tend to regulate total caloric intake, this may actually reflect low-protein intake.

In humans, it has been demonstrated that increasing the ratio of protein to carbohydrate in the diet stimulates oxidation of antipyrine and theophylline, while changing the ratio of fat to carbohydrate had no effect. In related studies, humans fed charcoal-broiled beef (food high in polycyclic hydrocarbon content) for several days had significantly enhanced activities of CYP1A1 and CYP1A2, resulting in enhanced metabolism of phenacetin, theophylline, and antipyrine. Studies of this nature indicate that there is significant interindividual variability in these observed responses.

8.2.3 Lipids

Dietary deficiencies in linoleic or in other unsaturated fats generally bring about a reduction in CYP and related monooxygenase activities in the rat. The increase in effectiveness of breast and colon carcinogens brought about in animals on high fat diets, however, appears to be related to events during the promotion phase rather than the activation of the causative chemical. Increases in dietary lipids have been shown to elevate CYP2E1 and CYP4A levels in humans. Lipids also appear to be necessary for the effect of inducers, such as phenobarbital, to be fully expressed.

8.2.4 Micronutrients

Vitamin deficiencies in general bring about a reduction in monooxygenase activity, although exceptions can be noted. Riboflavin deficiency causes an increase in CYP and aniline hydroxylation, although at the same time it causes a decrease in CYP reductase and benzo(a)pyrene hydroxylation. Ascorbic acid deficiency in the guinea pig not only causes a decrease in CYP and monooxygenase activity but also causes a reduction in microsomal hydrolysis of procaine. Deficiencies in vitamins A and E cause a decrease in monooxygenase activity, whereas thiamine deficiency causes an increase. The effect of these vitamins on different CYP isoforms has not been investigated. Changes in mineral nutrition have also been observed to affect monooxygenase activity. In the immature rat, calcium or magnesium deficiency causes a decrease, whereas, quite unexpectedly, iron deficiency causes an increase. This increase is not accompanied by a concomitant increase in CYP, however. An excess of dietary cobalt, cadmium, manganese, and lead all cause an increase in hepatic glutathione levels and a decrease in CYP content.

8.2.5 Starvation and Dehydration

Although in some animals starvation appears to have effects similar to those of protein deficiency, this is not necessarily the case. For example, in the mouse, monooxygenation is decreased, but reduction of *p*-nitrobenzoic acid is unaffected. In male rats, hexobarbital and pentobarbital hydroxylation as well as aminopyrine N-demethylation are decreased, but aniline hydroxylation is increased. All of these activities are stimulated in the female. Water deprivation in gerbils causes an increase in CYP and a concomitant increase in hexobarbital metabolism, which is reflected in a shorter sleeping time.

8.2.6 Nutritional Requirements in Xenobiotic Metabolism

Because xenobiotic metabolism involves many enzymes with different cofactor requirements, prosthetic groups, or endogenous co-substrates, it is apparent that many different nutrients are involved in their function and maintenance. Determination of the effects of deficiencies, however, is more complex because reductions in activity of any particular enzyme will be effective only if it affects a change in a rate-limiting step in a process. In the case of multiple deficiencies, the nature of the rate-limiting step may change with time.

Phase I Reactions Nutrients involved in the maintenance of the CYP-dependent monooxygenase system are shown in Figure 8.1. The B complex vitamins niacin and riboflavin are both involved, the former in the formation of NADPH, the latter in the formation of flavin adenine dinucleotied (FAD) and flavin mononucleotide (FMN). Essential amino acids are, of course, required for the synthesis of all of the proteins involved. The heme of the cytochrome requires iron, an essential inorganic nutrient. Other nutrients required in heme synthesis include pantothenic acid, needed for the synthesis of the coenzyme A (CoA) used in the formation of acetyl CoA, pyridoxine, a cofactor in heme synthesis and copper, required in the

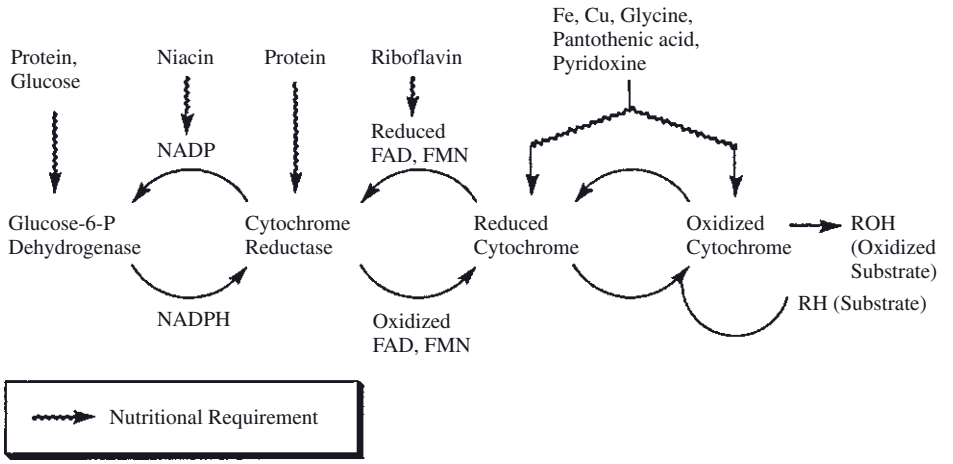


Figure 8.1 Nutritional requirements with potential effects on the cytochrome P450 monooxygenase system From: Donaldson, W. E. Nutritional factors. In *Introduction to Biochemical Toxicology,* 3rd ed., eds. E. Hodgson and R. C. Smart. New York: Wiley, 2001.

ferroxidase system that converts ferrous to ferric iron prior to its incorporation into heme. Although it is clear that dietary deficiencies could reduce the ability of the CYP system to metabolize xenobiotics, it is not clear how this effect would be manifested *in vivo* unless there is an understanding of the rate-limiting factors involved a considerable task in such a complex of interrelated reactions, and there is little reliable information on the effect of deficiencies in specific micronutrients.

Phase II As with Phase I reactions, Phase II reactions usually depend on several enzymes with different cofactors and different prosthetic groups and, frequently, different endogenous co-substrates. All of these many components can depend on nutritional requirements, including vitamins, minerals, amino acids, and others. Mercapturic acid formation can be cited to illustrate the principles involved. The formation of mercapturic acids starts with the formation of glutathione conjugates, reactions catalyzed by the glutathione S-transferases.

This is followed by removal of the glutamic acid and the glycine residues followed by acetylation of the remaining cysteine. Essential amino acids are required for the synthesis of the proteins involved, pantothenic acid for coenzyme A synthesis, and phosphorus for synthesis of the ATP needed for glutathione synthesis. Similar scenarios can be developed for glucuronide and sulfate formation, acetylation, and other Phase II reaction systems. As with Phase I enzymes, there is little reliable information on the effect of deficiencies in specific micronutrients.

8.3 PHYSIOLOGICAL EFFECTS

8.3.1 Development

Birth in mammals initiates an increase in the activity of many hepatic enzymes, including those involved in xenobiotic metabolism. The ability of the liver to carry

out monooxygenation reactions appears to be very low during gestation and to increase after birth, with no obvious differences being seen between immature males and females. This general trend has been observed in many species, although the developmental pattern may vary according to gender and genetic strain. The component enzymes of the CYP-dependent monooxygenase system both follow the same general trend although there may be differences in the rate of increase. In the rabbit, the postnatal increase in CYP and its reductase is parallel; in the rat, the increase in the reductase is slower than that of the cytochrome.

Phase II reactions may also be age dependent. Glucuronidation of many substrates is low or undetectable in fetal tissues but increases with age. The inability of newborn mammals of many species to form glucuronides is associated with deficiencies in both glucuronosyltransferase and its cofactor, uridine diphosphate glucuronic acid (UDPGA). A combination of this deficiency, as well as slow excretion of the bilirubin conjugate formed, and the presence in the blood of pregnanediol, an inhibitor of glucuronidation, may lead to neonatal jaundice. Glycine conjugations are also low in the newborn, resulting from a lack of available glycine, an amino acid that reaches normal levels at about 30 days of age in the rat and 8 weeks in the human. Glutathione conjugation may also be impaired, as in fetal and neonatal guinea pigs, because of a deficiency of available glutathione. In the serum and liver of perinatal rats, glutathione transferase is barely detectable, increasing rapidly until adult levels are reached at about 140 days (Figure 8.2). This pattern is not followed in all cases, because sulfate conjugation and acetylation appear to be fully functional and at adult levels in the guinea pig fetus. Thus, some compounds that are glucuronidated in the adult can be acetylated or conjugated as sulfates in the young.

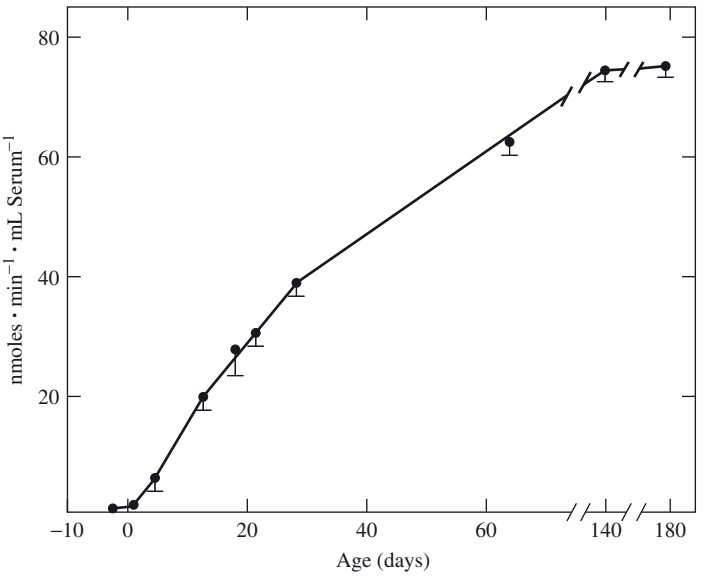


Figure 8.2 Developmental pattern of serum glutathione S-transferase activity in female rats. Adapted from Mukhtar and Bend. *Life Sci.* **21**:1277, 1977.

An understanding of how these effects may be related to the expression of individual isoforms is now beginning to emerge. It is known that in immature rats of either gender, CYP2A1, CYP2D6, and CYP3A2 predominate, whereas in mature rats, the males show a predominance of CYP2C11, CYP2C6, and CYP3A2, and the females CYP2A1, CYP2C6, and CYP2C12.

The effect of senescence on the metabolism of xenobiotics has yielded variable results. In rats, monooxygenase activity, which reaches a maximum at about 30 days of age, begins to decline some 250 days later, a decrease that may be associated with reduced levels of sex hormones. Glucuronidation also decreases in old animals, whereas monoamine oxidase activity increases. These changes in the monooxygenase activities are often reflected by changes in drug efficacy or overall toxicity.

In pediatric human populations, often there is a delayed increase in CYP enzyme expression and adult levels are not reached until 10 years of age or older. In elderly humans, age-related impairment of enzyme activity is highly controversial. Conflicting findings have found either age-related declines in or no changes with respect to the activity of CYP2C and CYP3A isoforms. Studies involving an erythromycin breath test in humans also suggested that there were no age-related declines associated with CYP3A4 activity. However, a study of CYP content and antipyrine clearance in liver biopsies obtained from 226 closely matched subjects indicated that subjects older than 70 had significantly less activity and clearance than younger subjects. Likewise, in older subjects, clearance of the drug omeprazole, a CYP2C19 substrate, was nearly half the rates observed in younger subjects. The effects of age on xenobiotic-metabolizing enzymes (XMEs) are complicated by other factors including liver mass, blood flow changes, and interindividual variability.

8.3.2 Gender Differences

Metabolism of xenobiotics may vary with the gender of the organism. Gender differences become apparent at puberty and are usually maintained throughout adult life. Adult male rats metabolize many compounds at rates higher than females, for example, hexobarbital hydroxylation, aminopyrine N-demethylation, glucuronidation of *o*-aminophenol, and glutathione conjugation of aryl substrates; however, with other substrates, such as aniline and zoxazolamine, no gender differences are seen. In other species, including humans, the gender difference in xenobiotic metabolism is less pronounced. The differences in microsomal monooxygenase activity between males and females have been shown to be under the control of sex hormones, at least in some species. Some enzyme activities are decreased by castration in the male, and administration of androgens to castrated males increases the activity of these sex-dependent enzyme activities without affecting the independent ones. Procaine hydrolysis is faster in male than female rats, and this compound is less toxic to the male. Gender differences in enzyme activity may also vary from tissue to tissue. Hepatic microsomes from adult male guinea pigs are less active in the conjugation of *p*-nitrophenol than are those from females, but no such gender difference is seen in the microsomes from lung, kidney, and small intestines.

Many differences in overall toxicity between males and females of various species are known (Table 8.1). Although it is not always known whether metabolism is the only or even the most important factor, such differences may be related to gender-related differences in metabolism. Hexobarbital is metabolized faster by male rats;

TABLE 8.1 Gender-Related Differences in Toxicity

Species	Toxicant	Susceptibility
Rat	EPN, warfarin, strychnine, hexobarbital, parathion	F > M
	Aldrin, lead, epinephrine, ergot alkaloids	M > F
Cat	Dinitrophenol	F > M
Rabbit	Benzene	F > M
Mouse	Folic acid	F > M
	Nicotine	M > F
Dog	Digitoxin	M > F

thus, female rats have longer sleeping times. Parathion is activated to the cholinesterase inhibitor paraoxon more rapidly in female than in male rats, and thus is more toxic to females. Presumably, many of the gender-related differences, as with the developmental differences, are related to quantitative or qualitative differences in the isoforms of the XMEs that exist in multiple forms, but this aspect has not been investigated extensively.

In the rat, sexually dimorphic CYPs appear to arise by programming, or imprinting, that occurs in neonatal development. This imprinting is brought about by a surge of testosterone that occurs in the male, but not the female, neonate and appears to imprint the developing hypothalamus so that in later development, growth hormone is secreted in a gender-specific manner. Growth hormone production is pulsatile in adult males with peaks of production at approximately 3-h intervals and more continuous in females, with smaller peaks. This pattern of growth hormone production and the higher level of circulating testosterone in the male maintain the expression of male-specific isoforms such as CYP2C11. The more continuous pattern of growth hormone secretion and the lack of circulating testosterone appears to be responsible for the expression of female-specific isoforms such as CYP2C12. The high level of sulfotransferases in the female appears to be under similar control, raising the possibility that this is a general mechanism for the expression of gender-specific XMEs or their isoforms. A schematic version of this proposed mechanism is seen in Figure 8.3.

Gender-specific expression is also seen in the flavin-containing monooxygenases. In mouse liver, FMO1 is higher in the female than in the male, and FMO3, present at high levels in female liver, is not expressed in male liver (Figure 8.4). No gender-specific differences are observed for FMO5. The important role of testosterone in the regulation of FMO1 and FMO3 was demonstrated in gonadectomized animals with and without testosterone implants. In males, castration increased FMO1 and FMO3 expression to levels similar to those observed in females, and testosterone replacement to castrated males resulted in ablation of FMO3 expression. Similarly, administration of testosterone to females caused ablation of FMO3 expression. Although these results clearly indicate a role for testosterone in the regulation of these isoforms, the physiological reasons for their gender-dependent expression remain unknown.

In humans, gender differences in the metabolism of some commonly used drugs have been observed, but overall, not much is known. These differences are thought to be due to expression levels of CYPs, such as CYP3A4, which leads to women generally being more susceptible to drug–drug interactions.

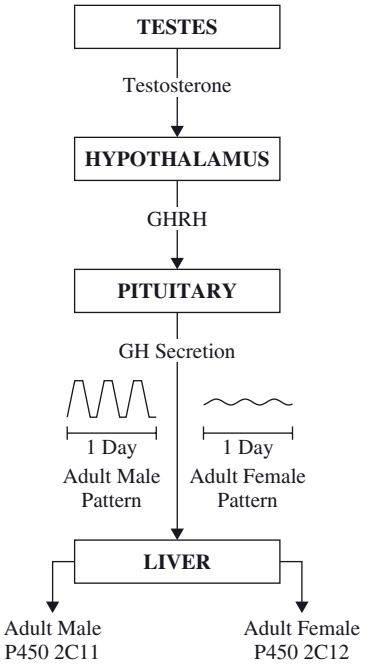


Figure 8.3 Hypothetical scheme for neonatal imprinting of the hypothalamus-pituitary-liver axis resulting in sexually dimorphic expression of hepatic enzymes in the adult rat. Neonatal surges of testosterone appear to play a role in imprinting. From Ronis, M. J. J. and H. C. Cunny. Physiological (endogenous) factors affecting the metabolism of xenobiotics. In *Introduction to Biochemical Toxicology*, 2nd ed., eds. E. Hodgson and P. E. Levi, p. 136. Norwalk, CT: Appleton & Lange, 1994.

8.3.3 Hormones

Hormones other than sex hormones are also known to affect the levels of XMEs, but these effects are much less studied or understood.

Thyroid Hormone Treatment of rats with thyroxin increases hepatic microsomal NADPH oxidation in both male and female rats, with the increase being greater in females. CYP content decreases in the male but not in the female.

Hyperthyroidism causes a decrease in gender-dependent monooxygenase reactions and appears to interfere with the ability of androgens to increase the activity of the enzymes responsible. Gender differences are not seen in the response of mice and rabbits to thyroxin. In mice, aminopyrine N-demethylase, aniline hydroxylase, and hexobarbital hydroxylase are decreased, whereas *p*-nitrobenzoic acid reduction is unchanged. In rabbits, hexobarbital hydroxylation is unchanged, whereas aniline hydroxylation and *p*-nitrobenzoic acid reduction increase. Thyroid hormone can also affect enzymes other than microsomal monooxygenases. For example, liver monoamine oxidase activity is decreased whereas the activity of the same enzymes in the kidney is increased.

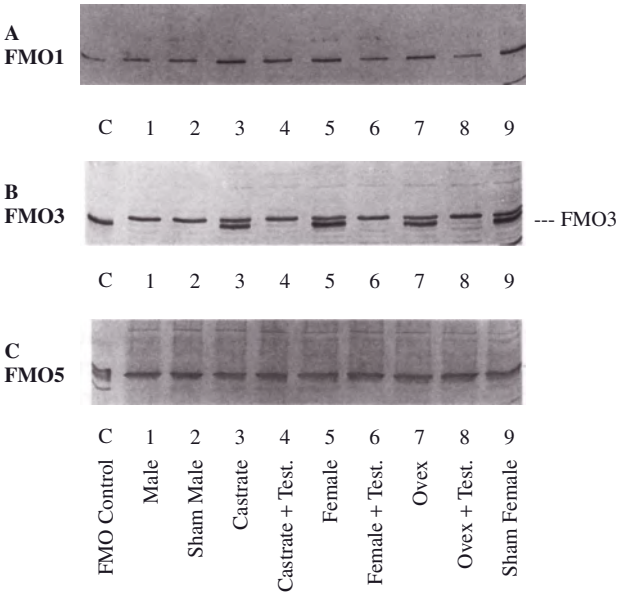


Figure 8.4 Immunoreactivity of liver microsomes from sexually intact control, sham control, gonadectomized mice, or mice undergoing gonadectomy and/or receiving testosterone implants (5 mg). From Falls, J. G., D.-Y. Ryu, Y. Cao, et al. *Arch. Biochem. Biophys.* **342**:212–223, 1997.

Adrenal Hormones Removal of adrenal glands from male rats results in a decrease in the activity of hepatic microsomal enzymes, impairing the metabolism of aminopyrine and hexobarbital, but the same operation in females has no effect on their metabolism of xenobiotics. Cortisone or prednisolone restores activity to normal levels.

Insulin The effect of diabetes on xenobiotic metabolism is quite varied and, in this regard, alloxan-induced diabetes may not be a good model for the natural disease. The *in vitro* metabolism of hexobarbital and aminopyrine is decreased in alloxan-diabetic male rats, but is increased in similarly treated females. Aniline hydroxylase is increased in both males and females with alloxan diabetes. The induction of CYP2D1 in diabetes (and in fasting) is believed to be due to the high circulating levels of endogenously generated ketones. Studies of activity of the enzymes mentioned show no gender differences in the mouse; both sexes show an increase. Some Phase II reactions, such as glucuronidation, are decreased in diabetic animals. This appears to be due to a lack of UDPGA caused by a decrease in UDPG dehydrogenase, rather than a decrease in transferase activity, and the effect can be reversed by insulin.

Other Hormones Pituitary hormones regulate the function of many other endocrine glands, and hypophysectomy in male rats results in a decrease in the activity of XMEs. Administration of adrenocorticotropic hormone (ACTH) also results in a decrease of those oxidative enzyme activities that are gender dependent. In

contrast, ACTH treatment of female rats causes an increase in aminopyrine N-demethylase but no change in other activities.

8.3.4 Pregnancy

Many XME activities decrease during pregnancy. Catechol *O*-methyltransferase and monoamine oxidase decrease, as does glucuronide conjugation. The latter may be related to the increasing levels of progesterone and pregnanediol, both known to be inhibitors of glucuronosyltransferase *in vitro*. A similar effect on sulfate conjugation has been seen in pregnant rats and guinea pigs. In some species, liver microsomal monooxygenase activity may also decrease during pregnancy, this decrease being accompanied by a concomitant decrease in CYP levels. An increased level of FMO2 is seen in the lung of pregnant rabbits.

8.3.5 Disease

Quantitatively, the most important site for xenobiotic metabolism is the liver; thus, effects on the liver are likely to have a pronounced effect on the organism's overall capacity in this regard. At the same time, effects on other organs can have consequences no less serious for the organism. Patients with acute hepatitis frequently have an impaired ability to oxidize drugs, with a concomitant increase in plasma half-life. Impaired oxidative metabolism has also been shown in patients with chronic hepatitis or cirrhosis. The decrease in drug metabolism that occurs in obstructive jaundice may be a consequence of the accumulation of bile salts, which are known inhibitors of some of the enzymes involved. Phase II reactions may also be affected, decreases in acetylation, glucuronidation, and a variety of esterase activities having been seen in various liver diseases. Hepatic tumors, in general, have a lower ability to metabolize foreign compounds than does normal liver tissue, although in some cases, the overall activity of tumor bearing livers may be no lower than that of controls. Kidney diseases may also affect the overall ability to handle xenobiotics because this organ is one of the main routes for elimination of xenobiotics and their metabolites. The half-lives of tolbutamide, thiopental, hexobarbital, and chloramphenicol are all prolonged in patients with renal impairment.

8.3.6 Diurnal Rhythms

Diurnal rhythms, both in CYP levels and in the susceptibility to toxicants, have been described, especially in rodents. Although such changes appear to be related to the light cycle, they may, in fact, be activity dependent because feeding and other activities in rodents are themselves markedly diurnal.

8.4 COMPARATIVE AND GENETIC EFFECTS

Comparative toxicology is the study of the variation in toxicity of exogenous chemicals toward different organisms, either of different genetic strains or of different taxonomic groups. Thus, the comparative approach can be used in the study of any

aspect of toxicology, such as absorption, metabolism, mode of action, and acute or chronic effects. Most comparative data for toxic compounds exist in two areas—acute toxicity and metabolism. Until recently, comparative toxicology was largely a descriptive listing of differences between species and strains. Given the recent advances in molecular biology, comparative toxicology is entering a new phase in which the reasons for this variation will be elucidated. However, the value of the comparative approach to date can be summarized under three headings:

1. *Selective Toxicity.* Comparative toxicology is necessary for the development of selective biocides, toxic to the target organism but less toxic to other organisms, particularly humans.
2. *Experimental Models.* Comparative studies of toxic phenomena are necessary to select the most appropriate surrogate for extrapolation to humans for the testing and development of drugs and biocides and for human health risk analysis.
3. *Environmental Xenobiotic Cycles.* Different organisms in the complex ecological food webs metabolize compounds at different rates and to different products; the metabolic end products are released back to the environment, either to be further metabolized by other organisms or to exert toxic effects of their own. In addition to field studies, laboratory micro ecosystems have been developed, and chemicals and their metabolites can be followed through the plants and terrestrial and aquatic animals involved.

It might also be noted that many aspects of comparative toxicology are also comparative biochemistry in the broader sense. If the proper role of comparative biochemistry is to put evolution on a molecular basis, detoxication enzymes, like other enzymes, are suitable subjects for study. XMEs were probably essential in the early stages of animal evolution because secondary plant products, even those of low toxicity, are frequently lipophilic and, as a consequence would, in the absence of such enzymes, accumulate in lipid membranes and lipid depots. The evolution of CYP isoforms, with more than 7000 cDNA sequences known, is proving a useful tool for the study of biochemical evolution.

8.4.1 Variations among Taxonomic Groups

There are few differences in xenobiotic metabolism that are specific for large taxonomic groups. The formation of glucosides by insects and plants rather than the glucuronides of other animal groups is one of the most distinct. Although differences between species are common and are of toxicological significance, they are usually quantitative rather than qualitative in nature and tend to occur within as well as between taxonomic groups. Although the ultimate explanation of such differences must be at the level of gene expression, they are manifested at many levels, the most important of which may be summarized as follows.

In Vivo *Toxicity* An example may be seen in the toxicity of pesticides. Toxicity is a term used to describe the adverse effects of chemicals on living organisms. Available data on the toxicity of selected pesticides to rats suggest that herbicide use, in

general, provides the greatest human safety factor by selectively killing plants. As the evolutionary position of the target species approaches that of humans, however, the human safety factor is narrowed considerably. Thus, as far as direct toxicity to humans and other mammals is concerned, pesticide toxicity seems to follow the progression: herbicides = fungicides < molluscicides < acaricides < nematocides < insecticides < rodenticides. However, this relationship is oversimplified because marked differences in lethality are observed when different members of a group of biocides are tested against laboratory test animals and target species.

Interspecific differences are also known for some naturally occurring poisons. Nicotine, for instance, is used as an insecticide and kills many insect pests at low doses, yet tobacco leaves constitute a normal diet for several species. As indicated earlier, most strains of rabbit eat Belladonna leaves without ill effects, whereas other mammals are easily poisoned. Natural tolerance to cyanide poisoning in millipedes and the high resistance to the powerful axonal blocking tetrodotoxin in puffer fish are examples of the tolerance of animals to the toxins they produce.

The specific organ toxicity of chemicals also exhibits wide species differences. Carbon tetrachloride, a highly potent hepatotoxicant, induces liver damage in many species, but chickens are almost unaffected by it. The eggshell thinning associated with dichlorodiphenyltrichloroethane (DDT) poisoning in birds is observed in falcons and mallard ducks, but not in gallinaceous species. Delayed neurotoxicity caused by organophosphates such as leptophos and tri-*o*-cresyl phosphate occurs in humans and chickens but not in most common laboratory mammals.

In Vivo *Metabolism* Many factors affect the rates of penetration, distribution, biotransformation, and excretion of chemicals, and thus govern their biological fate in the body. In general, the absorption of xenobiotics, their tissue distribution, and penetration across the blood–brain barrier and other barriers are dictated by their physicochemical nature and, therefore, tend to be similar in different animal species. The biological effect of a chemical depends on the concentration of its binding to tissue macromolecules. The biologic half-life is governed by the rates of metabolism and excretion and thus reflects the most important variable explaining interspecies differences in toxic response. Striking differences between species can be seen in the biologic half-lives of various drugs with humans, in general, metabolizing xenobiotics more slowly than various experimental animals. For example, phenylbutazone is metabolized slowly in humans, with a half-life averaging 3 days. In the monkey, rat, guinea pig, rabbit, dog, and horse, however, this drug is metabolized readily, with half-lives ranging between 3 and 6 h. The interdependence of metabolic rate, half-life, and pharmacological action is well illustrated in the case of hexobarbital. The duration of sleeping time is directly related to the biologic half-life and is inversely proportional to the *in vitro* degradation by liver enzymes from the respective species. Thus, mice inactivate hexobarbital readily, as reflected in a brief biologic half-life *in vivo* and short sleeping time, whereas the reverse is true in dogs.

Xenobiotics, once inside the body, undergo a series of biotransformations catalyzed by one or more XMEs acting either simultaneously or consecutively (see Chapter 6 for information on Phase I and Phase II XMEs). Because biotransformations are catalyzed by a large number of enzymes, it is to be expected that they will vary between species, with qualitative differences implying the involvement of

different enzymes, quantitative differences implying differences in enzyme levels, in the extent of competing reactions, or in the efficiency of enzymes capable of reversing the reaction.

Even in the case of a xenobiotic undergoing oxidation primarily by a single reaction, there may be remarkable species differences in relative rates. Aromatic hydroxylation of aniline is an example. In this case, both *ortho* and *para* positions are susceptible to oxidative attack yielding the respective aminophenols. The biological fate of aniline has been studied in many species, and striking selectivity in hydroxylation position has been noted. These data show a trend, in that carnivores generally display a high aniline *ortho*-hydroxylase ability with a *para/ortho* ratio of ≤1, whereas rodents exhibit a striking preference for the *para* position, with a *para/ortho* ratio from 2.5 to 15. 2-Acetylaminofluorene may be metabolized in mammals by two alternative routes: N-hydroxylation, yielding the carcinogenic N-hydroxy derivative; and aromatic hydroxylation, yielding the noncarcinogenic 7-hydroxy metabolite. The former is the metabolic route in the rat, rabbit, hamster, dog, and in humans in which the parent compound is known to be carcinogenic. In contrast, the monkey carries out aromatic hydroxylation and the guinea pig appears to deacetylate the N-hydroxy derivative; thus, both escape the carcinogenic effects of this compound.

The hydrolysis of esters by esterases and of amides by amidases constitutes one of the most common enzymatic reactions of xenobiotics in humans and other animal species. Because both the number of enzymes involved in hydrolytic attack and the number of substrates for them is large, it is not surprising to observe interspecific differences in the disposition of xenobiotics due to variations in these enzymes. In mammals, the presence of a carboxylesterase that hydrolyzes malathion but is generally absent in insects explains the remarkable selectivity of this insecticide. As with esters, wide differences exist between species in the rates of hydrolysis of various amides *in vivo*. The insecticide dimethoate is susceptible to the attack of both esterases and amidases, yielding nontoxic products. In the rat and mouse, both reactions occur, whereas sheep liver contains only the amidases and that of guinea pig only the esterases. The relative rates of these degradative enzymes in insects are very low as compared with those of mammals, however, and this correlates well with the high selectivity of dimethoate.

Phase II reactions are concerned with the conjugation of primary metabolites of xenobiotics produced by Phase I reactions, and factors that alter or govern the rates of Phase II reactions may play a role in interspecific differences in xenobiotic metabolism.

Interspecific differences in the magnitude of biliary excretion of a xenobiotic excretion product largely depend on molecular weight, the presence of polar groups in the molecule, and the extent of conjugation. Conjugates with molecular weights of less than 300 are poorly excreted in bile and tend to be excreted with urine, whereas the reverse is true for those with molecular weights higher than 300. The critical molecular weight appears to vary between species, and marked species differences are noted for biliary excretion of chemicals with molecular weights of about 300. Thus, the biliary excretion of succinylsulfathioazole is 20- to 30-fold greater in the rat and the dog than in the rabbit and the guinea pig, and more than 100-fold greater than in the pig and the rhesus monkey. The cat and sheep are intermediate and excrete about 7% of the dose in the bile.

TABLE 8.2 Occurrence of Common and Unusual Conjugation Reactions

Conjugating Group	Common	Unusual
Carbohydrate	Glucuronic acid (animals)	*N*-acetylglucosamine (rabbits)
	Glucose (insects, plants)	Ribose (rats, mice)
Amino acids	Glycine	Glutamine (insects, humans)
	Glutathione	Ornithine (birds)
	Methionine	Arginine (ticks, spiders)
		Glycyltaurine (cats)
		Glycylglycine (cats)
		Serine (rabbits)
Acetyl	Acetyl group from acetyl-0CoA	
Formyl		Formylation (dogs, rats)
Sulfate	Sulfate group from PAPS	
Phosphate		Phosphate monoester formation (dogs, insects)

Source: Modified from Kulkarni, A. P. and E. Hodgson. Comparative toxicology. In *Introduction to Biochemical Toxicology*, eds. E. Hodgson and F. E. Guthrie, p. 115. New York: Elsevier, 1980.

Available evidence suggests some relationship between the evolutionary position of a species and its conjugation mechanisms. In humans and most mammals, the principal mechanisms involve conjugations with glucuronic acid, glycine, glutamine, and sulfate, mercapturic acid synthesis, acetylation, methylation, and thiocyanate synthesis. In some species of birds and reptiles, ornithine conjugation replaces glycine conjugation; in plants, bacteria, and insects, conjugation with glucose instead of glucuronic acid results in the formation of glucosides. In addition to these predominant reactions, certain other conjugative processes are found involving specific compounds in only a few species. These reactions include conjugation with phosphate, taurine, *N*-acetylglucosamine, ribose, glycyltaurine, serine, arginine, and formic acids (Table 8.2).

From the standpoint of evolution, similarity might be expected between humans and other primate species as opposed to the nonprimates. This phylogenic relationship is apparent from the relative importance of glycine and glutamine in the conjugation of arylacetic acids. The conjugating agent in humans is exclusively glutamine, and the same is essentially true with Old World monkeys while New World monkeys use both the glycine and glutamine pathways. Most nonprimates and lower primates carry out glycine conjugation selectively. A similar evolutionary trend is also observed in the N-glucuronidation of sulfadimethoxine and in the aromatization of quinic acid; both reactions occur extensively in humans, and their importance decreases with increasing evolutionary divergence from humans.

Defective operation of Phase II reactions usually causes a striking species difference in the disposition pattern of a xenobiotic. The origin of such species variations is usually either the absence or a low level of the enzyme(s) in question and/or its cofactors. Glucuronide synthesis is one of the most common detoxication mechanisms in most mammalian species. The cat and closely related species have a defective glucuronide-forming system, however. Although cats form little or no glucuronide from *o*-aminophenol, phenol, *p*-nitrophenol, 2-amino-4-nitrophenol, 1- or 2-naphthol, and morphine, they readily form glucuronides from phenolphthalein, bilirubin, thyroxine, and certain steroids. Recently, polymorphisms of UDP-

glucuronosyltransferase have been demonstrated in rat and guinea pig liver preparations; thus, defective glucuronidation in the cat is probably related to the absence of the appropriate transferase rather than that of the active intermediate, UDPGA or UDP glucose dehydrogenase, which converts UDP glucose into UDPGA.

Studies on the metabolic fate of phenol in several species have indicated that four urinary products are excreted. Although extensive phenol metabolism takes place in most species, the relative proportions of each metabolite produced varies from species to species. In contrast to the cat, which selectively forms sulfate conjugates, the pig excretes phenol exclusively as the glucuronide. This defect in sulfate conjugation in the pig is restricted to only a few substrates, however, and may be due to the lack of a specific phenyl sulfotransferase because the formation of substantial amounts of the sulfate conjugate of 1-naphthol clearly indicates the occurrence of other forms of sulfotransferases.

In Vitro *Metabolism* Numerous variables simultaneously modulate the *in vivo* metabolism of xenobiotics; therefore, their relative importance cannot be studied easily. This problem is alleviated to some extent by *in vitro* studies of the underlying enzymatic mechanisms responsible for qualitative and quantitative species differences. Quantitative differences may be related directly to the absolute amount of active enzyme present and the affinity and specificity of the enzyme toward the substrate in question. Because many other factors alter enzymatic rates *in vitro*, caution must be exercised in interpreting data in terms of species variation. In particular, enzymes are often sensitive to the experimental conditions used in their preparation. Because this sensitivity varies from one enzyme to another, their relative effectiveness for a particular reaction can be sometimes miscalculated.

Species variation in the oxidation of xenobiotics, in general, is quantitative whereas qualitative differences, such as the apparent total lack of parathion oxidation by lobster hepatopancreas microsomes, are seldom observed. Although the amount of CYP or the activity of NADPH-CYP reductase seems to be related to the oxidation of certain substrates, this explanation is not always satisfactory because the absolute amount of CYP is not necessarily the rate-limiting characteristic. It is clear that there are multiple forms of CYP isoforms in each species, and that these forms differ from one species to another. Presumably, both quantitative and qualitative variations in xenobiotic metabolism depend in variations on the particular isoforms expressed and the extent of this expression.

Reductive reactions, like oxidation reactions, are carried out at different rates by enzyme preparations from different species. Microsomes from mammalian liver are 18 times or more higher in azoreductase activity and more than 20 times higher in nitroreductase activity than those from fish liver. Although relatively inactive in nitroreductase, fish can reduce the nitro group of parathion, suggesting multiple forms of reductase enzymes.

Hydration of epoxides catalyzed by epoxide hydrolase is involved in both detoxication and intoxication reactions. With high concentrations of styrene oxide as a substrate, the relative activity of hepatic microsomal epoxide hydrolase in several animal species is rhesus monkey > human = guinea pig > rabbit > rat > mouse. With some substrates, such as epoxidized lipids, the cytosolic hydrolase may be much more important than the microsomal enzyme.

The toxicity of the organophosphorus insecticide dimethoate depends on the rate at which it is hydrolyzed *in vivo*. This toxicant undergoes two main metabolic detoxication reactions, one catalyzed by an esterase and the other by an amidase. Although rat and mouse liver carry out both reactions, only the amidase occurs in sheep liver, and the esterase in guinea pig liver. The ability of liver preparations from different animal species to degrade dimethoate is as follows: rabbit > sheep > dog > rat > cattle > hen > guinea pig > mouse > pig, these rates being roughly inversely proportioned to the toxicity of dimethoate to the same species. Insects degrade this compound much more slowly than do mammals and hence are highly susceptible to dimethoate.

Glutathione S-transferase in liver cytosol from different animal species also shows a wide variation in activity. Activity is low in humans, whereas the mouse and guinea pig appear to be more efficient than other species. The ability of the guinea pig to form the initial glutathione conjugate contrasts with its inability to readily N-acetylate cysteine conjugates; consequently, mercapturic acid excretion is low in guinea pigs.

8.4.2 Selectivity

Selective toxic agents have been developed to protect crops, animals of economic importance, and humans from the vagaries of pests, parasites, and pathogens. Such selectivity is conferred primarily through distribution and comparative biochemistry.

Selectivity through differences in uptake permits the use of an agent toxic to both target and nontarget cells provided that lethal concentrations accumulate only in target cells, leaving nontarget cells unharmed. An example is the accumulation of tetracycline by bacteria, but not by mammalian cells, the result being drastic inhibition of protein synthesis in the bacteria, leading to death.

Certain schistosome worms are parasitic in humans and their selective destruction by antimony is accounted for by the differential sensitivity of phosphofructokinase in the two species, the enzyme from schistosomes being more susceptible to inhibition by antimony than is the mammalian enzyme.

Sometimes, both target and nontarget species metabolize a xenobiotic by the same pathways, but differences in rate determine selectivity. Malathion, a selective insecticide, is metabolically activated by CYPs to the cholinesterase inhibitor malaoxon. In addition to this activation reaction, several detoxication reactions also occur. Carboxylesterase hydrolyzes malathion to form the monoacid, phosphatases hydrolyze the P–O–C linkages to yield nontoxic products, and gluthathione *S*-alkyltransferase converts malathion to desmethylmalathion. Although all of these reactions occur in both insects and mammals, activation is rapid in both insects and mammals, whereas hydrolysis to the monoacid is rapid in mammals but slow in insects. As a result, malaoxon accumulates in insects but not in mammals, resulting in selective toxicity.

A few examples are also available, in which the lack of a specific enzyme in some cells in the human body has enabled the development of a therapeutic agent. For example, guanine deaminase is absent from the cells of certain cancers but is abundant in healthy tissue; as a result, 8-azaguanine can be used therapeutically.

Distinct differences in cells with regard to the presence or absence of target structures or metabolic processes also offer opportunities for selectivity. Herbicides such as phenylureas, simazine, and so on, block the Hill reaction in chloroplasts, thereby killing plants without harm to animals. This is not always the case, because paraquat, which blocks photosynthetic reactions in plants, is a pulmonary toxicant in mammals, due apparently to analogous free-radical reactions involving enzymes different from those involved in photosynthesis.

8.4.3 Genetic Differences

Just as the xenobiotic-metabolizing ability in different animal species seems to be related to evolutionary development and therefore to different genetic constitution, different strains within a species may differ from one another in their ability to metabolize xenobiotics. One reason for differences among strains is due to the fact that many genes are polymorphic or exist in multiple forms. A polymorphism is defined as an inherited monogenetic trait that exists in the population in at least two genotypes (two or more stable alleles) and is stably inherited. They arise as the result of a mutational event and generally result in an altered gene product. In humans, many polymorphisms are somewhat race specific, arising with greater frequency in one race than in another. Observed differences between strains of rats and mice as described below may also be the result of gene polymorphisms.

The effects of genetic polymorphisms can be observed at several different levels, as discussed below:

In Vivo *Toxicity* The toxicity of organic compounds has been found to vary between different strains of laboratory animals. For example, mouse strain C3H is resistant to histamine, the LD_{5O} being 1523 mg/kg in C3H/Jax mice as compared with 230 in Swiss/ICR mice; that is, the animals of the former strain are 6.6 times less susceptible to the effects of histamine. Striking differences in the toxicity of thiourea, a compound used in the treatment of hyperthyroidism, are seen in different strains of the Norway rat. Harvard rats were 11 times more resistant, and wild Norway rats were 335 times more resistant than were rats of the Hopkins strain.

The development of strains resistant to insecticides is an extremely widespread phenomenon that is known to have occurred in several hundred species of insects and mites, and resistance of up to several hundredfold has been noted. The different biochemical and genetic factors involved have been studied extensively and well characterized. Relatively few vertebrate species are known to have developed pesticide resistance and the level of resistance in vertebrates is low compared to that often found in insects. Susceptible and resistant strains of pine voles exhibit a 7.4-fold difference in endrin toxicity. Similarly, pine mice of a strain resistant to endrin were reported to be 12-fold more tolerant than a susceptible strain. Other examples include the occurrence of organochlorine insecticide-resistant and susceptible strains of mosquito fish, and resistance to Belladonna in certain rabbit strains.

Several genetic polymorphisms have been described and characterized with respect to CYP enzymes. The first and best-known example involves CYP2D6. In the course of a clinical trial for debrisoquine, a potential drug for use in lowering

blood pressure, Dr. Robert Smith, one of the investigators who used himself as a volunteer, developed severe orthostatic hypotension with blood pressure dropping to 70/50. The effects of the drug persisted for 2 days, while in other volunteers no adverse effects were noted. Urine analysis demonstrated that in Dr. Smith, debrisoquine was excreted unchanged, while in the other volunteers the primary metabolite was 4-hydroxy debrisoquine. Subsequent studies demonstrated that CYP2D6 was responsible for the formation of 4-hydroxy debrisoquine and that the polymorphic form of CYP2D6 is prevalent in Caucasians and African Americans, in which approximately 7% are poor metabolizers. In Asian populations, the frequency of poor metabolizers is only 1%.

Another well-known genetic polymorphism has been described in the metabolism of drugs such as isoniazid. "Slow acetylators" are homozygous for a recessive gene; this is believed to lead to the lack of the hepatic enzyme acetyltransferase, which in normal homozygotes or heterozygotes (rapid acetylators) acetylates isoniazid as a step in the metabolism of this drug. This effect is seen also in humans, the gene for slow acetylation showing marked differences in distribution between different human populations. It is very low in Eskimos and Japanese, with 80–90% of these populations being rapid acetylators, whereas 40–60% of Blacks and some European populations are rapid acetylators. Rapid acetylators often develop symptoms of hepatotoxicity and polyneuritis at the dosage necessary to maintain therapeutic blood levels of isoniazid.

Many other significant polymorphisms in XMEs have been described including those for several CYP genes, alcohol and aldehyde dehydrogenases, epoxide hydrolase, and paraoxonase. One interesting polymorphism affecting metabolism of dietary trimethylamines involves FMO3. Individuals with FMO3 polymorphisms have a condition known as fish odor syndrome, or trimethylaminurea. Individuals with this syndrome exhibit an objectionable body odor resembling rotting fish due to their inability to N-oxidize trimethylamine which are found in many foods including meat, eggs, and soybeans. This syndrome often leads to social isolation, clinical depression, and even suicide. Other toxicological implications of this polymorphism are still not known.

Metabolite Production Strain variations with respect to hexobarbital are often dependant on its degradation rate. For example, male mice of the AL/N strain are long sleepers, and this trait is correlated with slow inactivation of the drug. The reverse is true in CFW/N mice, which have short sleeping time due to rapid hexobarbital oxidation. This close relationship is further evidenced by the fact that the level of brain hexobarbital at awakening is essentially the same in all stains. Similar strain differences have been reported for zoxazolamine paralysis in mice.

Studies on the induction of aryl hydrocarbon hydroxylase by 3-methylcholanthrene have revealed several responsive and nonresponsive mouse strains, and it is now well established that the induction of this enzyme is controlled by a single gene. In the accepted nomenclature, aryl hydrocarbon $(Ah)^b$ represents the allele for responsiveness, whereas Ah^d denotes the allele for nonresponsiveness.

In rats, both age and gender seem to influence strain variation in xenobiotic metabolism. Male rats exhibit about twofold variation between strains in hexobarbital metabolism, whereas female rats may display up to sixfold variation. In

either gender, the extent of variations depends on age. The ability to metabolize hexobarbital is related to the metabolism of other substrates, and the interstrain differences are maintained.

A well-known interstrain difference in Phase II reactions is that of glucuronidation in Gunn rats. This is a mutant strain of Wistar rats that is characterized by a severe, genetically determined defect of bilirubin glucuronidation. Their ability to glucuronidate *o*-aminophenol, *o*-aminobenzoic acid, and a number of other substrates is also partially defective. This deficiency does not seem to be related to an inability to form UDPGA but rather to the lack of a specific UDP-glucuronosyltransferase. It has been demonstrated that Gunn rats can conjugate aniline by N-glucuronidation and can form the *O*-glucuronide of *p*-nitrophenol.

Rabbit strains may exhibit up to 20-fold variation, particularly in the case of hexobarbital, amphetamine, and aminopyrine metabolism. Relatively smaller differences between strains occur with chlorpromazine metabolism. Wild rabbits and California rabbits display the greatest differences from other rabbit strains in hepatic drug metabolism.

Enzyme Differences Variation in the nature and amount of constitutively expressed microsomal CYPs have not been studied extensively in different strains of the same vertebrate, although after induction, it has been shown that in addition to quantitative differences in the amount of CYP in different strains of mice, there may also be a qualitative difference in the CYP isoforms induced. (See Section 8.5.2, Induction.)

8.5 CHEMICAL EFFECTS

With regard to both logistics and scientific philosophy, the study of the metabolism and toxicity of xenobiotics must be initiated by considering single compounds. Unfortunately, humans and other living organisms are not exposed in this way; rather, they are exposed to many xenobiotics simultaneously, involving different portals of entry, modes of action, and metabolic pathways. An estimate of the number of chemicals in use in the United States is given in Table 8.3. Because it bears directly on the problem of toxicity-related interaction between different

TABLE 8.3 Estimates of the Number of Chemicals in Use in the United States

Number	Type	Source of Estimate[a]
1500	Active ingredients of pesticides	EPA
4000	Active ingredients of drugs	FDA
2000	Drug additives (preservatives, stabilizers, etc.)	FDA
2500	Food additives (nutritional value)	FDA
3000	Food additives (preservatives, stabilizers, etc.)	FDA
50,000	Additional chemicals in common use	EPA

[a] EPA, Environmental Protection Agency; FDA, Food and Drug Administration.

xenobiotics, the effect of chemicals on the metabolism of other exogenous compounds is one of the more important areas of biochemical toxicology.

Xenobiotics, in addition to serving as substrates for a number of enzymes, may also serve as inhibitors or inducers of these or other enzymes. Furthermore, many examples are known of compounds that first inhibit and subsequently induce enzymes such as the microsomal monooxygenases. The situation is even further complicated by the fact that although some substances have an inherent toxicity and are detoxified in the body, others without inherent toxicity can be metabolically activated to potent toxicants. The following examples are illustrative of the situations that might occur involving two compounds:

- Compound A, without inherent toxicity, is metabolized to a potent toxicant. In the presence of an inhibitor of its metabolism, there would be a reduction in toxic effect or, in the presence of an inducer, compound A would appear to be more toxic.
- Compound B, a toxicant, is metabolically detoxified. In the presence of an inhibitor of the detoxifying enzymes, there would be an increase in the toxic effect or, in the presence of an inducer, compound B would appear to be less toxic.

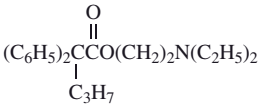
In addition, the toxicity of the inhibitor or inducer, as well as the time dependence of the effect, must also be considered because, as mentioned, many xenobiotics that are initially enzyme inhibitors ultimately become inducers. As the number of patients receiving multiple drugs has increased dramatically, there has been an increase in reports of drug–drug interactions resulting in adverse effects that has lead in some cases to the removal of drugs from the market. The U.S. Food and Drug Administration (FDA) has established guidelines for determining the ability of a drug to induce or inhibit xenobiotic enzymes and transporters. In the following sections, inhibition and induction will be discussed.

8.5.1 Inhibition

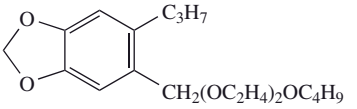
As previously indicated, inhibition of XMEs can cause either an increase or a decrease in toxicity. Several well-known inhibitors of such enzymes are shown in Figure 8.5 and are discussed in this section. Inhibitory effects can be demonstrated in a number of ways at different organizational levels.

Types of Inhibition: Experimental Demonstration

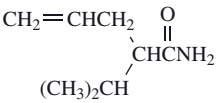
In Vivo *Symptoms* The measurement of the effect of an inhibitor on the duration of action of a drug *in vivo* was formerly a common method of demonstrating its action. These methods are open to criticism, however, because effects on duration of action can be mediated by systems other than those involved in the metabolism of the drug. Furthermore, they cannot be used for inhibitors that have pharmacological activity similar or opposite to the compound being used. At the present time, as a consequence of the availability of single expressed isoforms for direct studies of inhibitory mechanisms, these methods are used much less often although they are still valuable for human health risk analysis.



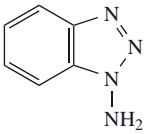
SKF-525A [P450]
2-(Diethylamino)ethyl-
2,2-diphenylpentanoate



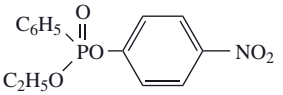
Piperonyl Butoxide [P450]
3,4-Methylenedioxy-6-propylbenzyl
N-butyl diethyleneglycol ether



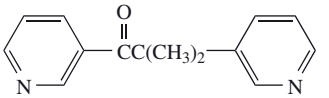
Allylisopropylacetamide
[P450]



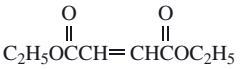
1-Aminobenzotriazole (1-ABT)
[P450]



EPN [esterases]
O-Ethyl-*O*-*p*-nitrophenyl
phenylphosphonothioate



Metyrapone
[P450]



Diethyl maleate
[glutathione *S*-transferase]

Disulfiram (Antabuse)
[aldehyde dehydrogenase]

Figure 8.5 Some common inhibitors of xenobiotic-metabolizing enzymes.

Previously, the most used and most reliable of these tests involved the measurement of effects on the hexobarbital or pentobarbital sleeping time and the zoxazolamine paralysis time. Both of these drugs are fairly rapidly deactivated by the hepatic microsomal monooxygenase system; thus, inhibitors of this system prolong their action. For example, treatment of mice with chloramphenicol 0.5–1.0 h before pentobarbital treatment prolongs the duration of the pentobarbital sleeping time in a dose-related manner; it is effective at low doses (<5 mg/kg) and has a greater than 10-fold effect at high doses (100–200 mg/kg). The well-known inhibitor of drug metabolism, SKF-525A, causes an increase in both hexobarbital sleeping time and zoxazolamine paralysis time in rats and mice, as do the insecticide synergists piperonyl butoxide and tropital, the optimum pretreatment time being about 0.5 h before the narcotic is given. In the case of activation reactions, such as the activation of the insecticide azinphosmethyl to its potent anticholinesterase oxon derivative, a decrease in toxicity is apparent when rats are pretreated with the CYP inhibitor SkF-525A.

Cocarcinogenicity may also be an expression of inhibition of a detoxication reaction, as in the case of the cocarcinogenicity of piperonyl butoxide, a CYP inhibitor, and the carcinogens, freons 112 and 113.

Distribution and Blood Levels Treatment of an animal with an inhibitor of xenobiotic metabolism may cause changes in the blood levels of an unmetabolized toxicant and/or its metabolites. This procedure may be used in the investigation of the inhibition of detoxication pathways; it has the advantage over *in vitro* methods of yielding results of direct physiological or toxicological interest because it is carried out in the intact animal. For example, if animals are first treated with either SKF-525A, glutethimide, or chlorcyclizine, followed in 1 h or less by pentobarbital, it can be shown that the serum level of pentobarbital is considerably higher in treated animals than in controls within 1 h of its injection. Moreover, the time sequence of the effects can be followed in individual animals, a factor of importance when inhibition is followed by induction—a not uncommon event.

Effects on Metabolism In Vivo A further refinement of the previous technique is to determine the effect of an inhibitor on the overall metabolism of a xenobiotic *in vivo*, usually by following the appearance of metabolites in the urine and/or feces. In some cases, the appearance of metabolites in the blood or tissue may also be followed. Again, the use of the intact animal has practical advantages over *in vitro* methods, although little is revealed about the mechanisms involved.

Studies of antipyrine metabolism may be used to illustrate the effect of inhibition on metabolism *in vivo*; in addition, these studies have demonstrated variation between species in the inhibition of the metabolism of xenobiotics. In the rat, a dose of piperonyl butoxide of at least 100 mg/kg was necessary to inhibit antipyrine metabolism, whereas in the mouse, a single intraperitoneal (IP) or oral dose of 1 mg/kg produced a significant inhibition. In humans, an oral dose of 0.71 mg/kg had no discernible effect on the metabolism of antipyrine.

Disulfiram (Antabuse(Odyssey Pharmaceuticals, Florham Park, NJ, USA)) inhibits aldehyde dehydrogenase irreversibly, causing an increase in the level of acetaldehyde, formed from ethanol by the enzyme alcohol dehydrogenase. This results in nausea, vomiting, and other symptoms in the human—hence its use as a deterrent in alcoholism. Inhibition by disulfiram appears to be irreversible, the level returning to normal only as a result of protein synthesis.

Use of specific metabolic enzyme inhibitors may often provide valuable information with respect to the metabolism of a particular drug. For example, quinidine is a potent and selective inhibitor of CYP2D6. This drug has been used in clinical studies as a pharmacological tool to mimic the lack of CYP2D6 in humans. By demonstrating that quinidine substantially slows the metabolism of trimipramine (a tricyclic antidepressant), investigators have implicated CYP2D6 in its metabolism.

Effects on In Vitro *Metabolism Following* In Vivo *Treatment* This method of demonstrating inhibition is of variable utility. The preparation of enzymes from animal tissues usually involves considerable dilution with the preparative medium during homogenization, centrifugation, and resuspension. As a result, inhibitors not tightly bound to the enzyme in question are lost, either in whole or in part, during the preparative processes. Therefore, negative results can have little utility because failure to inhibit and loss of the inhibitor give identical results. Positive results, however, not only indicate that the compound administered is an inhibitor but also provide a clear indication of excellent binding to the enzyme, most probably due to the formation of a covalent or slowly reversible inhibitory complex.

The inhibition of esterases following treatment of the animal with organophosphorus compounds, such as paraoxon, is a good example, because the phosphorylated enzyme is stable and is still inhibited after the preparative procedures. Inhibition by carbamates, however, is greatly reduced by the same procedures, because the carbamylated enzyme is unstable and, in addition, the residual carbamate is highly diluted.

Microsomal monooxygenase inhibitors that form stable inhibitory complexes with CYP, such as SKF-525A, piperonyl butoxide and other methylenedioxyphenyl compounds, and amphetamine and its derivatives, can be readily investigated in this way because the microsomes isolated from pretreated animals have a reduced capacity to oxidize many xenobiotics.

Another form of chemical interaction, resulting from inhibition *in vivo*, that can then be demonstrated *in vitro*, involves those xenobiotics that function by causing destruction of the enzyme in question, so-called suicide substrates. Exposure of rats to vinyl chloride results in a loss of CYP and a corresponding reduction in the capacity of microsomes subsequently isolated to metabolize foreign compounds. Allyl isopropylacetamide and other allyl compounds have long been known to have a similar effect.

In Vitro *Effects* *In vitro* measurement of the effect of one xenobiotic on the metabolism of another is by far the most common type of investigation of interactions involving inhibition. Although it is the most useful method for the study of inhibitory mechanisms, particularly when purified enzymes are used, it is of more limited utility in assessing the toxicological implications for the intact animal. The principal reason for this is that *in vitro* measurement does not assess the effects of factors that affect absorption, distribution, and prior metabolism, all of which occur before the inhibitory event under consideration. The use of human hepatocytes to study CYP inhibition can take into account some, but not all of these confounding factors, and has proven to provide data that is in good agreement with microsomal studies.

Although the kinetics of inhibition of XMEs can be investigated in the same ways as any other enzyme mechanism, a number of problems arise that may decrease the value of this type of investigation. They include the following:

- Inhibition of microsomal CYP-dependent oxidations and CYP isoforms have been investigated many times. However, as a result of using methods on particulate systems that were developed for single soluble enzymes, Lineweaver–Burk or other reciprocal plots are frequently curvilinear, and the same reaction may appear to have quite a different characteristic from laboratory to laboratory, species to species, and organ to organ.
- The nonspecific binding of substrate and/or inhibitor to membrane components is a further complicating factor affecting inhibition kinetics.
- Both substrates and inhibitors are frequently lipophilic, with low solubility in aqueous media.
- XMEs commonly exist in multiple forms (e.g., glutathione S-transferases and CYPs). These isoforms are all relatively nonspecific but differ from one another in the relative affinities of the different substrates.

The primary considerations in studies of inhibition mechanisms are reversibility and selectivity. The inhibition kinetics of reversible inhibition give considerable insight into the reaction mechanisms of enzymes and, for that reason, have been well studied. In general, reversible inhibition involves no covalent binding, occurs rapidly, and can be reversed by dialysis or, more rapidly, by dilution. Reversible inhibition is usually divided into competitive inhibition, uncompetitive inhibition, and noncompetitive inhibition. Because these types are not rigidly separated, many intermediate classes have been described.

Competitive inhibition is usually caused by two substrates competing for the same active site. Following classic enzyme kinetics, there should be a change in the apparent Michaelis constant (K_m), but not the maximum reaction rate (V_{max}). In microsomal monooxygenase reaction, type I ligands, which often appear to bind as substrates but do not bind to the heme iron, might be expected to be competitive inhibitors, and this frequently appears to be the case. Examples are the inhibition of the *O*-demethylation of *p*-nitroanisole by aminopyrine, aldrin epoxidation by dihydroaldrin, and *N*-demethylation of aminopyrene by nicotinamide. More recently, some of the polychlorinated biphenyls (PCBs), notably dichlorbiphenyl have been shown to have a high affinity as type I ligands for rabbit liver CYP and to be competitive inhibitors of the *O*-demethylation of *p*-nitroanisole.

Uncompetitive inhibition has seldom been reported in studies of xenobiotic metabolism. It occurs when an inhibitor interacts with an enzyme–substrate complex but cannot interact with free enzyme. Both K_m and V_{max} change by the same ratio, giving rise to a family of parallel lines in a Lineweaver–Burk plot.

Noncompetitive inhibitors can bind to both the enzyme and enzyme–substrate complex to form either an enzyme–inhibitor complex or an enzyme–inhibitor–substrate complex. The net result is a decrease in V_{max} but no change in K_m. Metyrapone (Figure 8.5), a well-known inhibitor of monooxygenase reactions, can also, under some circumstances, stimulate metabolism *in vitro*. In either case, the effect is noncompetitive, in that the K_m does not change, whereas V_{max} does, decreasing in the case of inhibition and increasing in the case of stimulation.

Irreversible inhibition, which is much more important toxicologically, can arise from various causes. In most cases, the formation of covalent or other stable bonds is involved or, more rarely, the disruption of the enzyme structure. In either of these cases, the effect cannot be readily reversed *in vitro* by either dialysis or dilution. The formation of stable inhibitory complexes may involve the prior metabolic formation of a reactive intermediate that then interacts with the enzyme, giving rise to the terms "mechanism-based inhibitor" or "suicide substrate." An excellent example of this type of inhibition is the effect of the insecticide synergist piperonyl butoxide (Figure 8.5) on hepatic microsomal monooxygenase activity. This methylenedioxyphenyl compound can form a stable inhibitory complex that blocks CO binding to CYP and also prevents substrate oxidation. This complex results from the formation of a reactive intermediate, which is shown by the fact that the type of inhibition changes from competitive to irreversible as metabolism, in the presence of NADPH and oxygen, proceeds. It appears probable that the metabolite in question is a carbene formed spontaneously by elimination of water following hydroxylation of the methylene carbon by the cytochrome (see Figure 6.8 for metabolism of methylenedioxyphenyl compounds). Piperonyl butoxide inhibits the *in vitro* metabolism of many substrates of the monooxygenase system, including

aldrin, ethylmorphine, aniline, and aminopyrine, as well as carbaryl, biphenyl, hexobarbital, *p*-nitroanisole, and many others. Although most of the studies carried out on piperonyl butoxide have involved rat or mouse liver microsomes, they have also been carried out on pig, rabbit, and carp liver microsomes, and in various preparations from houseflies, cockroaches, and other insects. Certain classes of monooxygenase inhibitors, in addition to methylenedioxyphenyl compounds, are now known to form "metabolite inhibitory complexes," including amphetamine and its derivatives, and SKF-525A and its derivatives.

The inhibition of the carboxylesterase that hydrolyzes malathion by organophosphorus compounds, such as EPN, is a further example of xenobiotic interaction resulting from irreversible inhibition because in this case, the enzyme is phosphorylated by the inhibitor.

Another class of irreversible inhibitors of toxicological significance consists of those compounds that bring about the destruction of the XMEs, hence such inhibitors are also designated "suicide substrates." The drug allylisopropylacetamide (Figure 8.5), as well as other allyl compounds, has long been known to cause the breakdown of CYP and the resultant release of heme. More recently, the hepatocarcinogen vinyl chloride has also been shown to have a similar effect, probably also mediated through the generation of a highly reactive intermediate. Much information has accumulated since the mid 1970s on the mode of action of the hepatotoxicant carbon tetrachloride, which affects a number of irreversible changes in both liver protein structure, such as urea, detergents, strong acids, and so on, are probably of significance only in *in vitro* experiments.

The importance of irreversible inhibition as opposed to competitive inhibition is illustrated in Table 8.4, which summarizes the inhibition of human Phase I reactions of both xenobiotic and endogenous metabolites by pesticides. Apart from inhibition of estradiol metabolism by pyrethroids and carbamates, all other examples are the result of mechanism-based irreversible inhibition.

Synergism and Potentiation The terms synergism and potentiation have been used and defined in various ways but, in any case, they involve a toxicity that is greater when two compounds are given simultaneously or are in close sequence than would be expected from a consideration of the toxicities of the compounds given alone. In medicine, synergism generally refers to the ability of two drugs to have a combined greater effect than the sum of the individual effects.

In an attempt to resolve the semantic difficulties and to make uniform the use of these terms, it is suggested that insofar as toxic effects are concerned, the terms be used as follows: *Both synergism and potentiation involve toxicity greater than would be expected from the toxicities of the compounds administered separately, but in the case of synergism, one compound has little or no intrinsic toxicity when administered alone, whereas in the case of potentiation, both compounds have appreciable toxicity when administered alone. It is further suggested that no special term is needed for simple additive toxicity of two or more compounds.*

An example of synergism has already been mentioned. Piperonyl butoxide, sesamex, and related compounds increase the toxicity of insecticides to insects by inhibiting insect CYP. Other insecticide synergists that interact with CYP include aryloxyalkylamines such as SKF-525A, Lilly 18947, and their derivatives; compounds containing acetylenic bonds such as aryl-2-propynyl phosphate esters

TABLE 8.4 Inhibition of Human Hepatic Phase I Metabolism by Pesticides

Substrate	Enzyme	Inhibitor(s)
Reference		
Xenobiotic substrates		
Carbaryl	Liver microsomes	Chlorpyrifos
Carbaryl	CYP2B6	Chlorpyrifos
Carbofuran	Liver microsomes	Chlorpyrifos
DEET	Liver microsomes	Chlorpyrifos
Fipronil	Liver microsomes	Chlorpyrifos
Fipronil	CYP3A4	Chlorpyrifos
Imipramine	Liver microsomes	Chlorpyrifos, azinphosphos methyl, parathion
Imipramine	CYPs 1A2, 3A4, 2C19	Chlorpyrifos, azinphosphos methyl, parathion
Nonane	Liver microsomes	Chlorpyrifos
Nonane	CYP2B6	Chlorpyrifos
Permethrin	Liver cytosol	Chlorpyrifos oxon, carbaryl
Endogenous substrates		
Estradiol	Liver microsomes	Chlorpyrifos, fonofos, carbaryl, naphthalene
Estradiol	CYP1A2	Chlorpyrifos, fonofos, carbaryl, naphthalene
Estradiol	CYP3A4	Chlorpyrifos, fonofos, deltamethrin, permethrin
Testosterone	Liver microsomes	Chlorpyrifos, phorate, fonofos
Testosterone	CYP3A4	Chlorpyrifos

containing propynyl functions; phosphorothionates; benzothiadiazoles; and some imidazole derivatives.

Antagonism In toxicology, antagonism may be defined as that situation in which the toxicity of two or more compounds administered together or sequentially is less than would be expected from a consideration of their toxicities when administered individually. Strictly speaking, this definition includes those cases in which the lowered toxicity results from induction of detoxifying enzymes (this situation is considered separately in Section 8.5.2). A part from the convenience of treating such antagonistic phenomena together with the other aspects of induction, they are frequently considered separately because of the significant time that must elapse between treatment with the inducer and subsequent treatment with the toxicant. The reduction of hexobarbital sleeping time and the reduction of zoxazolamine paralysis time by prior treatment with phenobarbital to induce drug-metabolizing enzymes are obvious examples of such induction effects at the acute level of drug action, whereas protection from the carcinogenic action of benzo(a)pyrene, aflatoxin B1**,** and diethylnitrosamine by phenobarbital treatment are examples of inductive effects at the level of chronic toxicity. In the latter case, the CYP isoforms induced by phenobarbital metabolize the chemical to less toxic metabolites.

Antagonism not involving induction is a phenomenon often seen at a marginal level of detection and is consequently both difficult to explain and is of marginal significance. In addition, several different types of antagonism of importance to toxicology that do not involve xenobiotic metabolism are known but are not appropriate for discussion in this chapter. They include competition for receptor sites, such as the competition between CO and O_2 in CO poisoning or situations in which one toxicant combines nonenzymatically with another to reduce its toxic effects, such as in the chelation of metal ions. Physiological antagonism, in which two agonists act on the same physiological system but produce opposite effects, is also of importance.

8.5.2 Induction

In the early 1960s, during investigations on the *N*-demethylation of aminoazo dyes, it was observed that pretreatment of mammals with the substrate or, more remarkably, with other xenobiotics, caused an increase in the ability of the animal to metabolize these dyes. It was subsequently shown that this effect was due to an increase in the microsomal enzymes involved. A symposium in 1965 and a landmark review by Conney in 1967 established the importance of induction in xenobiotic interactions. Since then, it has become clear that this phenomenon is widespread and nonspecific and involves enhanced gene transcription due to activation of receptors. Several hundred compounds of diverse chemical structure have been shown to induce monooxygenases and other enzymes. These compounds include drugs, pesticides, hydrocarbons, industrial chemicals, and many others; the only obvious common denominator is that they are organic and lipophilic. It has also become apparent that, even though all inducers do not have the same effects, the effects tend to be nonspecific to the extent that any single chemical can induce more than one xenobiotic-metabolizing gene. Other enzyme genes can also be induced, such as glutathione *S*-transferase, UDP-glucuronosyltransferases (UGTs), and epoxide hydrolases.

Specificity of Monooxygenase Induction The majority of studies involving *CYP* induction have been conducted in mammals. Mammals have at least 17 distinct *CYP* families, coding for as many as 50–60 individual *CYP* genes in any given species. Many of these *CYP* families are fairly specific for endogenous metabolic pathways and are not typically involved in metabolism of foreign chemicals. As discussed in Chapter 6, *CYP* families 1–4 are the predominant families involved in xenobiotic metabolism. These CYP enzyme families are also known for their ability to respond to xenobiotic challenges by increasing their protein levels. Many of the genes within families 1–4 are transcriptionally activated through one of four receptor-dependent mechanisms. Others, such as *CYP2E1* are regulated at the level of mRNA stabilization and/or protein stabilization. These mechanisms of regulation are discussed in Section 8.5.2.

Inducers of CYP genes act by similar mechanisms and are exemplified by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (inducer of *CYP1A1*), phenobarbital (inducer of the *CYP2B* and *CYP3A* families), rifampicin (inducer of *CYP3A* and *2C* families), and ethanol (inducer of *CYP2E1*). Inducers of the phenobarbital type tend to share few structural features other than lipophilicity, while TCDD-like inducers are primarily polycyclic hydrocarbons. Other inducers, such as ethanol, dexamethasone, and clofibrate are more specific. Many inducers require either

fairly high dose levels or repeated dosing to be effective, frequently >10 mg/kg and some as high as 100–200 mg/kg. Some insecticides, however, such as mirex, can induce at dose levels as low as 1 mg/kg, while the most potent inducer known, TCDD, is effective at 1 μg/kg in some species.

In the liver, phenobarbital-type inducers cause a marked proliferation of the smooth endoplasmic reticulum as well as an increase in the amount of CYP content. Often, these changes are sufficient to result in significant liver weight increases. Phenobarbital induction induces a wide range of oxidative activities including O-demethylation of *p*-nitroanisole, N-demethylation of benzphetamine, pentobarbital hydroxylation, and aldrin hydroxylation. *CYP* gene families that are primarily induced by phenobarbital and phenobarbital-like inducers include *CYP2B*, *CYP2C*, and *CYP3A* subfamilies.

In contrast with phenobarbital, induction by TCDD and polycyclic hydrocarbons does not cause proliferation of the endoplasmic reticulum although the CYP content is increased. CYP1A1 is the primary isoform induced, although other non-CYP proteins such as UGTs are also induced. Induction of CYP1A1 by polycyclic hydrocarbons results in the induction of a relatively narrow range of oxidative activities, consisting primarily of reactions involving aryl hydrocarbon hydroxylase, the best known reaction being the hydroxylation of benzo(a)pyrene.

Rifampicin and pregnenolone-16α-carbonitrile (PCN) induce members of the *CYP3A* family and represent a third type of inducer, in that the substrate specificity of the microsomes from treated animals differs from that of the microsomes from either phenobarbital-treated or TCDD-treated animals. Inducing substrates of this class include endogenous and synthetic glucocorticoids (e.g., dexamethasone (DEX)), pregnane compounds (e.g., PCN), and macrolide antiobiotics (e.g., rifampicin).

Ethanol and a number of other chemicals, including acetone and certain imidazoles, induce CYP2E1. Piperonyl butoxide, isosafrole, and other methylenedioxyphenyl compounds are known to induce CYP1A2 by a non-aryl hydrocarbon receptor (AhR)-dependent mechanism. Peroxisome proliferators, including the drug clofibrate, and the herbicide synergist tridiphane induce a CYP4A isozyme that catalyzes the ω-oxidation of lauric acid.

All inducers do not fall readily into one or the other of these classes. Some oxidative processes can be induced by either type of inducer, such as the hydroxylation of aniline and the *N*-demethylation of chlorcyclizine. Some inducers, such as the mixture of PCBs designated Arochlor 1254, can induce a broad spectrum of CYP isoforms. Many variations also exist in the relative stimulation of different oxidative activities within the same class of inducer, particularly of the phenobarbital type.

It appears reasonable that because several types of CYP are associated with the endoplasmic reticulum, various inducers may induce one or more of them. Because each of these types has a relatively broad substrate specificity, differences may be caused by variations in the extent of induction of different CYP isoforms. Now that methods are available for gel electrophoresis of microsomes and identification of specific isoforms by immunoblotting and isoforms-specific antibodies, the complex array of inductive phenomena is being more logically explained in terms of specific isozymes.

Although the bulk of published investigations of the induction of monooxygenase enzymes have dealt with the mammalian liver, induction has been observed in other mammalian tissues and in nonmammalian species, both vertebrate and inver-

tebrate. Many induced CYPs have now been cloned and/or purified from a variety of species. It is clear that many of these induced CYPs represent only a small percentage of the total CYP in the uninduced animal. For this reason, the "constitutive" isoforms, those already expressed in the uninduced animal, must be fully characterized because they represent the available xenobiotic-metabolizing capacity of the normal animal.

Mechanism and Genetics of Induction in Mammals Many different mechanisms may be involved in CYP induction. These include increased transcription of DNA, increased mRNA translation to protein, mRNA stabilization, and protein stabilization. Induction can only occur in intact cells and cannot be achieved by the addition of inducers directly to cell fractions such as microsomes. It has been known for some time that in most, but not necessarily all, cases of increase in monooxygenase activity, there is a true induction involving synthesis of new mRNA, and not an increase in activity of an enzyme already synthesized, since induction is generally prevented by inhibitors of protein synthesis. For example, the protein synthesis inhibitors such as puromycin, ethionine, and cycloheximide inhibit aryl hydrocarbon hydroxylase activity. A simplified scheme for gene expression and protein synthesis is shown in Figure 8.6.

Perhaps the best understood example of induction involves ligand activation of the AhR by compounds such as TCDD and 3-methylcholanthrene. The use of suitable inhibitors of RNA polymerase activity has shown that an inhibitor such as actinomycin D block increases in aryl hydrocarbon hydroxylase activity. Thus, it appears that the increase in enzyme activity is due to the induction of *CYP* genes and requires new RNA synthesis.

These findings indicate these compounds act as ligands for receptors that mediate the induction of genes encoding XMEs in a manner analogous to steroid hormones—namely, combining with a cytosolic receptor followed by movement into the nucleus and then increased transcription of the appropriate gene. In the case of the AhR, TCDD or some other appropriate ligand enters the cell through the plasma membrane and binds to the cytosolic Ah receptor protein (Figure 8.7). After ligand binding, the receptor translocates to the nucleus where it forms a dimer with another protein known as aryl hydrocarbon receptor nuclear translocator (ARNT).

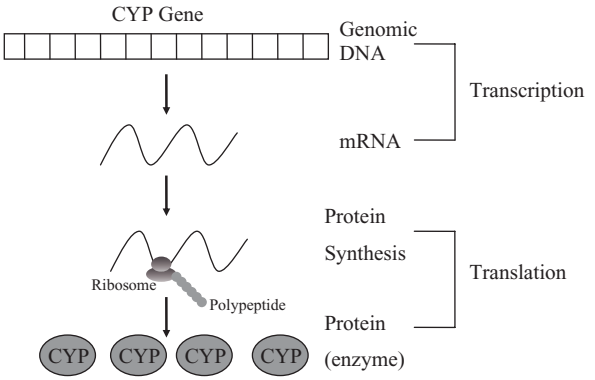


Figure 8.6 Simplified scheme for gene expression in animals of a *CYP* gene.

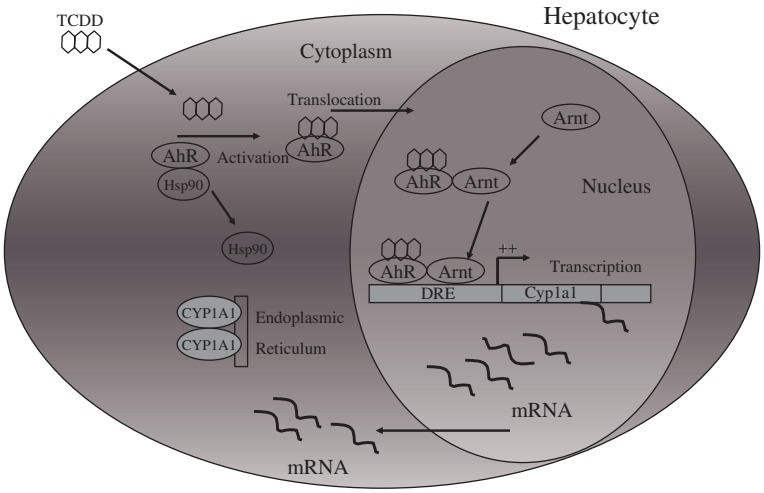


Figure 8.7 Proposed mechanism for TCDD-activated AhR translocation and DNA binding. Upon TCDD binding, activated AhR sheds chaperone proteins such as heat shock protein 90 (Hsp 90), translocates to the nucleus, and heterodimerizes with the ARNT protein. In the nucleus, the AhR–Arnt complex binds to dioxin response elements (DREs) such as those found in the *CYP1A1* gene promoter region. Activation of transcription of the *Cyp1a1* gene leads to formation of new RNA and an increase in *CYP1A1* protein levels.

Figure 8.8 The mouse *Cyp1a1* and *Cyp1a2* genes are located on chromosome 9 and contain 8 dioxin response elements (DREs) that are responsible for the AhR-mediated induction. A DRE cluster (DREC) is located 1.4 kb upstream of the *Cyp1a1* gene which also plays a role in the induction of *Cyp1a2*. (Source: Adapted from Nukaya, M., S. Moran, and C. A. Bradfield. The role of the dioxin-response element cluster between Cyp1a1 and Cyp1a2 loic in aryl hydrocarbon receptor biology. *Proc. Natl. Acad. Sci. USA* **106**:4923–4928, 2009.)

In the nucleus, the activated receptor acts as a transcription factor and interacts with specific sequences of DNA known as dioxin responsive elements (DREs) such as those found near the mouse *CYP1A1* gene (Figure 8.7). On mouse chromosome 9 multiple DREs, known as a DRE cluster (DREC), are located upstream from the transcriptional start site in the 5′ flanking region of the mouse *CYP1A1* gene, which also have been shown to influence CYP1A2 expression (Figure 8.8). The protein–DNA interaction that occurs at the DREs and along with interaction with coactivator proteins results in and increased transcription followed by increased protein synthesis. Activation of the AhR signaling pathway by TCDD-type inducers has been determined to induce a number of Phase I and Phase II genes.

Although phenobarbital induction has been studied for many years, the mechanism for induction has only recently been established.

The major advance in understanding phenobarbital induction came from a study using rat primary hepatocytes where phenobarbital responsiveness was demonstrated to be associated with a 163 bp DNA sequence at –2318 through –2155 bp of the *CYP2B2* gene. Subsequent studies using *in situ* transfection of *CYP2B2* promoter-luciferase constructs into rat livers confirmed this, as did similar studies involving the mouse *CYP2b10* gene. Additional deletion assays have narrowed phenobarbital responsiveness down to a minimum sequence of 51 bp from –2339 through –2289 of the *Cyp2b10* gene, now known as the phenobarbital-responsive enhancer module (PBREM). The PBREM sequence has also been found in rat *CYP2B1*, *CYP2B2*, and human *CYP2B6* genes. Multiple cis-acting elements within this fragment cooperate to bring about increased DNA transcription that include two receptor DR4 sites (NR1 and NR2).

The transcription factor that interacts with the PBREM is the nuclear receptor (NR) known as constitutive androstane receptor (CAR) or NR1I3 (NR subfamily 1, group I, member 3). CAR binds to each of the PBRE NR sites as a heterodimer with the retinoid X receptor (RXR), a common heterodimerization partner for many orphan NRs. Mouse CAR is normally found within the cytoplasm, but phenobarbital exposure results in a change in CAR's phosphorylation state, dissociation from chaperone proteins, and subsequently, translocation to the nucleus. Translocation to the nucleus leads to RXR association, DNA binding to the PBREM, and association coactivator proteins to form an activate transcriptional complex. Initial studies indicated that CAR was constitutively active, but this was most likely due to cell culture systems that did not contain adequate levels of co-chaperones to maintain CAR with the cytoplasm. Recent studies using CAR knockout mice indicate that many drug metabolizing genes are under CAR regulation, including *CYP2B*, *CYP3A*, NADPH-CYP reductase, and enzymes involved in sulfotransferase metabolism.

In the early 1980s, a distinct group of *CYPs* was described by several groups, which was characterized principally by its inducibility by glucocorticoids, by a mechanism not involving the glucocorticoid receptor, and by a wide variety of other chemicals with no structural similarities. Specifically, the *CYP3A* subfamily, which is well-known for the diversity of substrates which it is capable of metabolizing, was highly induced. In humans, the specific isoform, CYP3A4, is responsible not only for the metabolism of endogenous compounds such as testosterone, but also is credited for the metabolism of the largest number of currently used drugs. Many CYP3A substrates are also known for their ability to induce their own metabolism as well as the metabolism of other CYP3A substrates, resulting in patients with the potential for dangerous drug–drug type interactions. Regulation of the CYP3A family is primarily through enhanced transcription as demonstrated in studies using the RNA inhibitor actinomycin D and the translation inhibitor cycloheximide. Several studies in rats and human hepatocytes have identified several elements on the 5′ upstream promoter region as well as receptors involved in CYP3A regulation (Figure 8.9). Deletion studies involving transient transfections of various chimeric reporter gene constructs into primary cultures of rat hepatocytes demonstrated the presence of a dexamethasone/PCN response element within the first 164 bp at the start of transcription. Subsequent studies demonstrated that several CYP3A isoforms from different species contained NR binding sites that are activated by DEX/PCN but exhibit low activation by rifampicin. Further work identified an

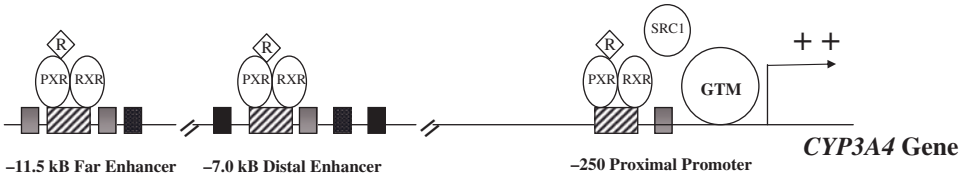


Figure 8.9 Illustration depicting the multiple pregnane X receptor (PXR) response elements found in the human *CYP3A4* gene. The activation of the human pregnane X receptor (PXR) by binding to the ligand rifampicin (R) leads to heterodimerization with the retinoid X receptor (RXR), subsequent binding to PXR response elements located in the proximal promoter, distal enhancer, and far enhancer. Transcriptional activation by PXR is aided by interaction with co-activators, such as the steroid receptor co-activator-1 (SRC-1), that help facilitate formation of the general transcriptional machinery (GTM), which includes RNA polymerase and associated proteins.

additional 230 bp distal element called the xenobiotic-responsive enhancer module (XREM) located at –7836 through –7607 of the CYP3A4 that conferred responsiveness to both rifampicin and dexamethasone when combined with the proximal promoter region. The XREM contains two PXR binding sites, one DR3 motif (dNR1) and one ER6 motif (dNR2), neither of which is solely responsible for the activity of XREM. An additional proximal promoter PXR binding site, known as dNR3, contains an ER6 motif and, most recently, a far distal enhancer was identified that contains aER6 site located 11.5 kb from the start of transcription, and both appear to have important roles in CYP3A4 induction.

The nuclear orphan receptor, pregnane X receptor (PXR), also known as NR1I2 (NR subfamily 1, group I, member 2), is the major determinant of *CYP3A* gene regulation by xenobiotics. Several lines of evidence support PXR involvement with *CYP3A* induction. First, both PXR and CYP3A isoforms are predominantly expressed in liver and intestine, with less expression found in lung and kidneys. Second, PXR binds to human and rat *CYP3A* promoter regions and can activate expression of *CYP3A4* promoter in transfection assays. Third, many of the same inducers of *CYP3A* isoforms also activate PXR. Fourth, interspecies differences in response to CYP3A inducers have been demonstrated to be due to the ability of these inducers to activate PXR in these species. Fifth, disruption of the mouse PXR gene eliminated induction of *CYP3A* by PCN, and transgenic mice "humanized" with the PXR gene were able to respond to rifampicin induction. PXR has a unique ligand binding domain (LBD) compared with other receptors, and X-ray crystallography of the LBD has shown that it is much larger, and thus can accommodate a wide variety of ligands. PXR has been shown to play a key role in the induction of a growing number of metabolizing genes including Phase I, Phase II, and xenobiotic transporters.

Peroxisome proliferators, including hypolipidemic drugs such as clofibrate, phthalate plasticizers, and herbicides bring about the induction of a CYP4A isoform that catalyzes the oxidation of many biologically important fatty acids, including arachidonic acid and other eicosanoids. *CYP4A* expression is part of a pleiotropic response in the rodent liver which includes increased liver weight, proliferation of peroxisomes, and the elevation of several peroxisomal enzymes such as catalase. Peroxisome proliferators are often epigenetic carcinogens in rodents but, since the

effect is primarily seen in rodents, its significance for other species such as humans is unclear. The receptor protein, peroxisome proliferator-activated receptor-α (PPARα), was first cloned in 1990. PPARα knockout mice exposed to chemicals which normally induce CYP4A as well as peroxisome proliferation do not exhibit these characteristics, demonstrating the essential nature of PPARα for these responses. Like PXR, PPARα also binds to DNA as a heterodimer with RXR in response to peroxisome proliferating chemicals.

Other CYPs are induced by similar mechanisms involving other transcription factors including farnesoid X receptor (FXR), liver X receptor (LXR), hepatic nuclear factor (HNF) family members, GR, and CCAAT/enhancer-binding proteins (C/EBPs). Many of the previously mentioned inductions of *CYP1A1*, *CYP2B*, and *CYP3A* family members also involve these transcription factors and overlap exists in these regulatory pathways as genes may contain multiple transcription factor binding sites.

CYP2E1 catalyzes metabolism of several low molecular weight xenobiotics including drugs (e.g., acetaminophen), solvents (e.g., ethanol, carbon tetrachloride), and procarcinogens (e.g., *N*-nitrosodimethylamine). Induction of *CYP2E1* can occur as a result of exposure to several xenobiotics including ethanol, acetone, and imidazole, or alternatively, as a result of physiological conditions such as starvation and diabetes. Its induction by either fasting or diabetes is believed to be due to the high levels of ketones likely to be present in either of these conditions. It might also be noted that although *CYP2E1* is in the same family as *2B1* and *2B2*, it is not induced by phenobarbital-type inducers. In contrast to many other inducible *CYPs*, *CYP2E1* induction is not accompanied by high levels of *CYP2E1* mRNA, suggesting that regulation is by means of a posttranscriptional mechanism.

The regulation of *CYP2E1* gene expression involves several mechanisms that do not primarily include increased transcription. Recent studies demonstrated that rapid increases in CYP2E1 protein levels following birth are due to stabilization of preexisting proteins by ketone bodies released at birth. Rats treated with ethanol or acetone can have three- to sixfold increases in CYP2E1 protein in the absence of increased *CYP2E1* mRNA. Other studies have demonstrated that substrates including ethanol, imidazole, and acetone had little effects on *CYP2E1* transcript content and that these substrates tend to prevent protein degradation. Thus, increased protein expression levels in response to these substrates is due to enhanced translation and also protein stabilization a result of the inhibition of the proteasome ubiquitin degradation pathway of proteolysis. The ubiquitination process normally tags proteins with a chain of multiple ubiquitin moieties, thus targeting proteins for selective degradation by a cytosolic 26S protease, known as the proteasome. In recent studies, an antibody prepared against a putative ubiquitination-target site on the CYP2E1 protein quenched ubiquitination in a concentration-dependent manner. These results provide a plausible mechanistic explanation for the observation that substrate binding protects the CYP2E1 protein from ubiquitin-dependent proteolysis.

In other observations, diabetes is known to increase CYP2E1 expression at both the mRNA and protein levels in both chemically induced and spontaneous diabetic rats. Elevation of mRNA levels as a result of diabetes has been attributed to mRNA stabilization which can be reversed by daily insulin treatment. Recent research has shown that insulin destabilizes *CYP2E1* mRNA by binding to a 16 bp sequence

within the 5′ coding sequence of *CYP2E1*. The mechanism for regulation by this means is still uncertain although other genes have also been reported with similar destabilizing sequences within their coding sequences.

Effects of Induction The effects of inducers are usually the opposite of those of inhibitors; thus, their effects can be demonstrated by much the same methods, that is, by their effects on pharmacological or toxicological properties *in vivo* or by the effects on enzymes *in vitro* following prior treatment of the animal with the inducer. *In vivo* effects are frequently reported; the most common ones are the reduction of the hexobarbital sleeping time or zoxazolamine paralysis time. Also, *in vivo* effects of CYP3A inducers can be determined by pharmacokinetic studies by administering mice the CYP3A substrate midazolam (MDZ). After removing small amounts of blood over a time coure, the disappearance of midazolam, or the formation of MDZ metabolite can be measured by liquid chromatography mass spectrometry/mass spectrometry (LC-MS/MS) methods. These effects have been reported for numerous inducers and can be quite dramatic. For example, in the rat, the paralysis time resulting from a high dose of zoxazolamine can be reduced from 11 h to 17 min by treatment of the animal with benzo(a)pyrene 24 h before the administration of zoxazolamine.

The induction of CYP enzymes may be protective, as demonstrated by the creation of a DREC-deficient mouse model where loss of Cyp1a1 and Cyp1a2 induction lead to enhanced dioxin-induced hepatotoxicity. The induction of monooxygenase activity may also protect an animal from the effect of carcinogens by increasing the rate of detoxication. This has been demonstrated in the rat with a number of carcinogens including benzo(a)pyrene, *N*-2-fluorenylacetamide, and aflatoxin B_1. Effects on carcinogenesis may be expected to be complex because some carcinogens are both activated and detoxified by monooxygenase enzymes, while epoxide hydrolase, which can also be involved in both activation and detoxication, may also be induced. For example, the toxicity of the carcinogen 2-naphthylamine, the hepatotoxic alkaloid monocrotaline, and the cytotoxin cyclophosphamide are all increased by phenobarbital induction—an effect mediated by the increased population of reactive intermediates.

Organochlorine insecticides are also well-known inducers. Treatment of rats with either DDT or chlordane, for example, will decrease hexobarbital sleeping time and offer protection from the toxic effect of warfarin. Persons exposed to DDT and lindane metabolized antipyrine twice as fast as a group not exposed, whereas those exposed to DDT alone had a reduced half-life for phenylbutazone and an increased excretion of 6-hydroxycortisol.

Effects on xenobiotic metabolism *in vivo* are also widely known in both humans and animals. Cigarette smoke, as well as several of its constituent polycyclic hydrocarbons, is a potent inducer of aryl hydrocarbon hydroxylase in the placenta, liver, and other organs. The average content of CYP1A1 in liver biopsies from smokers was approximately fourfold higher than that from nonsmokers. Hepatic activity of CYP1A1 as measured by phenacetin *O*-demethylation, was also increased from 54 pmol/min/mg of protein in nonsmokers to 230 nmol/min/mg of protein in smokers. Examination of the term placentas of smoking human mothers revealed a marked stimulation of aryl hydrocarbon hydroxylase and related activities—remarkable in an organ that, in the uninduced state, is almost inactive toward foreign chemicals. These *in vitro* differences in metabolism are also observed *in vivo*, as smokers have

been demonstrated to have increased clearance rates for several drugs metabolized principally by CYP1A1 including theophylline, caffeine, phenacetin, fluvoxamine, clozapine, and olanzapine.

Induction of XMEs Other than Monooxygenases Although less well studied, XMEs other than those of the CYP system have been shown to be induced in recent studies, frequently by the same inducers that induce the oxidases. These include glutathione S-transferases, epoxide hydrolase, UDP-glucuronosyltransferase, and sulfotransferases, and the mechanisms of induction involve receptors such as AhR, PXR, and CAR. The selective induction of one pathway over another can greatly affect the metabolism of a xenobiotic.

8.5.3 Biphasic Effects: Inhibition and Induction

Many inhibitors of mammalian monooxygenase activity can also act as inducers. Inhibition of microsomal monooxygenase activity is fairly rapid and involves a direct interaction with the cytochrome, whereas induction is a slower process. Therefore, following a single injection of a suitable compound, an initial decrease due to inhibition would be followed by an inductive phase. As the compound and its metabolites are eliminated, the levels would be expected to return to control values. Some of the best examples of such compounds are the methylenedioxyphenyl synergists, such as piperonyl butoxide. Because CYP combined with methylenedioxyphenyl compounds in an inhibitory complex cannot interact with CO, the CYP titer, as determined by the method of Omura and Sato (dependent upon CO-binding to reduced cytochrome), would appear to follow the same curve.

It is apparent from extensive reviews of the induction of monooxygenase activity by xenobiotics that many compounds other than methylenedioxyphenyl compounds have the same effect. It may be that any synergist that functions by inhibiting microsomal monooxygenase activity could also induce this activity on longer exposure, resulting in a biphasic curve as described previously for methylenedioxyphenyl compounds. This curve has been demonstrated for NIA 16824 (2-methylpropyl-2-propynyl phenylphosphonate) and WL 19255 (5,6-dichloro-1,2,3-benzothiadiazole), although the results were less marked with R05-8019 [2,(2,4,5-trichlorophenyl)-propynyl ether] and MGK 264 [*N*-(2-ethylhexyl)-5-norbornene-2,3-dicarboximide].

8.6 ENVIRONMENTAL EFFECTS

Because the *in vitro* effects of light, temperature, and so on, on XMEs are not different from their effects on other enzymes or enzyme systems, we are not concerned with them at present. This section deals with the effects of environmental factors on the intact animal as they relate to *in vivo* metabolism of foreign compounds.

8.6.1 Temperature

Although it might be expected that variations in ambient temperature would not affect the metabolism of xenobiotics in animals with homeothermic control, this is not the case. Temperature variations can be a form of stress and thereby produce

changes mediated by hormonal interactions. Such effects of stress require an intact pituitary-adrenal axis and are eliminated by either hypophysectomy or adrenalectomy. There appears to be two basic types of temperature effect on toxicity; either with increase in toxicity at both high and low temperature, or an increase in toxicity with an increase in temperature. For example, both warming and cooling increases the toxicity of caffeine to mice, whereas the toxicity of *D*-amphetamine is lower at reduced temperatures and shows a regular increase with increases in temperature.

In many studies, it is unclear whether the effects of temperature are mediated through metabolism of the toxicant or via some other physiological mechanism. In other cases, however, temperature clearly affects metabolism. For example, in cold-stressed rats, there is an increase in the metabolism of 2-naphthylamine to 2-amino-1-naphthol.

8.6.2 Ionizing Radiation

In general, ionizing radiation reduces the rate of metabolism of xenobiotics both *in vivo* and in enzyme preparations subsequently isolated. This has occurred in hydroxylation of steroids, in the development of desulfuration activity toward azinphosmethyl in young rats, and in glucuronide formation in mice. Pseudocholinesterase activity is reduced by ionizing radiation in the ileum of both rats and mice.

8.6.3 Light

Because many enzymes, including some of those involved with xenobiotic metabolism, show a diurnal pattern that can be keyed to the light cycle, light cycles rather than light intensity would be expected to affect these enzymes. In the case of hydroxyindole-*O*-methyltransferase in the pineal gland, there is a diurnal rhythm with greatest activity at night; continuous darkness causes maintenance of the high level. CYP and the microsomal monooxygenase system show a diurnal rhythm in both the rat and the mouse, with greatest activity occurring at the beginning of the dark phase.

8.6.4 Moisture

No moisture effect has been shown in vertebrates, but in insects, it was noted that housefly larvae reared on diets containing 40% moisture had four times more activity for the epoxidation of heptachlor than did larvae reared in a similar medium saturated with water.

8.6.5 Altitude

Altitude can either increase or decrease toxicity. It has been suggested that these effects are related to the metabolism of toxicants rather than to the physiological mechanisms involving the receptor system, but in most examples, this has not been demonstrated clearly. Examples of altitude effects include the observations that at altitudes of ≥5000 ft, the lethality of digitalis or strychnine to mice is decreased, whereas that of *D*-amphetamine is increased.

8.6.6 Other Stress Factors

Noise has been shown to affect the rate of metabolism of 2-napthylamine, causing a slight increase in the rat. This increase is additive with that caused by cold stress.

8.7 SUMMARY AND CONCLUSIONS

It is apparent from the material presented in this chapter and the previous chapters related to metabolism that the metabolism of xenobiotics is complex, involving many enzymes; that it is susceptible to a large number of modifying factors, both physiological and exogenous; and that the toxicological implications of metabolism are important. In spite of the complexity, summary statements of considerable importance can be abstracted:

1. Phase I metabolism generally introduces a functional group into a xenobiotic, which enables conjugation to an endogenous metabolite to occur during Phase II metabolism.
2. The conjugates produced by Phase II metabolism are considerably more water soluble than either the parent compound or the Phase I metabolite(s) and hence are more easily excreted.
3. During the course of metabolism, and particularly during Phase I reactions, reactive intermediates that are much more toxic than the parent compound may be produced. Thus, xenobiotic metabolism may be either a detoxication or an activation process.
4. Because the number of enzymes involved in Phase I and Phase II reactions is large and many different sites on organic molecules are susceptible to metabolic attack, the number of potential metabolites and intermediates that can be derived from a single substrate is frequently very large.
5. Because both qualitative and quantitative differences exist between species, strains, individual organs, and cell types, a particular toxicant may have different effects in different circumstances.
6. Because exogenous chemicals can be inducers and/or inhibitors of the XMEs of which they are substrates; such chemicals may interact to bring about toxic sequelae different from those that might be expected from any of them administered alone.
7. Because endogenous factors also affect the enzymes of xenobiotic metabolism, the toxic sequelae to be expected from a particular toxicant will vary with developmental stage, nutritional statue, health or physiological status, stress, or environment.
8. It has become increasingly clear that most enzymes involved in xenobiotic metabolism occur as several forms, which coexist within the same individual and, frequently, within the same subcellular organelle. An understanding of the biochemistry and molecular genetics of these isoforms may lead to an understanding of the variation between species, individuals, organs, sexes, developmental stages, and so on.

BIBLIOGRAPHY AND SUGGESTED READING

Anderson, K. E. and A. Kappas. Dietary regulation of cytochrome P450. *Annu. Rev. Nutr.* **11**:141–167, 1991.

Benedetti, M. S., R. Whomsley, and M. Canning. Drug metabolism in the paediatric population and in the elderly. *Drug Discov. Today* **12**:599–610, 2007.

Brown, H. S., A. Chadwick, and J. B. Houston. Use of isolated hepatocyte preparations for cytochrome P450 inhibition studies: Comparison with microsomes for ki determination. *Drug Metab. Dispos.* **35**:2119–2126, 2007.

Conney, A. H. Induction of drug-metabolizing enzymes: A path to the discovery of multiple cytochromes P450. *Annu. Rev. Pharmacol. Toxicol.* **43**:1–30, 2003.

Donaldson, W. E. Nutritional factors. In *Introduction to Biochemical Toxicology*, 3rd ed., eds. E. Hodgson and R. C. Smart, pp. 255–276. Hoboken, NJ: Wiley, 2001.

Falls, J. G., D.-Y. Ryu, Y. Cao, et al. Regulation of mouse liver flavin-containing monooxygenases 1 and 3 by sex steroids. *Arch. Biochem. Biophys.* **342**:212–223, 1997.

Goodwin, B., M. R. Redinbo, and S. A. Kliewer. Regulation of CYP3A gene transcription by the pregnane X receptor. *Annu. Rev. Pharmacol. Toxicol.* **42**:1–23, 2002.

Guengerich, F. P. Cytochrome P-450 3A4: Regulation and role in drug metabolism. *Annu. Rev. Pharmacol. Toxicol.* **39**:1–17, 1999.

Hodgson, E. and E. Croom. Phase I—Toxicogenetics. In *Molecular and Biochemical Toxicology*, 4th ed., eds. E. Hodgson and R. C. Smart. Hoboken, NJ: Wiley, 2008.

Huang, S. M., J. M. Strong, L. Zhang, et al. New era in drug interaction evaluation: U.S. Food and Drug Administration update on CYP enzymes, transporters, and the guidance process. *J. Clin. Pharmacol.* **48**:662–670, 2008.

Kakizaki, S., Y. Yamamoto, A. Ueda, et al. Phenobarbital induction of drug/steroid-metabolizing enzymes and nuclear receptor CAR. *Biochim. Biophys. Acta.* **1619**:239–242, 2003.

Kocarek, T. A., R. C. Zanger, and R. F. Novak. Post-transcriptional regulation of rat CYP2E1 expression: Role of CYP2E1 mRNA untranslated regions in control of translational efficiency and message stability. *Arch. Biochem. Biophys.* **376**:180–190, 2000.

Kulkarni, A. P. and E. Hodgson. Comparative toxicology. In *Introduction to Biochemical Toxicology*, eds. E. Hodgson and F. E. Guthrie, p. 115. New York: Elsevier, 1980.

Lee, C. and S. L. Werlin. The induction of hepatic cytochrome P450 3A in rats: Effects of age. *Proc. Soc. Exp. Biol. Med.* **210**:134–139, 1995.

Lin, J. H. CYP induction-mediated drug interactions: In vitro assessment and clinical implications. *Pharm. Res.* **23**:1089–1116, 2006.7

Mukhtar, H. and J. R. Bend. Serum glutathione S-transferases: perinatal development, sex difference, and effect of carbon tetrachloride administration on enzyme activity in the rat. *Life Sci.* **21**:1277–1286, 1977.

Murray, M. Altered CYP expression and function in response to dietary factors: Potential roles in disease pathogenesis. *Curr. Drug Metab.* **7**:67–81, 2006.

Newton, D. J., R. W. Wang, and A. Y. H. Lu. Cytochrome P450 inhibitors: Evaluation of specificities in the in vitro metabolism of therapeutic agents by human liver microsomes. *Drug Metab. Dispos.* **23**:154–158, 1995.

Nukaya, M., S. Moran, and C. A. Bradfield. The role of the dioxin-responsive element cluster between the Cyp1a1 and Cyp1a2 loci in aryl hydrocarbon receptor biology. *Proc. Natl. Acad. Sci. USA* **106**:4923–4928, 2009.

Parkinson, A., D. R. Mudra, C. Johnson, et al. The effects of gender, age, ethnicity, and liver cirrhosis on cytochrome P450 enzyme activity in human liver microsomes and inducibility in cultured human hepatocytes. *Toxicol. Appl. Pharmacol.* **199**:193–209, 2004.

Ronis, M. J. J. and H. C. Cunny. Physiological (endogenous) factors affecting the metabolism of xenobiotics. In *Introduction to Biochemical Toxicology*, 2nd ed., eds. E. Hodgson and P. E. Levi, p. 136. Norwalk, CT: Appleton & Lange, 1994.

Ronis, M. J. J. and H. C. Cunny. Developmental effects on xenobiotic metabolism. In *Molecular and Biochemical Toxicology*, 4th ed., eds. E. Hodgson and R. C. Smart, Hoboken, NJ: Wiley, 2008.

Sueyoshi, T. and M. Negishi. Phenobarbital response elements of cytochrome P450 genes and nuclear receptors. *Annu. Rev. Pharmacol. Toxicol.* **41**:123–143, 2001.

Timsit, Y. E. and M. Negishi. CAR and PXR: The xenobiotic-sensing receptors. *Steroids* **72**:231–246, 2007.

Yao, M., M. Zhu, M. W. Sinz, et al. Development and full validation of six inhibition assays for five major cytochrome P450 enzymes in human liver microsomes using an automated 96-well microplate incubation format and LC-MS/MS analysis. *J. Pharm. Biomed. Anal.* **44**:211–223, 2007.

SAMPLE QUESTIONS

1. Chemical A has no inherent toxicity but is metabolized to a potent toxicant while chemical B, an inherently potent toxicant is metabolically detoxified. What would be the effect of treatment of the animal with either an inhibitor or an inducer of the enzymes involved in their metabolism?

2. What are the advantages and disadvantages of demonstrating inhibition of the metabolism of a toxicant by observation of (a) *in vivo* symptoms or (b) *in vitro* effects of the inhibitor on the xenobiotic-metabolizing enzyme involved.

3. Define "mechanism-based inhibitor" (also known as "suicide inhibitor") citing two examples.

4. Discuss the time course in the expected change in enzyme activity (i.e., the shape of the curve) following treatment of an animal with a synergist such as piperonyl butoxide.

5. Compound Z is being studied for its ability to alter the metabolism of the cytochrome P450 (CYP) substrate testosterone. You are presented with the *in vitro* metabolism data below. The rate of testosterone metabolism to the metabolite 6β-OH testosterone was studied using increasing concentrations of testosterone (0–8 μM) with a constant concentration of Compound Z (2 μM). What does the data tell you about how compound Z is altering testosterone metabolism? Briefly, describe how you would study the *in vivo* effects of Compound Z on liver metabolism.

6. After eating two charcoal-broiled hotdogs and one very well done hamburger at a picnic, you have significantly induced the levels of the metabolizing enzyme CYP1A1 in your liver. Name and briefly discuss the receptor pathway inducing CYP1A1 in the liver due to the polycyclic hydrocarbons (TCDD-like compounds) in the cooked meat ingested. Name two nutritional effects and two physiological effects that can alter the levels of xenobiotic metabolism.

CHAPTER 9

Elimination of Toxicants

GERALD A. LEBLANC

9.1 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.

As organisms increased in complexity:

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.

9.1.1 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.

A Textbook of Modern Toxicology, *Fourth Edition*. Edited by Ernest Hodgson

9.1.2 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.

9.1.3 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 (e.g., adipose tissue contains a significant amount of lipid; whereas blood contains a significant amount of water), chemicals are faced with the challenge to be mobile in these various environments.

9.1.4 Lipid Content

As a general though not universal rule, organisms have the ability 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.

9.1.5 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 the organisms 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., coevolved) with biotransformation processes that render chemicals amenable to these modes of elimination (see Chapter 6).

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 or returned to the blood for renal elimination. 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 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 dichlorodiphenyltrichloroethane (DDT) metabolite dichlorodiphenyldichloroethylene (DDE), the flame retardant mirex, and the polychlorinated biphenyls (PCBs) have been measured at significant levels in mother's milk from Arctic Inuit populations. While lactation may provide a benefit to the mother by the elimination of toxic chemicals, transfer of these toxicants to the suckling infant may pose health risk to the infant.
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 analysis is often used as a marker of exposure to such materials.

9.2 TRANSPORT

For a chemical to be eliminated from the body at a site of elimination (e.g., kidney) that is distant from the site of storage (e.g., adipose tissue) or toxicity (e.g., 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.

9.3 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.

9.3.1 Size

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

9.3.2 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 6) 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 9.1). 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 9.1). 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 reabsorbed from the filtrate and returned to the blood. Much of this reabsorbtion occurs in the proximal tubules (Figure 9.1). Cells lining the proximal tubules contain fingerlike projections that extend 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 reabsorption occurs in the descending portion of the loop resulting in concentration of the filtrate. Water reabsorption does not occur in the ascending portion of the loop. Rather, the remaining, concentrated ions such as sodium, chloride, and potassium are reabsorbed. Those materials retained in the

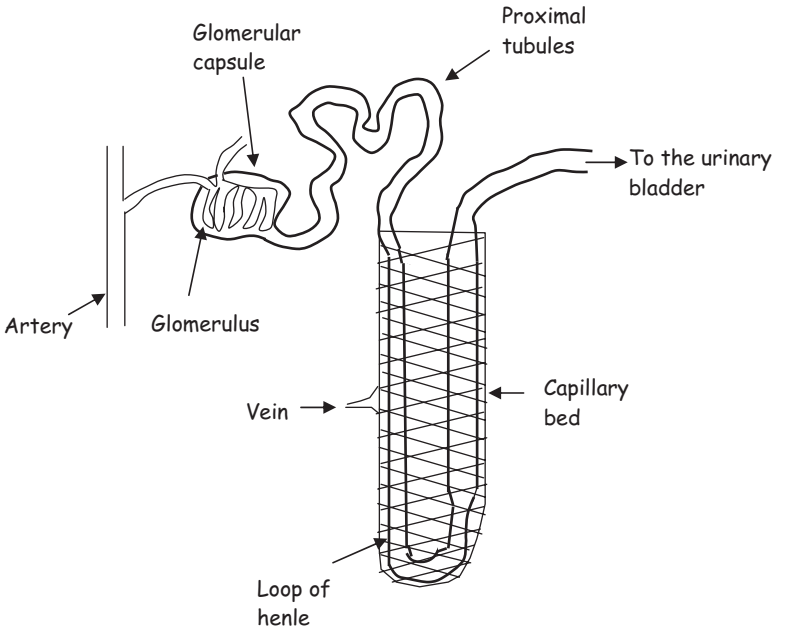


Figure 9.1 The nephron of the kidney. The nephron is the functional unit of the kidney that is responsible for the removal of water-soluble wastes and foreign compounds from the blood.

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.

9.4 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, typically 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 9.2). Sinusoids are cavernous spaces among the hepatocytes which 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 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, then undergo endocytosis (Figure 9.3).

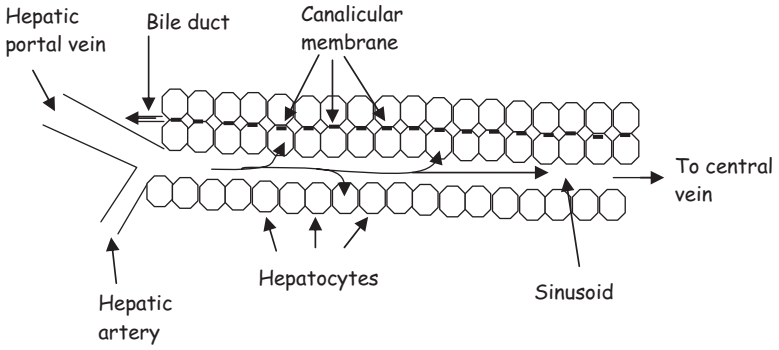


Figure 9.2 Diagrammatic representation of the basic architecture of the liver.

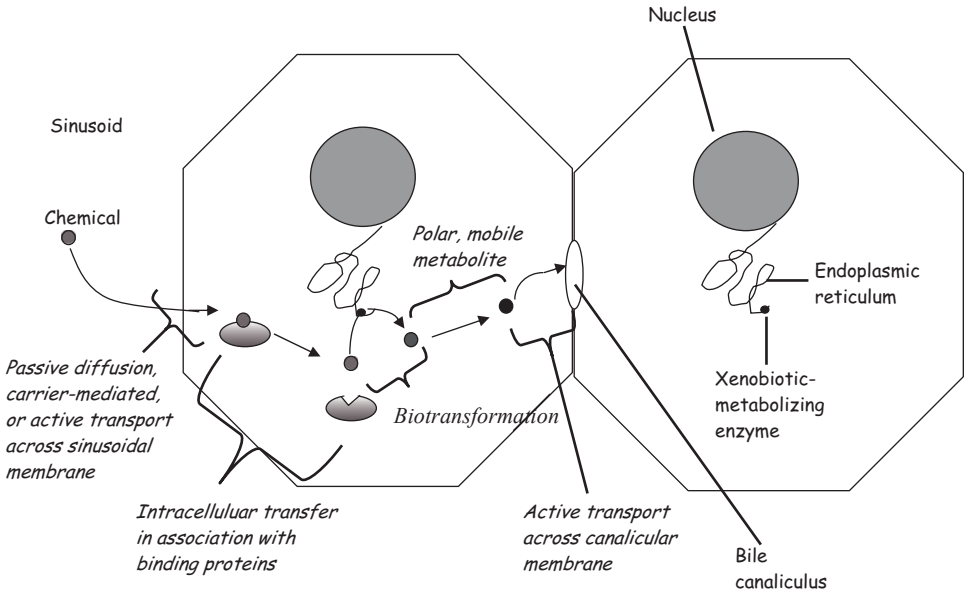


Figure 9.3 Vectorial transport of a chemical from the liver sinusoid, through the hepatocyte, to the cannalicular space.

Lipophilic materials require intracellular carrier proteins to be optimally mobilized, just as they required transport proteins in the blood (Figure 9.3). Several intracellular carrier proteins that mobilize specific endogenous chemical have been characterized. Many of these proteins have been shown to bind xenobiotics; however, less is known of their actual contribution to intracellular xenobiotic mobilization. The intracellular lipid binding proteins are a major family in intracellular carrier proteins. Within this family are the fatty acid binding proteins (FABP), the cellular retinoic acid binding proteins (CRABP), and the bile acid binding proteins (BABP). Some of the cytosolic glutathione S-transferase proteins also have been shown to non-catalytically 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 6). 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 9.3). 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.

9.4.1 Entero-Hepatic Circulation

Upon biliary elimination into the gastrointestinal tract, chemicals that have undergone conjugation reactions in the liver may be subject to the action of hydrolytic enzymes that deconjugate the molecule. Deconjugation results in increased lipophilicity of the molecule and renders them once again subject to passive uptake. Reabsorbed chemicals reenter the circulation via the hepatic portal vein which shunts the chemical back to the liver, where the chemical can be reprocessed (i.e., biotransformed) and eliminated. This process is called entero-hepatic circulation (Figure 9.4). A chemical may undergo several cycles of entero-hepatic circulation

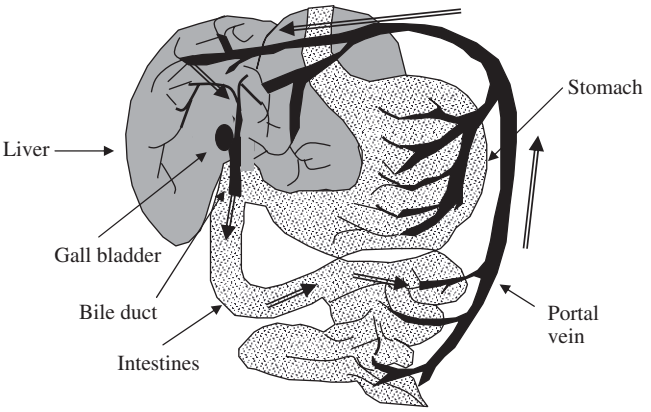


Figure 9.4 Enterohepatic circulation (as indicated by ⟹). Polar xenobiotic conjugates are secreted into the intestine via the bile duct and gall bladder. Conjugates are hydrolyzed in the intestines, released xenobiotics are reabsorbed and transported back to the liver via the portal vein.

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 (Chapter 7). Chemical toxicity to the liver is discussed elsewhere (Chapter 13).

9.4.2 Active Transporters of the Bile Canaliculus

The bile canaliculi are minute tubes that form among hepatocytes. These tubes are the product of aggregates of specialized membrane (canalicular membrane) on the basolateral surfaces the hepatocytes (Figure 9.3). Bile passes from the hepatocytes into these tubes which merge to form larger bile ductules and eventually the bile duct. The bile canaliculus constitutes only ~13% of the contiguous surface membrane of the hepatocyte yet 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 multigene superfamily of proteins know 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 Kow < 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 (e.g., glutathione, glucuronic acid, 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.

9.5 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, membranous 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.

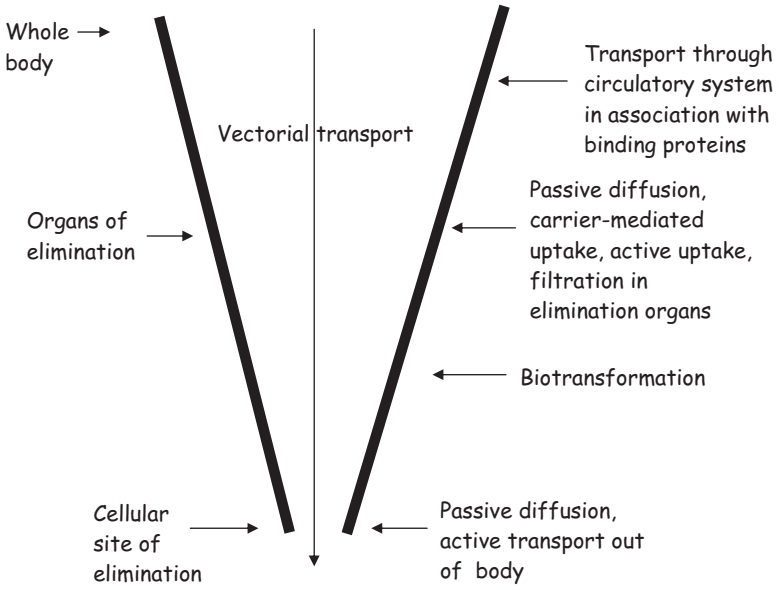


Figure 9.5 Processes involved in the vectorial transport of xenobiotics from the whole body point of origin to the specific site of elimination.

9.6 CONCLUSION

Many processes function coordinately to ensure that chemicals that are distributed throughout the body are efficiently eliminated at distinct and highly specialized locations. This directional transfer of chemicals from the site of origin (i.e., site of absorption, storage, and toxicity) to the site of elimination is a form of vectorial transport (Figure 9.5). 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 coevolution of complexity in form from unicellular to multiorgan organisms.

BIBLIOGRAPHY AND SUGGESTED READING

Kester, J. E. Liver. In *Encyclopedia of Toxicology*, Vol. 2, ed. P. Wexler, pp. 253–261. New York: Academic Press, 1998.

Miller, D. S. Cellular transport and elimination. In *Molecular and Biochemical Toxicology*, 4th ed., eds. R. C. Smart and E. Hodgson, pp. 273–286. Hoboken, NJ: Wiley, 2008.

Rankin, G. O. Kidney. In *Encyclopedia of Toxicology*, Vol. 2, ed. P. Wexler, pp. 198–225. New York: Academic Press, 1998.

Rozman, K. K. and C. D. Klassen. Absorption, distribution, and excretion of toxicants. In *Casarett & Doull's Toxicology*, 6th ed., ed. C. D. Klaassen, pp. 107–132. New York: McGraw-Hill, 2001.

SAMPLE QUESTIONS

1. What is meant by "vectorial transport" with respect to the elimination of toxicants from the body?

2. How do hepatic metabolic conjugation reactions contribute to the vectorial elimination of toxicants?

3. What are the bile canaliculi?

4. What is the role of serum binding proteins such as albumin and sex steroid binding protein in the elimination of toxicants?