CHAPTER 7

# Metabolism of Toxicants

RANDY L. ROSE and ERNEST HODGSON

## 7.1 INTRODUCTION

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 wide arrays of 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.

Most xenobiotic metabolism occurs in the liver, an organ devoted to the synthesis of many important biologically functional proteins and thus with 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 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. Following the addition of a polar group, conjugating enzymes typically add much more bulky substituents, such as sugars, sulfates, or amino acids that result in a substantially increased water solubility of the xenobiotic, making it 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.

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 8.

*A Textbook of Modern Toxicology, Third Edition,* edited by Ernest Hodgson
ISBN 0-471-26508-X 

## 7.2 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 7.1.

### 7.2.1 The Endoplasmic Reticulum, Microsomal Preparation, and Monooxygenations

Monooxygenation of xenobiotics are catalyzed either by the cytochrome P450 (CYP)-dependent monooxygenase system or by flavin-containing monooxygenases (FMO).

**Table 7.1 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, imiprimine, thiourea, methimazole |
| Desulfuration | Fonofos |
| Prostaglandin synthetase cooxidation | |
| Dehydrogenation | Acetaminophen, benzidine, epinephrine |
| *N*-Dealkylation | Benzphetamine, dimethylaniline |
| Epoxidation/hydroxylation | Benzo(a)pyrene, 2-aminofluorene, phenylbutazone |
| Oxidation | FANFT, ANFT, 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 |

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 of the components of the CYP-dependent monooxygenase system, the specific activity of the smooth type is usually higher.

The preparation of microsomal fractions, S9, and cytosolic fractions from tissue homogenates involves the use of two to 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 minutes. The resulting supernatant, often referred to as the S9 fraction, can be used in studies where both microsomal and cytosolic enzymes are desired. More often, however, the S9 fraction is centrifuged at $100{,}000 \times g$ for 60 minutes to yield a microsomal pellet and a cytosolic supernatant. The pellet is typically resuspended in a volume of buffer, which will give 20 to 50 mg protein/ml and stored at $-20$ to $-70^{\circ}C$. Often, the microsomal pellet is resuspended a second time and resedimented at $100{,}000 \times g$ for 60 minutes 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 NADPH cytochrome P450 reductase for CYP. Enzymes found in the cytosolic fraction (derived from the supernatant of the first $100{,}000 \times g$ spin) include hydrolases and most of the conjugating enzymes such as glutathione transferases, glucuronidases, sulfotransferases, methyl transferases, and acetylases. It is important to note that some cytosolic enzymes can also be found in microsomal fractions, although the opposite is not generally the case.

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):

$$RH + O_2 + NADPH + H^+ \longrightarrow NADP^+ + ROH + H_2O.$$

### 7.2.2 The Cytochrome P450-Dependent Monooxygenase System

The CYPs, the carbon monoxide-binding pigments of microsomes, are heme proteins of the b cytochrome type. Originally described as a single protein, there are now known to be more than 2000 CYPs widely distributed throughout animals, plants, and microorganisms. A system of nomenclature utilizing the prefix CYP has been devised

for the genes and cDNAs corresponding to the different forms (as discussed later in this section), although P450 is still appropriate as a prefix for the protein products. Unlike most cytochromes, the name CYP 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 $\alpha$-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 phosphatidylchloline, 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 this spectrum. 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 to 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 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-cytochrome P450 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 oxidations. This reductase is an essential component in CYP-catalyzed enzyme systems reconstituted from purified components. Moreover antibodies prepared from purified reductase are inhibitors of microsomal

monooxygenase reactions. The reductase is a flavoprotein of approximately 80,000 daltons that contain 2 mole each of flavin mononucleotide (FMN) and flavinadenine dinucleotide (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 mechanism of CYP function has not been established unequivocally; however, the generally recognized steps are shown in Figure 7.1. The initial step consists of the binding of substrate to oxidize CYP followed by a one electron reduction catalyzed by NADPH-cytochrome P450 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

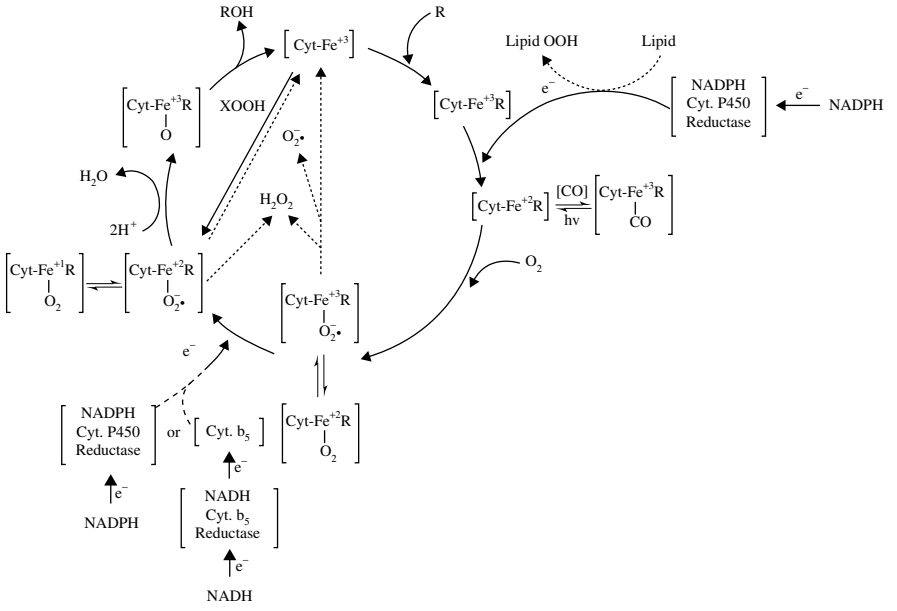



**Figure 7.1** Generalized scheme showing the sequence of events for P450 monooxygenations.

CYP-dependent monooxygenations because many occur in systems reconstituted from NADPH, $O_2$, phosphatidylchloline, and highly purified CYP and NADPH-cytochrome P450 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 cytochrome P450 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 of Cytochrome P450.*** 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 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 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 $\omega$-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, an observation that may be of considerable importance in studies of the metabolic activation of carcinogens.

***Multiplicity of Cytochrome P450, Purification, and Reconstitution of Cytochrome P450 Activity.*** Even before appreciable purification of CYP had been accomplished, it was 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 has been largely resolved. The problem of instability on solubilization was resolved by the use of glycerol and dithiothreitol as protectants, and the problem of reaggregation by maintaining a low concentration of a suitable detergent, such as Emulgen 911 (Kao-Atlas, Tokyo), throughout the procedure. Multiple CYP isoforms, as discussed previously, may be separated from each other and purified as separate entities, although individual isoforms are now routinely cloned and expressed as single entities. The lengthy processes of column purification of CYPs have now been largely superceded by the cloning and expression of transgenic isoforms in a variety of expression systems.

Systems reconstituted from purified CYP, NADPH-cytochrome P450 reductase and phosphatidylchloline will, in the presence of NADPH and $O_2$, oxidize xenobiotics such as benzphetamine, 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. Rather, it appears that many of the cytochromes isolated are still relatively nonspecific. The relative activity toward different substrates does nevertheless vary greatly from one CYP isoform to another even when both are relatively nonspecific. This lack of specificity is illustrated in Table 7.2, using human isoforms as examples.

***Classification and Evolution of Cytochrome P450.*** The techniques of molecular biology have been applied extensively to the study of CYP. More than 1925 genes have been characterized as of 2002, and the nucleotide and derived amino acid sequences compared. In some cases the location of the gene on a particular chromosome has been determined and the mechanism of gene expression investigated.

A system of nomenclature proposed in 1987 has since been updated several times, most recently in 1996. The accepted guidelines from nomenclature designate cytochrome P450 genes as CYP (or cyp in the case of mouse genes). The CYP designation is followed by an Arabic numeral to denote the gene family, followed by a letter designating the subfamily. The individual isoform is then identified using a second Arabic numeral following the subfamily designation. Polymorphic isoforms of genes are indicated by an asterisk followed by an arabic numeral. If there are no subfamilies or if there is only a single gene within the family or subfamily, the letter and/or the second numeral may be omitted (e.g., CYP17). The name of the gene is italicized, whereas the protein (enzyme) is not.

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

**Table 7.2 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,[3] aflatoxin, estradiol | Caffeine, [acetanilide, methoxyresorufin, ethoxyresorufin] |
| 2A6 | Coumarin, nicotine | Aflatoxin, diethylnitrosamine, NNK[3] | Coumarin |
| 2B6 | Cyclophosphamide, ifosphamide, nicotine | 6 Aminochrysene, aflatoxin, NNK[3] | [7-ethoxy-4-trifluoro-methyl coumarin] |
| 2C8 | Taxol, tolbutamide, carbamazepine | — | [Chloromethyl fluorescein diethyl ether] |
| 2C9 | Tienilic acid, tolbutamide, warfarin, phenytoin, THC, hexobarbital, diclofenac | — | [Diclofenac (4′ -OH)] |
| 2C19 | *S*-Mephenytoin, diazepam, phenytoin, omeprazole, indomethacin, impramine, propanolol, proguanil | — | [*S*-Mephentoin (4′ -OH)] |
| 2D6 | Debrisoquine, sparteine, bufuralol, propanolol, thioridazine, quinidine, phenytoin, fluoxetine | NNK[3] | Dextromethorphan, [bufuralol (4′ -OH) |
| 2E1 | Chlorzoxazone, isoniazid, acetaminophen, halothane, enflurane, methoxyflurane | Dimethylnitrosamine, benzene, halogenated alkanes (eg, $CCl_4$) 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-$\beta$)] |
| 4A9/11 | (Very few drugs) | Fatty acids, prostaglandins, thromboxane, prostacyclin | [Lauric acid] |

*Note*: NNK[3] = 4(methylnitrosamino)-1-(3-pyridl)-1-butanone, a nitrosamine specific to tobacco smoke.

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 analogue is found is 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). Relationships between different CYP families and subfamilies are related to the rate and extent of CYP evolution.

Figure 7.2 demonstrates some of the evolutionary relationships between CYP genes between some of the earliest vertebrates and humans. This dendogram compares CYP genes from the puffer fish (fugu) and 8 other fish species with human CYPs (including 3 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.

The gene products, the CYP isoforms, may still be designated P450 followed by the same numbering system used for the genes, or the CYP designation may be used, for example, P4501A1 or CYP1A1.

As of May 16, 2002, a total of 1925 CYP sequences have been "named" with several others still awaiting classification. Of these, 977 are animal sequences, 607 from plants, 190 from lower eukaryotes and 151 are bacterial sequences. These sequences fall into more than 265 CYP families, 18 of which belong to mammals. Humans have 40 sequenced CYP genes. As the list of CYPs is continually expanding, progress in this area can be readily accessed via the internet at the Web site of the P450 Gene Superfamily Nomenclature Committee (*http://drnelson. utmem.edu/nelsonhomepage.html*) or at another excellent Web site (*http://www.icgeb.trieste.it/p450*).

***Cytochrome P450 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 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$\alpha$-position, while CYP17 and 21 catalyze the 17$\alpha$ and 21-hydroxylations of progesterone, respectively. CYP19 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.

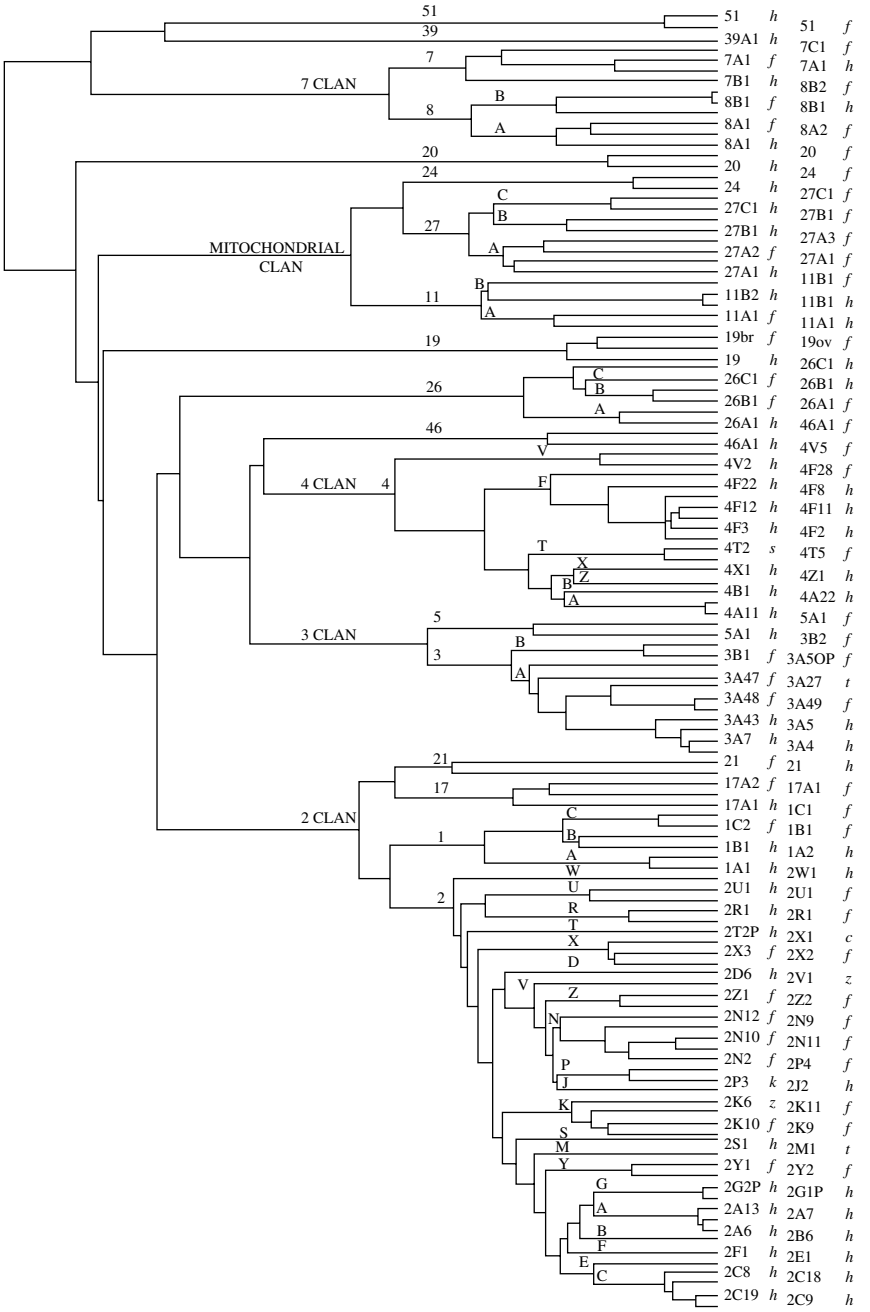



**Figure 7.2** UPGMA tree of 54 puffer fish (fugu), 60 human, and 8 other fish P450s. Species are indicated by *f*, *h*, *z*, *c*, *k*, *s*, and *t* for fugu, human, zebrafish, catfish, killifish, seabass, and trout, respectively. (Reprinted from D. R. Nelson, *Archives of Biochemistry and Biophysics* 409, pp. 18–24. 2003, with permission from Academic Press.)

Because these two highly homologous forms are so highly conserved among species, it is thought that both may possess important endogenous functions that have yet to be elucidated. 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), and the latter preferring 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, $\beta$-naphthylamine, 4-aminobiphenyl, 2-acetylaminoflourene, and benzidine. Figure 7.3 illustrates a typical reaction sequence leading to the formation of epoxide and the 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,

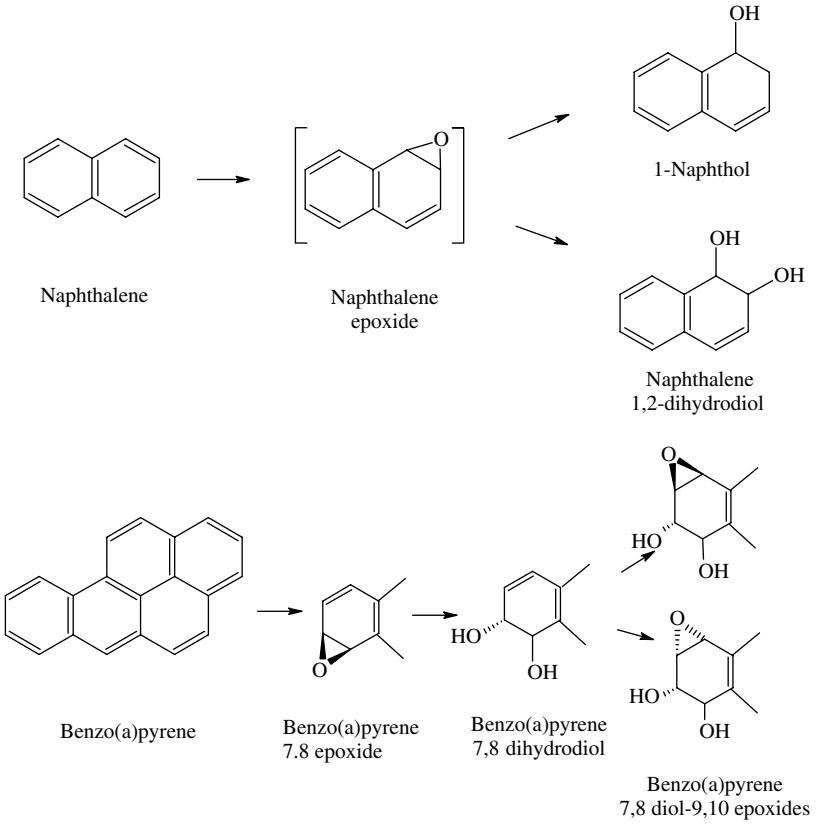



**Figure 7.3** Examples of epoxidation reactions.

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 humans.

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, -2B6, -2C8, -2C9, -2C19, -2D6, and -2E1. 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-acetylaminofluorene, methoxyflurane, halothane, valproic acid, and disulfiram. Precarcinogens likely activated by 2A6 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 2A6 polymorphisms have reduced risk of lung cancers. Although theoretically individuals lacking 2A6 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 2A6 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*-mephobarbitol, artemisinin, bupropion, propofol, ifosfamide, ketamine, selegiline, and methadone. CYP2B6 has also been demonstrated to have a role in the activation of the organophosphate, chlorpyrifos, and in the degradation of the commonly used insecticide repellant, diethyl toluamide (DEET). Historically it was thought that CYP2B6 is found in 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 2B6, though greater than 20-fold differences in levels of protein have been observed.

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 first described polymorphic effects, that involving mephenytoin metabolism. This 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 anti-ulcer 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, -2C9, and -2C18. 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 -2C8, 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 to 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, accounting for nearly 30% of the total amount, and is known to metabolize many important drugs including cyclosporine A, nifedipine, rapamycin, ethinyl estradiol, quinidine, digitoxin, lidocaine, erythromycin, midazolam, triazolam, lovastatin, and tamoxifen. Other important oxidations ascribed to the CYP3 family include many steroid hormones, macrolide antibiotics, alkaloids, benzodiazepines, dihydropyridines, warfarin, polycyclic hydrocarbon-derived dihydrodiols, and aflatoxin $B_1$. Many chemicals are also capable of inducing 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.

***Cytochrome P450 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 enzyme activities are therefore 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 7.1 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 carcinogenesis 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 7.3, 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 to yield the glutathione conjugate, which is ultimately metabolized to a mercapturic acid. These 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 7.3). 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, and *n*-hexane, as well as alicylcic compounds such as cyclohexane, are known to be oxidized to alcohols. Likewise alkyl side chains of aromatic compounds such as cyclohexane, are known to be oxidized to alcohols, but alkyl side chains of aromatic compounds are more readily oxidized, often at more than one position, and so provide good examples of this type of oxidation. 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 $\omega$-oxidation, benzylmethyl carbinol ($C_6H_5CH_2CHOHCH_3$) by $\omega$-1 oxidation, and ethyl-phenylcarbinol ($C_6H_5CHOHCH_2CH_3$) by $\alpha$-oxidation. Further oxidation of these alcohols is also possible.

*Aliphatic Epoxidation.* Many aliphatic and alicylcic compounds containing unsaturated carbon atoms are thought to be metabolized to epoxide intermediates (Figure 7.4). 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 7.5).

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

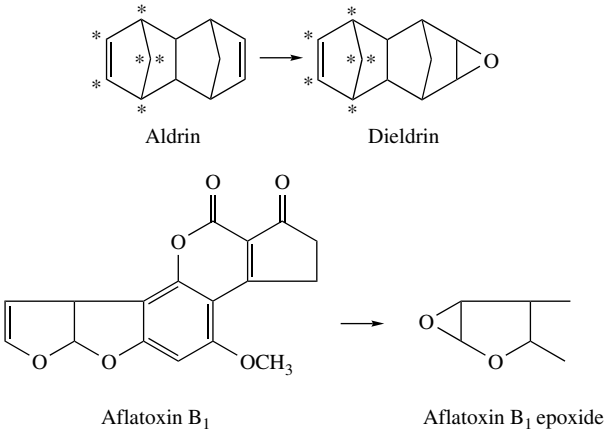



**Figure 7.4** Examples of aliphatic epoxidation. * denote Cl atoms.

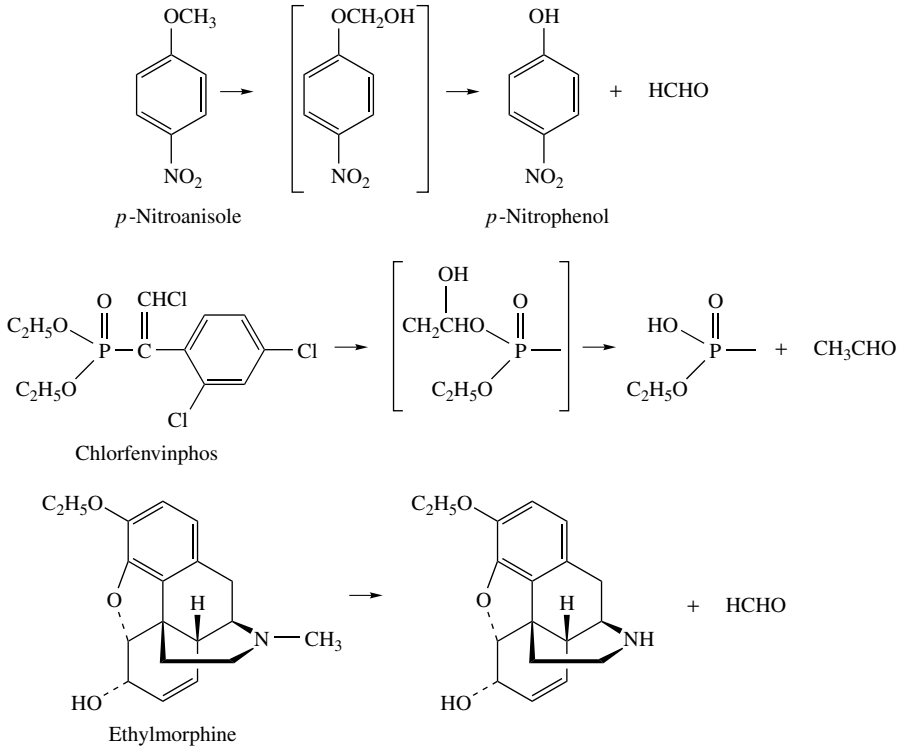



**Figure 7.5** Examples of dealkylation.

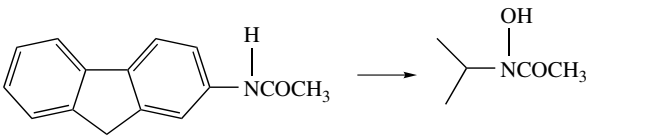
first described for the insecticide chlorfenvinphos and is known to occur with a wide variety of vinyl, phenyl, phenylvinyl, and naphthyl phosphate and thionophosphate triesters (Figure 7.5).

*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 flavin-containing monooxygenase (see the discussion below) it is possible that the initial attack is through sulfoxidation mediated by FMO rather than CYP.

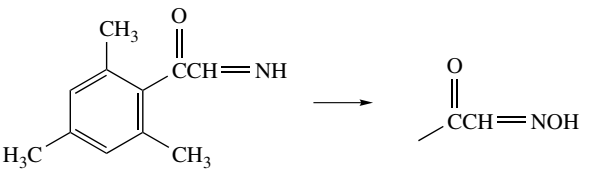
*N-Oxidation.* *N*-oxidation can occur in a number of ways, including hydroxylamine formation, oxime formation, and *N*-oxide formation, although the latter is primarily dependent on the FMO enzyme. Hydroxylamine formation occurs with a number of amines such as aniline and many of its substituted derivatives. In the case of 2-acetylaminofluorene the product is a potent carcinogen, and thus the reaction is an activation reaction (Figure 7.6).

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 7.6).




2-Acetylaminofluorene N-Hydroxy-2-acetylaminofluorene

(*a*) Hydroxylamine formation




Trimethylacetophenone imine Trimethylacetophenone oxime

(*b*) Oxime formation

**Figure 7.6** Examples of *N*-oxidation.

*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 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. The carbinol is thus formed by two different routes:

$$R_2C(OH)NH2 \xrightarrow{-H_2O} R_2C{=}NH \xrightarrow{+O} R_2CNOH \xrightarrow{+H_2O} R_2C{=}O$$

*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, organophosphates, and chlorinated hydrocarbons. Recent work suggests that members of the CYP2C family are highly involved in sulfoxidation of several organophosphate compounds including phorate, coumaphos, demeton, and others. 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. Thus, a reexamination of earlier work in which many of these reactions were ascribed to CYP is required.

*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 too is now known to be catalyzed by the FMO also.

*Desulfuration and Ester Cleavage.* The phosphorothionates $[(R^1O)_2P(S)OR^2)]$ and phosphorodithioate $[(R^1O)_2P(S)SR^2]$ 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 11 for a discussion of the mechanism of cholinesterase inhibition). This reaction has been described for many organophosphorus compounds but has been studied most intensively in the case of parathion. 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 7.7). Some organophosphorus insecticides, all phosphonates, are activated by the FMO as well s the CYP.

*Methylenedioxy (Benzodioxole) Ring Cleavage.* Methylenedioxy-phenyl 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 7.8).

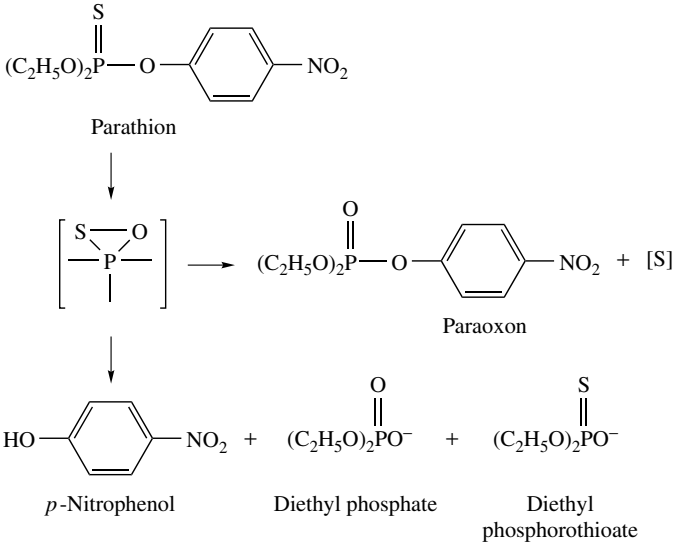



**Figure 7.7** Desulfuration and oxidative dearylation.

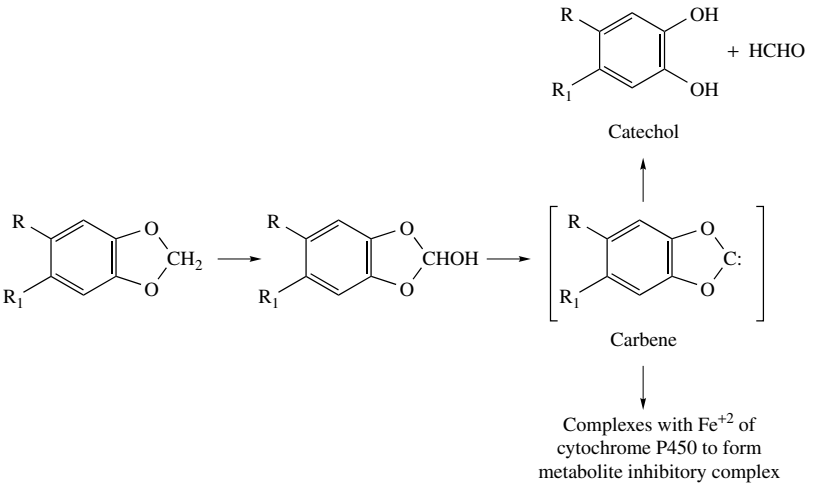



**Figure 7.8** Monooxygenation of methylenedioxyphenyl compounds.

### 7.2.3 The Flavin-Containing Monooxygenase (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 flavin-containing monooxygenase (FMO), is also dependent 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 orthologues 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 perhaps also in the Hispanic population. FMO3, the predominant human FMO, is poorly expressed in neonatal humans but is expressed in most individuals by one 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, trimethylamineuria, 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 are 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 7.9). A short list of 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-4phenyl-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

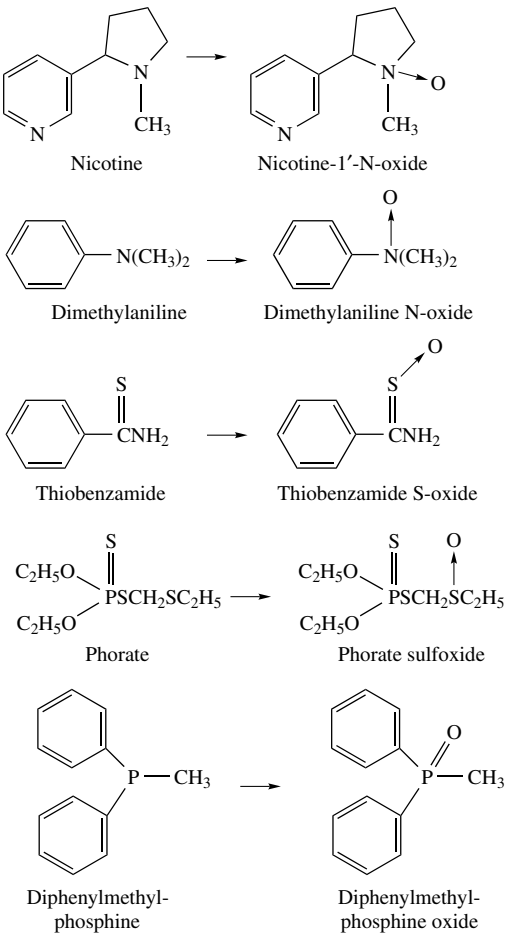



**Figure 7.9** Examples of oxidations catalyzed by the flavin-containing monooxygenase (FMO).

metabolic contribution of the other. Since FMOs are generally heat labile, heating the microsomal preparation to 50°C for one minute 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 cytochrome P450 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 9.

### 7.2.4 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:

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

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.

This enzyme 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 whose subunits can occur in several forms 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.

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 produces aldehydes. Aldehydes are highly reactive electrophilic compounds; they may react with thiol and amino groups to produce a variety of effects. Some aldehydes produce therapeutic effects, but more often the effects are cytotoxic, genotoxic, mutagenic, and 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:

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

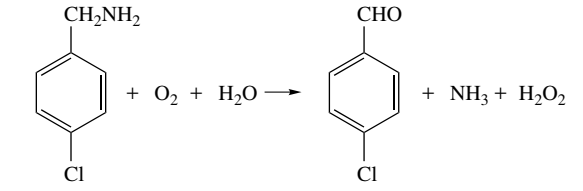
The aldehyde gene superfamily is large with more than 330 aldehyde dehydrogenase genes in prokaryote and eukaryotic species. The eukaryotic aldehyde dehydrogenase gene superfamily consists of 20 gene families, 9 of which contain 16 human genes and 3 pseudogenes. The importance of some of these genes 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 7.10.

*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




*p*-Chlorobenzylamine *p*-Chlorobenzaldehyde

(*a*) Monoamine oxidase

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

Cadaverine

(*b*) Diamine oxidase

**Figure 7.10** Examples of oxidations catalyzed by amine oxidases.

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 $\alpha$-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 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.

### 7.2.5 Cooxidation by Cyclooxygenases

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, cyclooxygenase (COX), also known as prostaglandin synthase (Figure 7.11). 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 daltons, containing one heme per subunit. During the second step of the previous sequence (peroxidase), many xenobiotics can be cooxidized, 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 cyclooxygenase (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 cooxidation, 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.

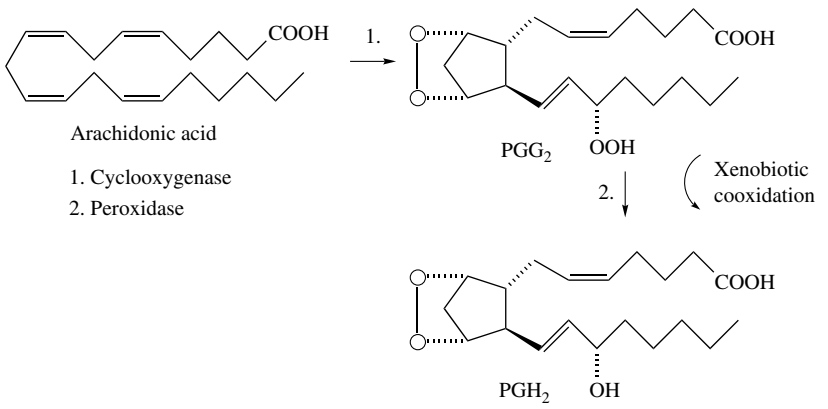



**Figure 7.11** Cooxidation during prostaglandin biosynthesis.

To differentiate between xenobiotic oxidations by COX and CYP, in vitro microsomal incubations of the xenobiotic may be performed either in the presence of 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.

### 7.2.6 Reduction Reactions

A number of functional groups, such as nitro, diazo, carbonyl, disulfide sulfoxide, alkene, and pentavalent arsenic, 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 bound in cinnamic acid ($C_6H_5CH{=}CHCOOH$), the reaction has been attributed to the intestinal microflora. Examples of reduction reactions are shown in Figure 7.12.

*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 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

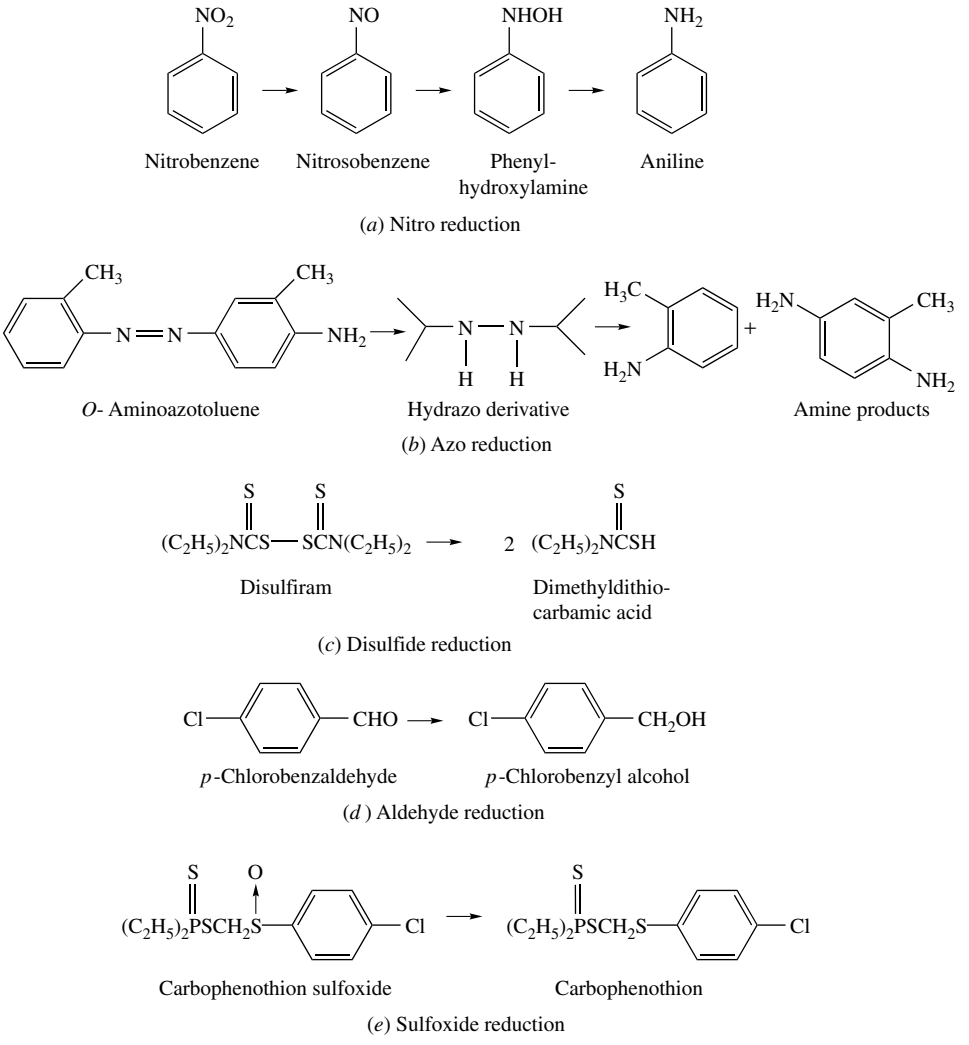



**Figure 7.12** Examples of metabolic reduction reactions.

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

$$\text{RSSR} + \text{GSH} \longrightarrow \text{RSSG} + \text{RSH}$$

$$\text{RSSG} + \text{GSH} \longrightarrow \text{GSSG} + \text{RSH}$$

$$\text{GSSG} + \text{NADPH} + \text{H}^+ \longrightarrow 2\text{GSH} + \text{NADP}^+$$

*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.

### 7.2.7 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:

| | |
|---|---|
| $RC(O)OR' + H_2O \longrightarrow RCOOH + HOR'$ | Carboxylester hydrolysis |
| $RC(O)NR'R'' + H_2O \longrightarrow RCOOH + HNR'R''$ | Carboxyamide hydrolysis |
| $RC(O)SR' + H_2O \longrightarrow RCOOH + HSR'$ | 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 fractions, 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 devized 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 insecticide paraoxon 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 active site that is phosphorylated by this inhibitor. 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 7.13. 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.

### 7.2.8 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

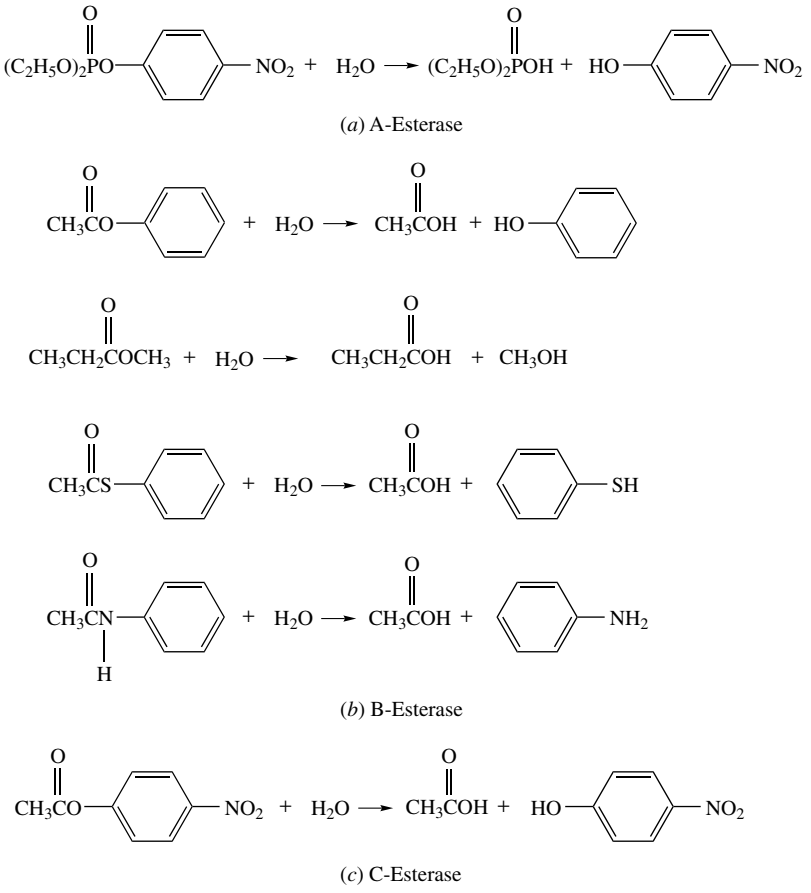



**Figure 7.13** Examples of esterase/amidase reactions involving xenobiotics.

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 7.14.

### 7.2.9 DDT Dehydrochlorinase

DDT-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 and occurs in the soluble fraction of tissue homogenates. Although the reaction requires glutathione, it apparently serves in a catalytic role

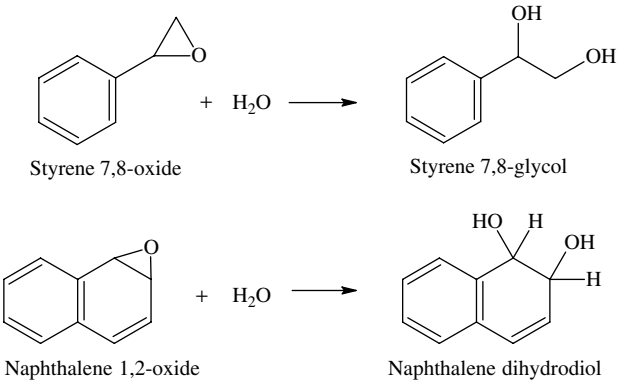



**Figure 7.14** Examples of epoxide hydrolase reactions.

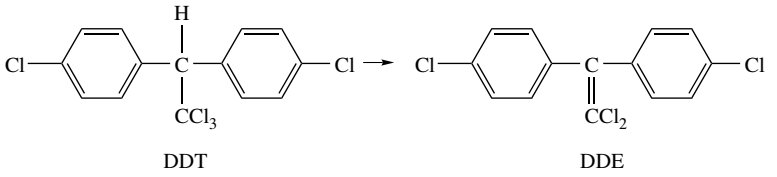



**Figure 7.15** DDT-dehydrochlorinase.

because it does not appear to be consumed during the reaction. The Km for DDT is $5 \times 10^{-7}$ mol/L with optimum activity at pH 7.4. The monomeric form of the enzyme has a molecular mass of about 36,000 daltons, 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 analogues. In all cases the *p,p* configuration is required, *o,p*, and other analogues are not utilized as substrates. The reaction is illustrated in Figure 7.15.

## 7.3 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, glutathione, sulfate, and so on. Conjugation products, with only rare exceptions, are more polar, less toxic, and more readily excreted than are their parent compounds.

Conjugation reactions usually involve metabolite 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.

### 7.3.1 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–$NH_2$, 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 daltons, apparently containing carbohydrate, whose activity 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. UDPGA is in the $\alpha$-configuration whereas, due to the inversion, the glucuronide formed is in the $\beta$-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 7.16.

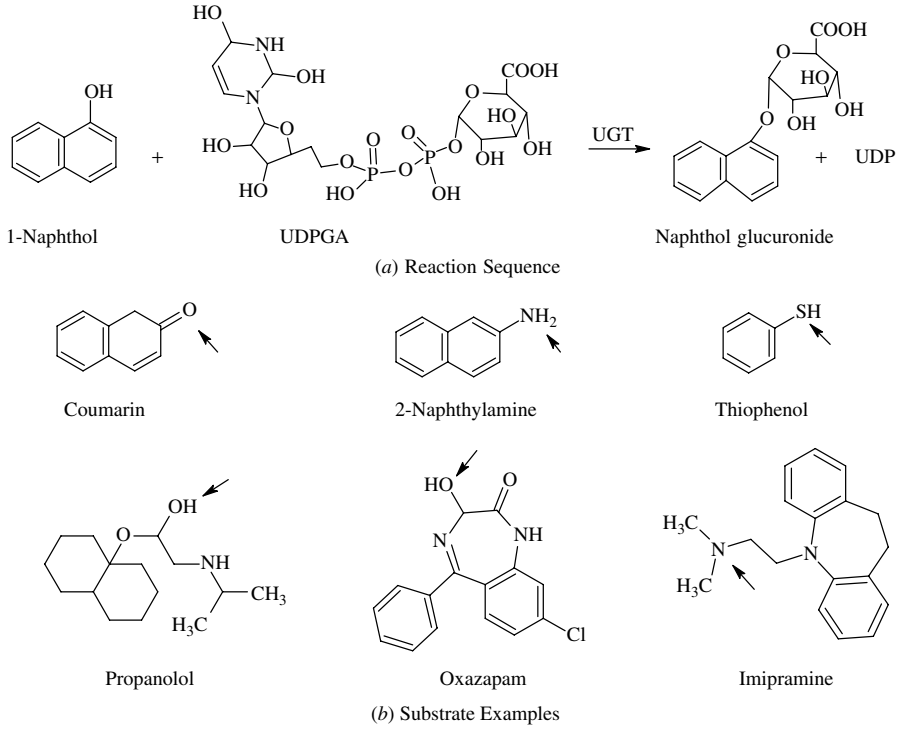



**Figure 7.16** Reaction sequences of uridine diphospho glucuronosyl transferase and chemical structures of compounds that form glucuronides. Arrows indicate the position on each molecule where glucuronidation occurs.

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 now many examples where glucuronide conjugation results in greater toxicity. Perhaps the best-known example involves the bioactivation of *N*-hydroxy-2-acetylaminofluorine. This substrate, unlike 2-acetylaminofluorine, is unable to bind to DNA in the absence of metabolism. 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. 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 hyperbilirubineamias 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.

### 7.3.2 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.

### 7.3.3 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 alcohols, arylamines, and phenols, result in the production of water soluble sulfate esters that 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 7.17, 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 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

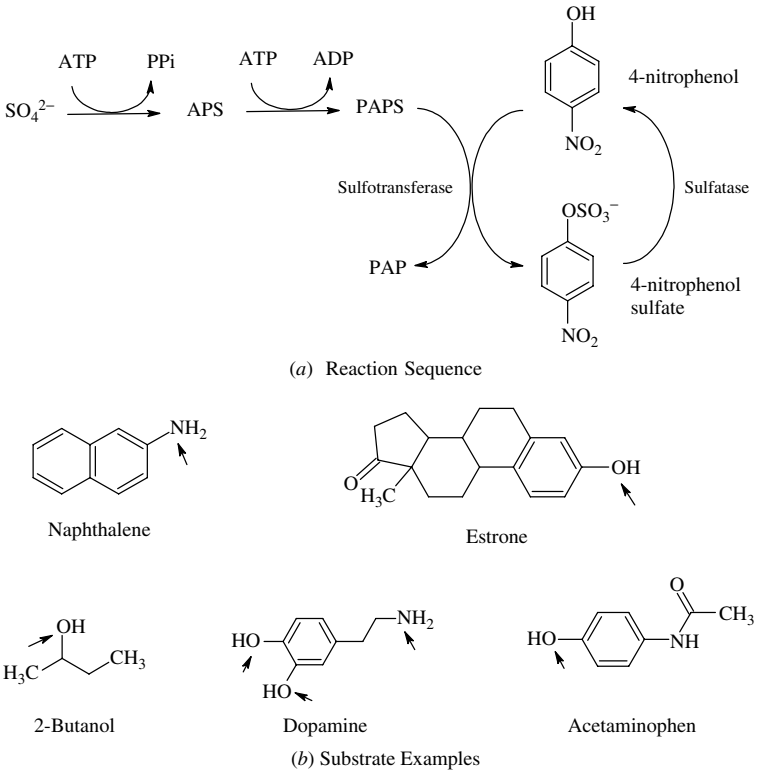



**Figure 7.17** Reaction sequence of sulfotransferases and chemical structures of compounds that form sulfates. Arrows indicate positions on each molecule where sulfotransferases may attack.

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 hydroxysteriod SULT (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 to 64,000 daltons.

Hydroxysteroid sulfotansferase 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. Hydroxysteroid sulfotransferase will react with hydroxysterols and primary and secondary alcohols but not with hydroxyl groups in the aromatic rings of steroids.

### 7.3.4 Methyltransferases

A large number of both endogenous and exogenous compounds can be methylated by several *N*-, *O*-, and *S*-methyl transferases. The most common methyl donor is *S*-adenosyl methionine (SAM), which is 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 7.18.

*N-Methylation.* Several enzymes are known that catalyze *N*-methylation reactions. They include histamine *N*-methyltransferase, a highly specific enzyme that occurs in

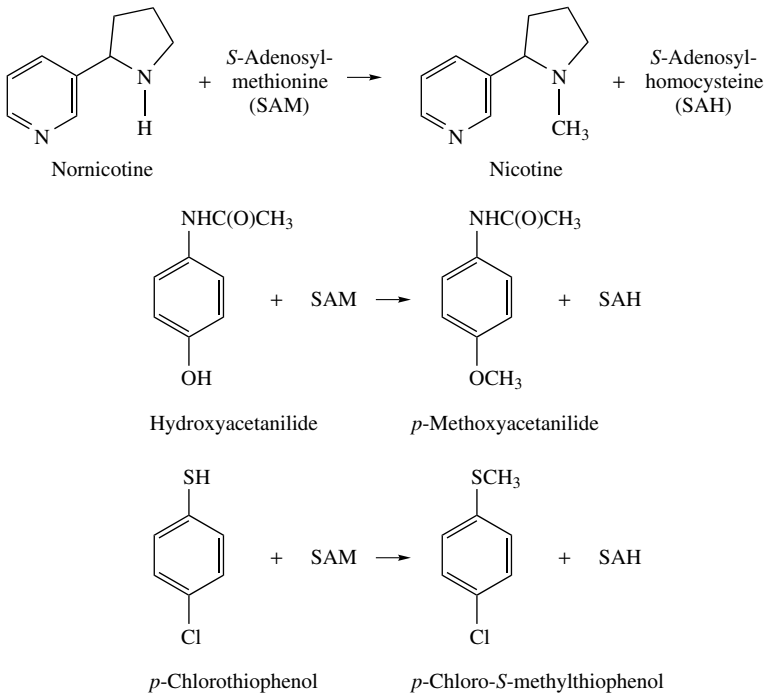



**Figure 7.18** Examples of methyl transferase reactions.

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 tryptamine 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 daltons, 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*-ethyl-maleimide and *p*-chloromercuribenzoate. A hydroxyindole *O*-methyltransferase, which methylates *N*-acetyl-serotonin 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 methyl transferases, utilizes *S*-adenosylmethionine. It has been purified from rat liver and is a monomer of about 28,000 daltons. A wide variety of substrates are methylated, including thiacetanilide, mercaptoethanol, and diphenylsulfide. 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 dimethylsulfide.

Methylthiolation, or the transfer of a methylthio (CH3S–) group to a foreign compound may occur through the action of another recently discovered enzyme, cysteine conjugate $\beta$-lyase. This enzyme acts on cysteine conjugates of foreign compounds as follows:

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

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:

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

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.

### 7.3.5 Glutathione *S*-Transferases (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 (glutathione) and the enzymes required for the formation of these acids been identified and characterized. The overall pathway is shown in Figure 7.19.

The initial reaction is the conjugation of xenobiotics having electrophilic substituents with glutathione, a reaction catalyzed by one of the various forms of GST. This is followed by transfer of the glutamate by $\gamma$-glutamyltranspeptidase, 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 glutathione but nonspecific with respect to xenobiotic 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,

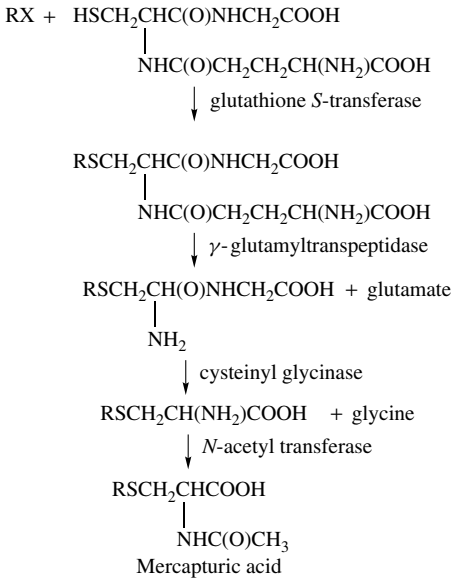



**Figure 7.19** Glutathione transferase reaction and formation of mercapturic acids.

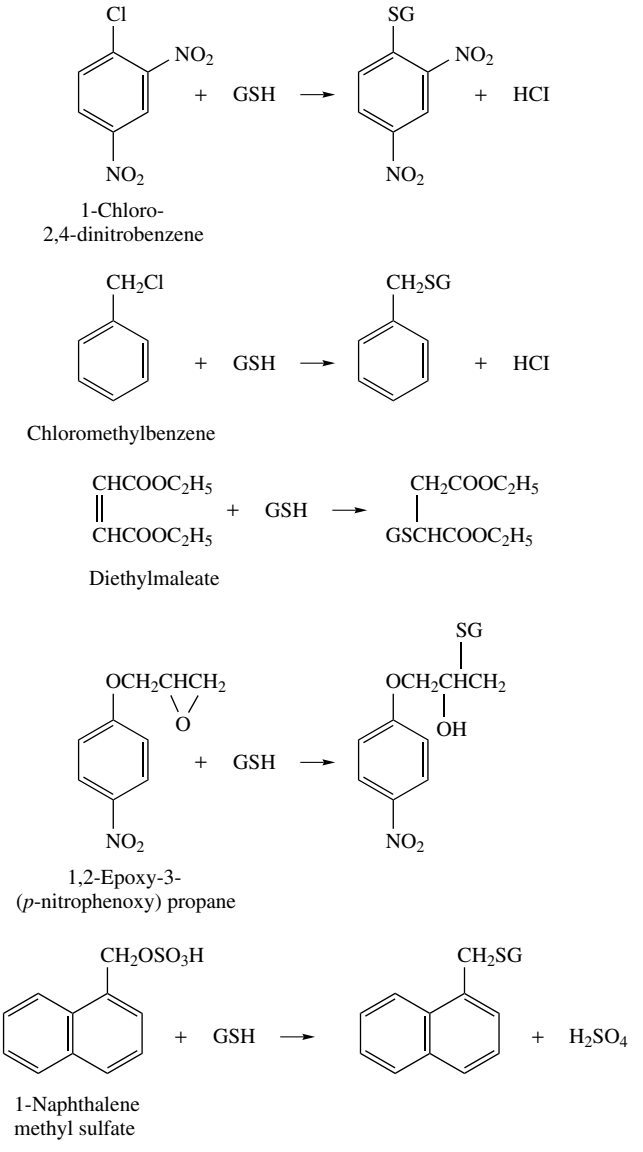



**Figure 7.20** Examples of glutathione transferases reactions.

aryltransferase, aralkyltransferase, alkenetransferase, and epoxidetransferase. Examples are shown in Figure 7.20.

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 and 50,000 daltons. 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 $\alpha$ (alpha), $\kappa$ (kappa), $\mu$ (mu), $\pi$ (pi), $\sigma$ (sigma), and $\theta$ (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 $\alpha$, K for $\kappa$, 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 multi-drug resistance (mdr) protein. Prior to excretion, the metabolites are usually processed by multiple enzymes to release the substrate conjugated to a mercapturic acid (Figure 7.19). The enzymes involved in this process are $\gamma$-glutamyltranspeptidase, cysteinyl glycinase, and *N*-acetyl transferase.

$\gamma$-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 to 90,000 daltons, and the enzyme appears to consist of two unequal subunits; the different forms appear to differ in the degree of sialalylation. This enzyme, which exhibits wide specificity toward $\gamma$-glutamyl peptides and has a number of acceptor amino acids, catalyzes two types of reactions:

| | | |
|---|---|---|
| Hydrolysis | $\gamma$-Glu-R + $H_2O$ | Glu + HR |
| Transpeptidation | $\gamma$-Glu-R + Acceptor | $\gamma$-Glu-Acceptor + HR |
| | $\gamma$-Glu-R + $\gamma$-Glu-R | $\gamma$-Glu$\gamma$-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 daltons. 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.

### 7.3.6 Cysteine Conjugate $\beta$-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 daltons. 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.

### 7.3.7 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

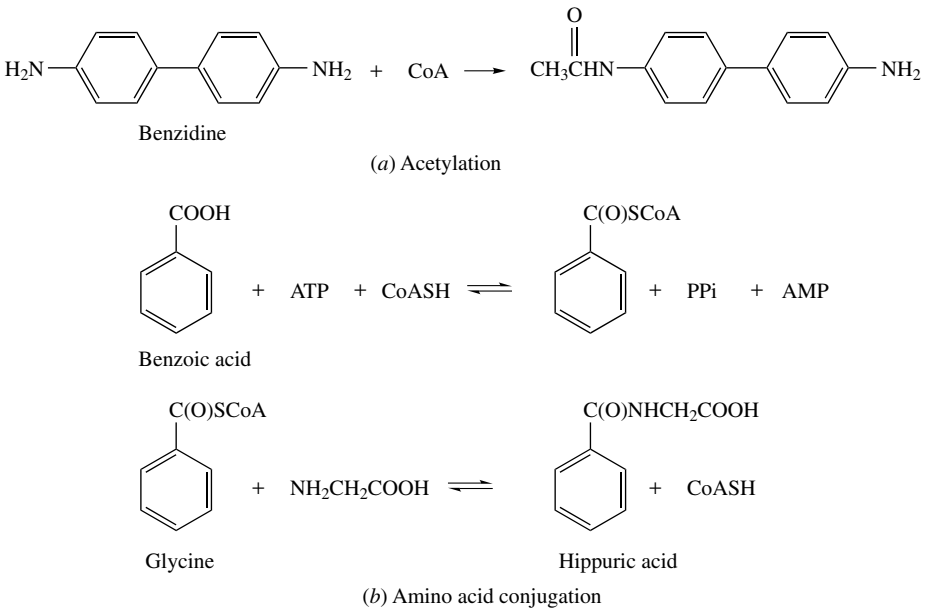



**Figure 7.21** Examples of acylation reactions.

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 7.21.

*Acetylation.* Acetylated derivatives of foreign exogenous amines are acetylated by *N*-acetyl transferase, 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 7.22. The *N*-acyl group of 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-acetylaminofluorine.

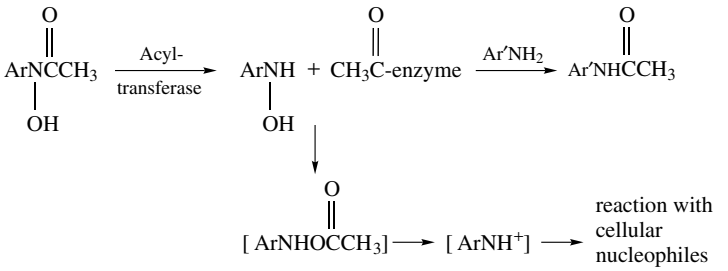



**Figure 7.22** *N*-, *O*-Acyltransferase reactions of arylhydroxamic acid. Ar = aryl group.

Despite 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 derivative 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 (AMP) 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*-acyl-transferase. 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.

### 7.3.8 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.

## SUGGESTED READING

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

Coughtrie, M. W. H., S. Sharp, K. Maxwell, and N. P. Innes. Biology and function of the reversible sulfation pathway catalyzed by human sulfotransferases and sulfatases. *Chemico-Biol. Inter.* **109**: 3–27, 1998.

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

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., and J. A. Goldstein. Metabolism of toxicants: Phase I reactions and pharmacogenetics. In *Introduction to Biochemical Toxicology*, 3rd ed., E. Hodgson and R. C. Smart, eds. New York: Wiley, 2001, pp. 67–113.

Koukouritaki, S. B., P. Simpson, C. K. Yeung, A. E. Rettie, and R. N. Hines. 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, C. T. Dolphin, A. A. Elfarra, R. N. Hines, E. Hodgson, T. Kimura, J. Ozols, I. R. Phillips, R. M. Philpot, L. L. Poulsen, A. E. Rettie, E. A. Shephard, D. E. Williams and D. M. Ziegler. 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., and W. A. Dauterman. Conjugation and elimination of toxicants: In *Introduction to Biochemical Toxicology*, 3rd edn., E. Hodgson and R. C. Smart, eds. New York: Wiley, 2001, pp. 115–135.

Nelson, D. R., L. Koymans, T. Kamataki, J. J. Stegeman, R. Feyereisen, D. J. Waxman, M. R. Waterman, O. Gotoh, M. J. Coon, R. W. Estabrook, I. C. Gunsalus, and D. W. Nebert. P450 superfamily: Update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics* **6**: 1–42, 1996.

Oesch, F, and M. Arand. Xenobiotic metabolism. In *Toxicology*, H. Marquardt, S. G. Shafer, R. McClellan, and F. Welsch, eds. New York: Academic Press, 1999, pp. 83–109.

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

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

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

CHAPTER 8

# Reactive Metabolites

RANDY L. ROSE and PATRICIA E. LEVI

## 8.1 INTRODUCTION

Between uptake from the environment and excretion from the body, many exogenous compounds (xenobiotics) 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. This biotransformation of relatively inert chemicals to highly reactive intermediary metabolites is commonly referred to as metabolic activation or bioactivation, and it is known to be the initial event in many 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, is on toxicants requiring metabolic activation and to those 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 (DAB), 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 8.1. As illustrated by this diagram, xenobiotic metabolism can produce not only nontoxic metabolites, which are more polar and 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. In general, reactive metabolites are electrophiles (molecules containing positive centers). These electrophiles in turn can react with cellular nucleophiles (molecules containing negative centers), such as glutathione, proteins, and nucleic acids. Other reactive metabolites may be free radicals or act as radical generators that interact with oxygen to produce reactive oxygen species that are capable of causing damage to membranes, DNA, and other macromolecules.

*A Textbook of Modern Toxicology, Third Edition,* edited by Ernest Hodgson
ISBN 0-471-26508-X 

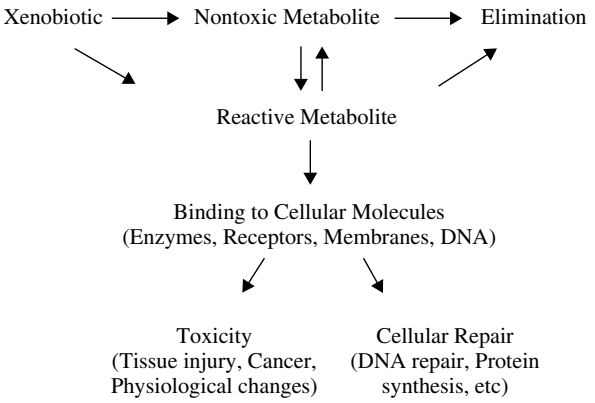



**Figure 8.1** The relationship between metabolism, activation, detoxication, and toxicity of a chemical.

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 and DNA.

## 8.2 ACTIVATION ENZYMES

Whereas most, if not all, of the enzymes involved in xenobiotic metabolism can form reactive metabolites (Table 8.1), the enzyme systems most frequently involved in the activation of xenobiotics are those which catalyze oxidation reactions. The cytochrome P450 monooxygenases (CYP) are by far the most important enzymes involved in the oxidation of xenobiotics. This is because of the abundance of CYP (especially in the liver), the numerous isozymes of CYP, and the ability of CYP to be induced by xenobiotic compounds.

Although the CYP enzymes are the 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 isozymes with different substrate specificities, the presence or absence of a particular CYP isozyme may contribute to tissue-specific toxicities. Many drugs and other foreign compounds are known to induce one or more of the CYP isozymes, resulting in an increase, decrease, or an alteration in the metabolic pathway of chemicals metabolized by the CYP isozymes involved. Specific examples of these types of interactions are given later in this section.

In addition to activations catalyzed by CYPs and FMOs, phase two conjugations, cooxidation by COX during prostaglandin biosynthesis, and metabolism by intestinal

**Table 8.1 Enzymes Important in Catalyzing Metabolic Activation Reactions**

| Type of Reaction | Enzyme |
|---|---|
| Oxidation | Cytochrome P450s |
| | Prostaglandin synthetase |
| | Flavin-containing monooxygenases |
| | Alcohol and aldehyde dehydrogenases |
| Reduction | Reductases |
| | Cytochromes P450 |
| | Gut microflora |
| Conjugation | Glutathione transferases |
| | Sulfotransferases |
| | Glucuronidases |
| Deconjugation | Cysteine *S*-conjugate $\beta$-lyase |
| Hydrolysis | Gut microflora, hydrolyses |

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.

## 8.3 NATURE AND STABILITY OF REACTIVE METABOLITES

Reactive metabolites include such diverse groups as epoxides, quinones, free radicals, reactive oxygen species, and unstable conjugates. Figure 8.2 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 divided into several categories depending on their half-life under physiological conditions and how far they may be transported from the site of activation.

### 8.3.1 Ultra-short-lived Metabolites

These are metabolites that bind primarily to the parent enzyme. This category includes substrates that form enzyme-bound intermediates that react with the active site of the enzyme. Such chemicals are known as "suicide substrates." A number of compounds are known to react in this manner with CYP, and such compounds are often used experimentally as CYP inhibitors (see the discussion of piperonyl butoxide, Section 7.2.2). 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.

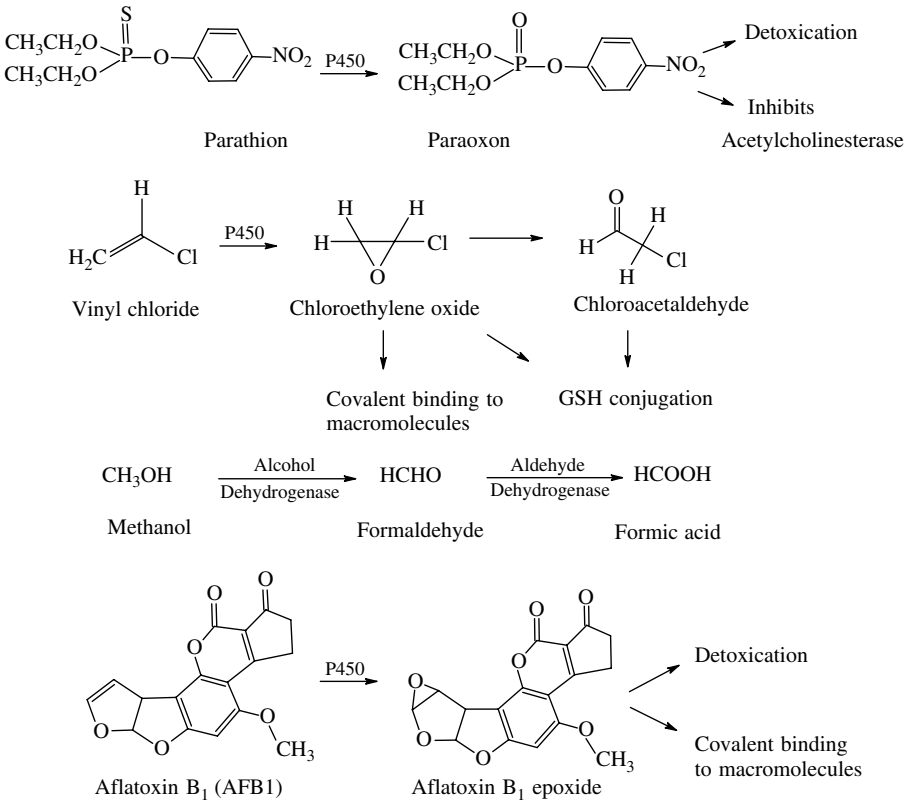



**Figure 8.2** Examples of some activation reactions.

### 8.3.2 Short-lived Metabolites

These metabolites 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 at 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.

### 8.3.3 Longer-lived Metabolites

These 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 in 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.

## 8.4 FATE OF REACTIVE METABOLITES

If production of reactive metabolites is the initial process in the role of reactive metabolites in toxicity, then the fate of these reactive metabolites is the next step to understand in the process. Within the tissue a variety of reactions may occur depending on the nature of the reactive species and the physiology of the organism.

### 8.4.1 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 the chapters in Parts IV and V.

### 8.4.2 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 8.3). Because of the critical nature of various cellular membranes (nuclear, mitochondrial, lysosomal, etc.), lipid peroxidation can be a pivotal event in cellular necrosis.

### 8.4.3 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

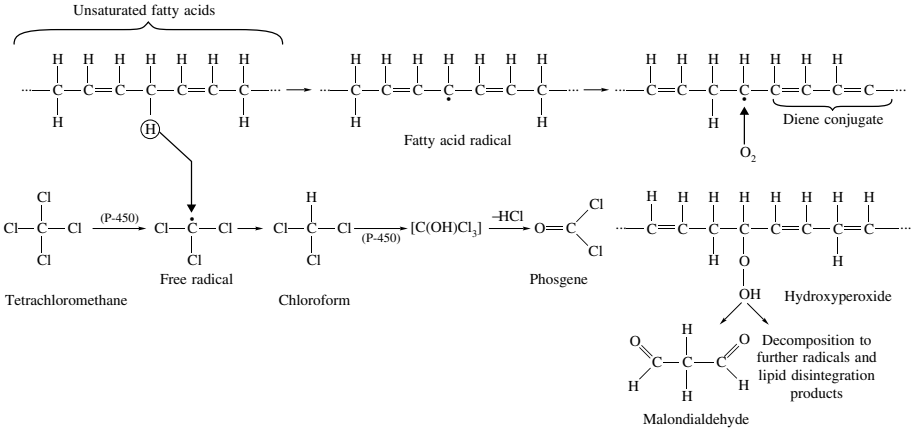



**Figure 8.3** Metabolism of tetrachloromethane. Upon metabolic activation a $CCl_3$ radical is formed. This radical extracts protons from unsaturated fatty acids to form a free fatty-acid radical. This leads to diene conjugates. At the same time, $O_2$ forms a hydroperoxide with the C radical. Upon 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 H. M. Bolt and J. T. Borlak, in *Toxicology*, pp. 645–657, copyright 1999, with permission from Elsevier.)

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 sulfotransferases 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.

## 8.5 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 9.

### 8.5.1 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 forms of cytochrome P450. Frequently the CYP forms 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.

### 8.5.2 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.

### 8.5.3 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.

## 8.6 EXAMPLES OF ACTIVATING REACTIONS

The following examples have been selected to illustrate the various concepts of activation and detoxication discussed in the previous sections.

### 8.6.1 Parathion

Parathion is one of several organophosphorus insecticides that has had great economic importance worldwide for several decades. Organophosphate toxicity is the result of excessive stimulation of cholinergic nerves, which is dependent on their ability to inhibit acetylcholinesterases. Interestingly the parent organophosphates are relatively poor inhibitors of acetylcholinesterases, requiring metabolic conversion of a P=S bond to a P=O bond for acetylcholinesterase inhibition (Figure 8.2; see Chapters 11 and 16 for a discussion of the mechanism of acetylcholinesterase inhibition). In vitro studies of rat and human liver have demonstrated that CYP is inactivated by the electrophilic sulfur atom released during oxidation of parathion to paraoxon. Some have shown that the specific isoforms responsible for the metabolic activation of parathion are destroyed in the process. For example, preincubations of NADPH-supplemented human liver microsomes with parathion resulted in the inhibition of some isoform-specific metabolites including testosterone (CYP3A4), tolbutamide (CYP2C9), and 7-ethylresorufin (CYP1A2) but not aniline (CYP2E1). These losses of metabolic activity were also associated with the loss of CYP content as measured by the CO-difference spectra. These results suggest that parathion 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 parathion metabolism is CYP3A4, which is the dominant CYP in humans; accounting for between 30–50% of the total liver CYP. Because of this enzyme's importance in drug metabolism, the strong potential for inhibition by organophosphate compounds may have serious consequences in individuals undergoing drug therapy.

### 8.6.2 Vinyl Chloride

A second 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 8.2).

### 8.6.3 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 have severe visual loss or blindness, and one-third have died. Methanol itself is not responsible for the toxic effects but is rapidly metabolized in humans by alcohol dehydrogenase to formaldehyde, which is subsequently metabolized by aldehyde dehydrogenase to form

the highly toxic formic acid (Figure 8.2). The aldehyde dehydrogenase is so efficient in its metabolism of formaldehyde that it is actually difficult to detect formaldehyde in post mortem 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 the production of formic acid by competing with methanol for the alcohol dehydrogenase pathway.

### 8.6.4 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 8.2). 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. Table 8.2 shows the relative rates of AFB1 metabolism by liver microsomes from different species. 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. In Table 8.2, for example, it can be seen 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.

### 8.6.5 Carbon Tetrachloride

Carbon tetrachloride has long been known to cause fatty acid accumulation and hepatic necrosis. Extraction of a chlorine atom by CYP from carbon tetrachloride results in

**Table 8.2 Formation of Aflatoxin $B_1$ Dihydrodiol by Liver Microsomes**

| Source of Microsomes | Dihydrodiol Formation[a] |
|---|---|
| Rat | 0.7 |
| C57 mouse | 1.3 |
| Guinea pig | 2.0 |
| Phenobarbital-induced rat | 3.3 |
| Chicken | 4.8 |

*Source*: Adapted from G. E. Neal et al., *Toxicol. Appl. Pharmacol.* **58**: 431–437, 1981.

[a] μg formed/mg microsomal protein/30 min.

the formation of a trichloromethyl radical that extracts protons from esterified desaturated fatty acids resulting in the production of chloroform (Figure 8.3). 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 8.3). 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 8.3).

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.

### 8.6.6 Acetylaminofluorene

In the case of the hepatocarcinogen, 2-acetylaminofluorene (2-AAF), two activation steps are necessary to form the reactive metabolites (Figure 8.4). 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.

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

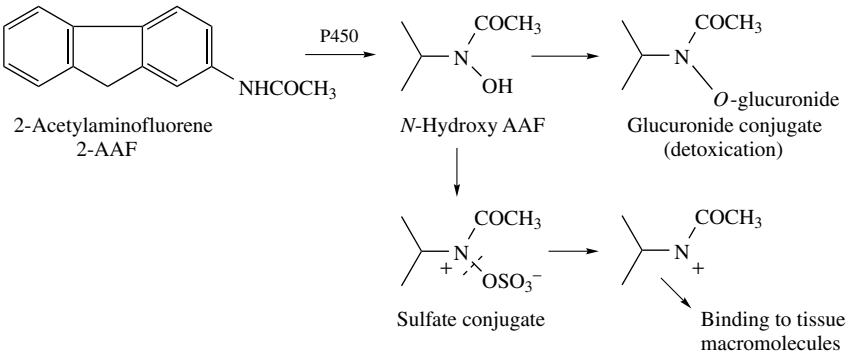



**Figure 8.4** Bioactivation of 2-acetylaminofluorene.

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.

### 8.6.7 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 8.5). Interestingly only one of these isomers(+)-benzo(a)pyrene 7,8-diol-9,10 epoxide-2 has significant carcinogenic potential. Comparative studies with 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.

### 8.6.8 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 quinoneimine, are formed by the CYP enzymes (Figure 8.6).

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

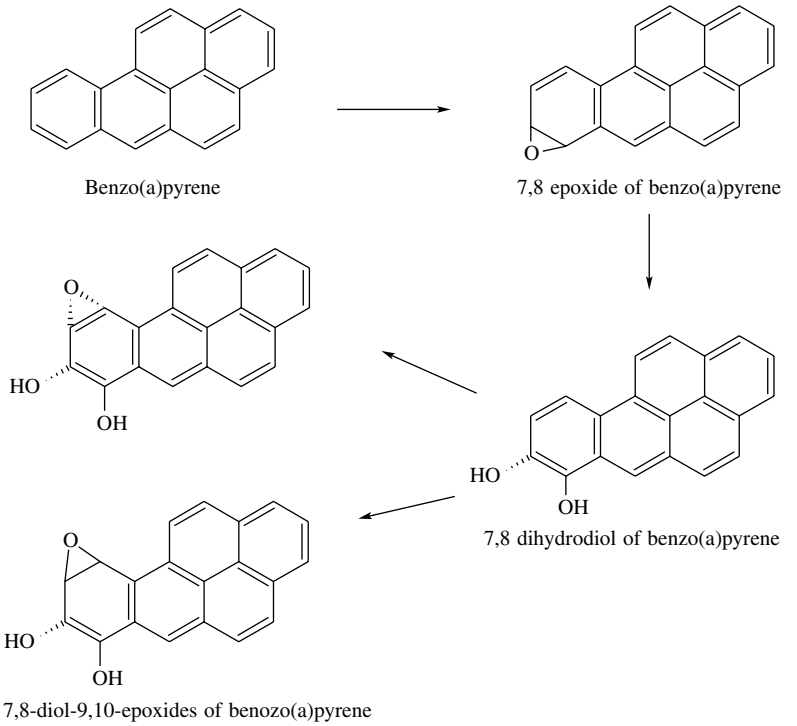



**Figure 8.5** Selected stages of biotransformation of benzo(a)pyrene. The diol epoxide can exist in four diastereoisomeric forms of which the key carcinogenic metabolite is (+)-benzo(a)pyrene 7,8-diol-9,10-epoxide.

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, 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.

### 8.6.9 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 $\beta$-glucoside of methylazoxymethanol (Figure 8.7). 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, $\beta$-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 $\beta$-glucosidases are not present in mammalian tissues but are present in the gut. However, it can be demonstrated that the metabolite, methylazoxymethanol, will lead to tumors in both normal and germ-free animals regardless of the route of administration.

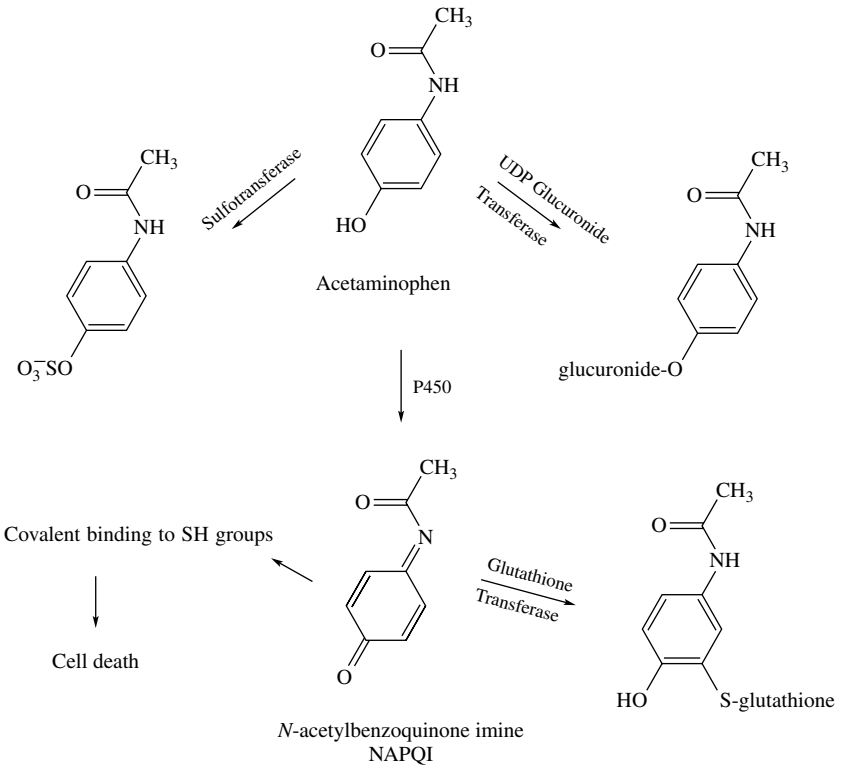



**Figure 8.6** Metabolism of acetaminophen and formation of reactive metabolites.

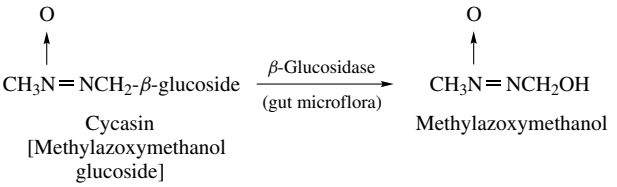



**Figure 8.7** Bioactivation of cycasin by intestinal microflora to the carcinogen methylazoxymethanol.

## 8.7 FUTURE DEVELOPMENTS

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 postmitochondrial 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.

## SUGGESTED READING

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

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

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

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

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

Levi, P. E., and E. Hodgson. Reactive metabolites and toxicity. In *Introduction to Biochemical Toxicology*, 3rd ed., E. Hodgson and R. C. Smart, eds. New York: Wiley, 2001, pp. 199–220.

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.

Rinaldi, R., E. Eliasson, S. Swedmark, and R. Morganstern. Reactive intermediates and the dynamics of glutathione transferases. *Drug Metabol. Disp.* **30**: 1053–1058, 2002.

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

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

CHAPTER 9

# Chemical and Physiological Influences on Xenobiotic Metabolism

RANDY L. ROSE and ERNEST HODGSON

## 9.1 INTRODUCTION

The metabolism of toxicants and their overall toxicity can be modified by many factors both extrinsic and intrinsic to the normal functioning of the organism. It is entirely possible that many changes in toxicity are due to changes in metabolism, because most sequences of events that lead to overt toxicity involve activation and/or detoxication of the parent compound. In many cases the chain of cause and effect is not clear, due to the difficulty of relating single events measured in vitro to the complex and interrelated effects that occur in vivo. This relationship between in vitro and in vivo studies is important and is discussed in connection with enzymatic inhibition and induction (see Section 9.5). 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. These studies indicate that similar effects may occur in humans or other animals, but not that they must occur or that they occur at the same magnitude in all species if they occur at all.

## 9.2 NUTRITIONAL EFFECTS

Many nutritional effects on xenobiotic metabolism have been noted, but the information is scattered and often appears contradictory. This is one of the most important of several neglected areas of toxicology. This section 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 (see Section 9.5).

### 9.2.1 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 hydroxylation are all

*A Textbook of Modern Toxicology, Third Edition,* edited by Ernest Hodgson
ISBN 0-471-26508-X 

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 and NADPH-cytochrome P450 reductase that are also noted. One might speculate that the gender and other variations are due to differential effects on P450 isozymes. 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 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.

### 9.2.2 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 cytochrome P450 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 CYPs 1A1 and 1A2, resulting in enhanced metabolism of phenacetin, theophylline, and antipyrine. Studies of this nature indicate that there is significant interindividual variability in these observed responses.

### 9.2.3 Lipids

Dietary deficiencies in linoleic or in other unsaturated fats generally bring about a reduction in P450 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.

Lipids also appear to be necessary for the effect of inducers, such as phenobarbital, to be fully expressed.

### 9.2.4 Micronutrients

Vitamin deficiencies, in general, bring about a reduction in monooxygenase activity, although exceptions can be noted. Riboflavin deficiency causes an increase in P450 and

aniline hydroxylation, although at the same time it causes a decrease in P450 reductase and benzo(a)pyrene hydroxylation. Ascorbic acid deficiency in the guinea pig not only causes a decrease in P450 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 P450 isozymes 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 P450, however. An excess of dietary cobalt, cadmium, manganese, and lead all cause an increase in hepatic glutathione levels and a decrease in P450 content.

### 9.2.5 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 P450 and a concomitant increase in hexobarbital metabolism, which is reflected in a shorter sleeping time.

### 9.2.6 Nutritional Requirements in Xenobiotic Metabolism

Because xenobiotic metabolism involves many enzymes with different cofactor requirements, prosthetic groups, or endogenous cosubstrates, 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 cytochrome P450 monooxygenase system are shown in Figure 9.1. The B complex vitamins niacin and riboflavin are both involved, the former in the formation of NADPH and the latter in the formation of FAD and 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 used in the formation of acetyl Co-A, pyridoxine, a cofactor in heme synthesis and copper, required in the 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 P450 system to metabolize xenobiotics, it is not clear how this effect will be manifested in vivo unless there is an understanding of the rate-limiting factors involved, which is a considerable task in such a complex of interrelated reactions. Similar considerations

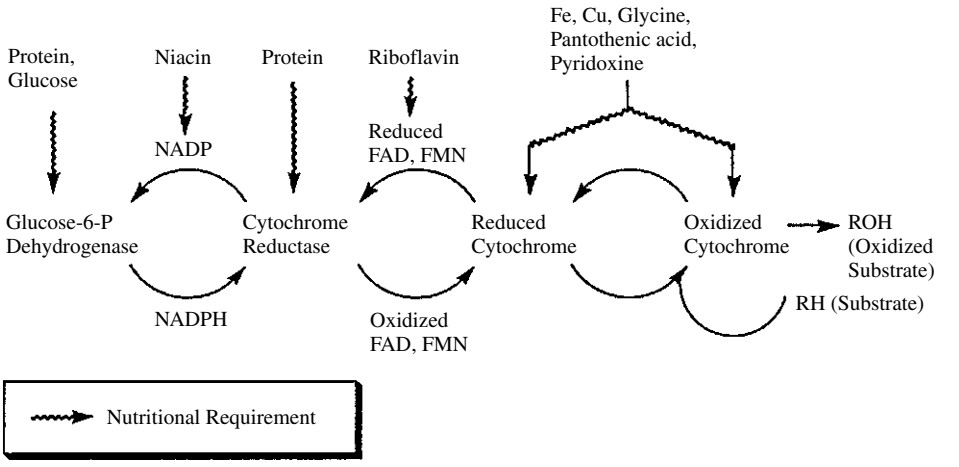



**Figure 9.1** Nutritional requirements with potential effects on the cytochrome P450 monooxygenase system (From W. E. Donaldeson Nutritional factors, in *Introduction to Biochemical Toxicology*, 3rd ed., E. Hodgson and R. C. Smart, Wiley, 2001.)

could be made for other phase I reaction systems such as arachidonic acid cooxidations, the glutathione peroxidase system, and so on.

***Phase II Reactions.*** As with phase I reactions, phase II reactions usually depend on several enzymes with different cofactors and different prosthetic groups and, frequently, different endogenous cosubstrates. 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, which is 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.

## 9.3 PHYSIOLOGICAL EFFECTS

### 9.3.1 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 P450 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 P450 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 all adult levels are reached at about 140 days (Figure 9.2). 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.

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, P450s 2A1, 2D6, and 3A2 predominate, whereas in mature rats, the males show a predominance of P450s 2C11, 2C6, and 3A2 and the females P450s 2A1, 2C6, and 2C12.

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

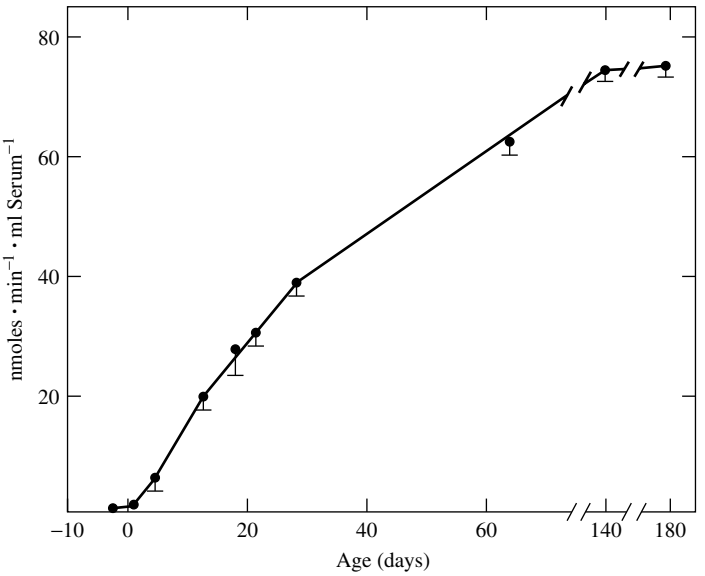



**Figure 9.2** Developmental pattern of serum glutathione *S*-transferase activity in female rats. (Adapted from H. Mukhtar and J. R. Bend, *Life Sci.* **21**: 1277, 1977.)

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 humans, age-related impairment of enzyme activity is highly controversial. Age-related declines in activity were not detected with respect to the activity of CYP2C and CYP3A isoforms among 54 liver samples from donors ranging in age from 9 to 89 years. 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.

### 9.3.2 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 9.1). Although it is not always known whether metabolism is the only or even the most important factor, such differences may be due to gender-related differences in metabolism. Hexobarbital is metabolized faster by male rats; 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 isozymes of the xenobiotic-metabolizing enzymes that exist in multiple forms, but this aspect has not been investigated extensively.

In the rat, sexually dimorphic P450s 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 the growth hormone

**Table 9.1 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 |

is secreted in a gender-specific manner. Growth hormone production is pulsatile in adult males with peaks of production at approximately 3-hour 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 P450 2C11. 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 P450 2C12. 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 xenobiotic-metabolizing enzymes or their isoforms. A schematic version of this proposed mechanism is seen in Figure 9.3.

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 9.4). 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.

### 9.3.3 Hormones

Hormones other than sex hormones are also known to affect the levels of xenobiotic metabolizing enzymes, 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. Cytochrome P450 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

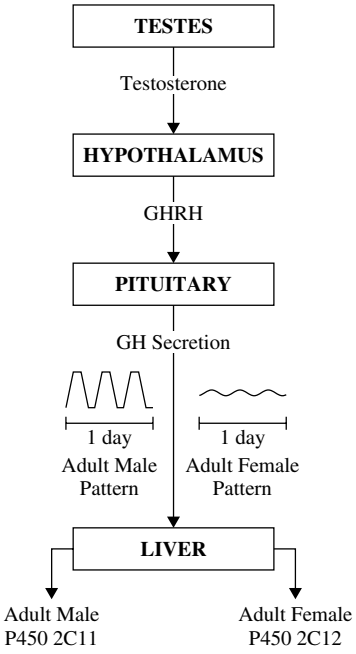



**Figure 9.3** Hypothetical scheme for neonatal imprinting of the hypothalamus–pituitary–liver axis resulting in sexually dimorphic expression of hepatic enzymes in the adult rat. Neonatal surges of testosterone appear to play a role in imprinting. (From M. J. J. Ronis and H. C. Cunny, in *Introduction to Biochemical Toxicology*, 2nd ed. E. Hodgson and P. E. Levi, eds., Appleton and Lange, 1994, p. 136.)

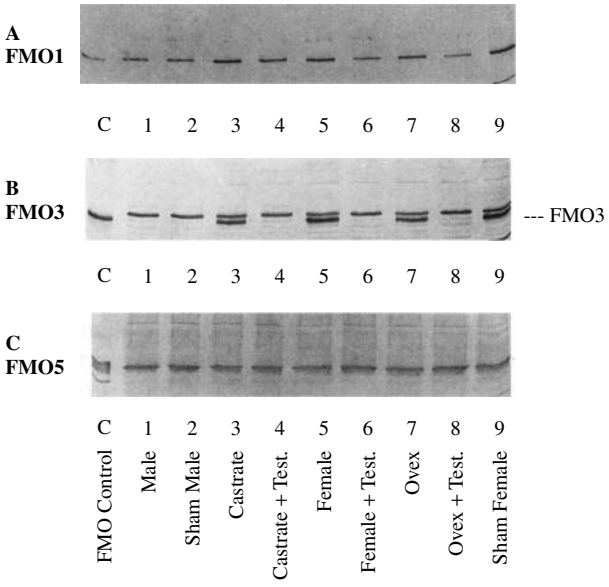



**Figure 9.4** Immunoreactivity of liver microsomes from sexually intact control, sham control, gonadectomized mice, or mice undergoing gonadectomy and/or receiving testosterone implants (5 mg). (From J. G. Falls et al., *Arch. Biochem. Biophys.* **342**: 212–223, 1997.)

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.

***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. 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 P450 2D1 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 xenobiotic metabolizing enzymes. 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.

### 9.3.4 Pregnancy

Many xenobiotic metabolizing enzyme 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 P450 levels. An increased level of FMO2 is seen in the lung of pregnant rabbits.

### 9.3.5 Disease

Quantitatively, the most important site for xenobiotic metabolism is the liver; thus effects on the liver are likely to be pronounced in 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.

### 9.3.6 Diurnal Rhythms

Diurnal rhythms, both in P450 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.

## 9.4 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. The value of the comparative approach can be summarized under four headings:

1. *Selective toxicity.* If toxic compounds are to be used for controlling diseases, pests, and parasites, it is important to develop 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 model for extrapolation to humans and for testing and development of drugs and biocides. Taxonomic proximity does not necessarily indicate which will be the best experimental animal because in some cases primates are less valuable for study than are other mammals.
3. *Environmental xenobiotic cycles.* Much concern over toxic compounds springs from their occurrence in the environment. 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. Clearly, it is desirable to know the range of metabolic processes possible.

Laboratory micro ecosystems have been developed, and with the aid of $^{14}C$-labeled compounds, chemicals and their metabolites can be followed through the plants and terrestrial and aquatic animals involved.

4. *Comparative biochemistry.* Some researchers believe that the proper role of comparative biochemistry is to put evolution on a molecular basis, and that detoxication enzymes, like other enzymes, are suitable subjects for study. Xenobiotic-metabolizing enzymes 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 cytochrome P450 isoforms, with more than 2000 isoform cDNA sequences known, is proving a useful tool for the study of biochemical evolution.

### 9.4.1 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 among species are common and of toxicologic 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 biochemical genetics, they are manifested at many other levels, the most important of which are summarized in the following sections.

***In vivo Toxicity.*** Toxicity is a term used to describe the adverse effects of chemicals on living organisms. Depending on the degree of toxicity, an animal may die, suffer injury to certain organs, or have a specific functional derangement in a subcellular organelle. Sublethal effects of toxicants may be reversible. 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 the direct toxicity to humans and other mammals of biocide toxicity seems to be in the following progression: herbicides = fungicides < molluscicides < acaricides < nematocides < insecticides < rodenticides. This formula is obviously oversimplified because marked differences in lethality are observed when different members of each group of biocides is tested against laboratory test animals and target species. One should also bear in mind that any chemical can be environmentally dangerous if misused because many possible targets are interrelated in complex ecological systems.

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. Dinitrophenol causes cataracts in humans, ducks, and chickens but not in other experimental animals. The eggshell thinning associated with DDT poisoning in birds is observed in falcons and mallard ducks, whereas this reproductive toxicity is not observed in gallinaceous species. Delayed neurotoxicity caused by organophosphates such as leptophos and tri-*o*-cresyl phosphate occurs in humans and can be easily demonstrated in chickens, but can be produced only with difficulty in most common laboratory mammals.

***In vivo Metabolism.*** Many ecological and physiological 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 various animal species. The biologic effect of a chemical depends on the concentration of its binding to tissue macromolecules. Thus substantial differences in these variables should confer species specificity in the biologic response to any metabolically active xenobiotic. The biologic half-life is governed by the rates of metabolism and excretion and thus reflects the most important variables explaining interspecies differences in toxic response. Striking differences among species can be seen in the biologic half-lives of various drugs. Humans, in general, metabolize xenobiotics more slowly than do 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 hours. The interdependence of metabolic rate, half-life, and pharmacologic 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 of 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. Those reactions that introduce a new functional group into the molecule by oxidation, reduction, or hydrolysis are designated phase I reactions, whereas the conjugation reactions by which phase I metabolites are combined with endogenous substrates in the body are referred to as phase II reactions. Chemicals may undergo any one of these reactions or any combination of them, either simultaneously or consecutively. Because biotransformations are catalyzed by a large number of enzymes, it is to be expected that they will vary among species. Qualitative differences imply the occurrence of different enzymes, whereas quantitative differences imply variations in the rate of biotransformation along a common metabolic pathway, the variations resulting from 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. Thus in humans, rats, and guinea pigs, the major route of papaverine metabolism is *O*-demethylation to yield phenolic products, but very little of these products is formed in dogs. Aromatic hydroxylation of aniline is another 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 (Table 9.2). These data show a trend,

**Table 9.2 In vivo Hydroxylation of Aniline in Females of Various Species**

| | Percent Dose Excreted as Aminophenol | | |
|---|---|---|---|
| Species | *Ortho* | *Para* | *P/O* Ratio |
| Dog | 18.0 | 9.0 | 0.5 |
| Cat | 32.0 | 14.0 | 0.4 |
| Ferret | 26.0 | 28.0 | 1.0 |
| Rat | 19.0 | 48.0 | 2.5 |
| Mouse | 4.0 | 12.0 | 3.0 |
| Hamster | 5.5 | 53.0 | 10.0 |
| Guinea pig | 4.2 | 46.0 | 11.0 |
| Rabbit | 8.8 | 50.0 | 6.0 |
| Hen | 10.5 | 44.0 | 4.0 |

*Source*: Adapted from D. V. Parke, *Biochem. J.* **77**: 493, 1960.

in that carnivores generally display a high aniline *ortho*-hydroxylase ability with a *para/ortho* ratio of $\leq 1$ whereas rodents exhibit a striking preference for the *para* position, with a *para/ortho* ratio of from 2.5 to 11. Along with extensive *p*-aminophenol, substantial quantities of *o*-aminophenol are also produced from aniline administered to rabbits and hens. The major pathway is not always the same in any two animal species. 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 human 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 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. Fluoracetamide is less toxic to mice than to the American cockroach. This is explained by the faster release of the toxic fluoroacetate in insects as compared with mice. 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 esterase. 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.

The various phase II reactions are concerned with the conjugation of primary metabolites of xenobiotics produced by phase I reactions. Factors that alter or govern the rates of phase II reactions may play a role in interspecific differences in xenobiotic metabolism. Xenobiotics, frequently in the form of conjugates, can be eliminated

through urine, feces, lungs, sweat, saliva, milk, hair, nails, or placenta, although comparative data are generally available only for the first two routes. Interspecific variation in the pattern of biliary excretion may determine species differences in the relative extent to which compounds are eliminated in the urine or feces. Fecal excretion of a chemical or its metabolites tends to be higher in species that are good biliary excretors, such as the rat and dog, than in species that are poor biliary excretors, such as the rabbit, guinea pig, and monkey. For example, the fecal excretion of stilbestrol in the rat accounts for 75% of the dose, whereas in the rabbit about 70% can be found in the urine. Dogs, like humans, metabolize indomethacin to a glucuronide but, unlike humans that excrete it in the urine, dogs excrete it primarily in the feces—apparently due to inefficient renal and hepatic blood clearance of the glucuronide. These differences may involve species variation in enterohepatic circulation, plasma level, and biologic half-life.

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.

The evidence reported in a few studies suggests some relationship between the evolutionary position of a species and its conjugation mechanisms (Table 9.3). In humans and most mammals, the principal mechanisms involve conjugations with glucuronic acid, glycine, glutamine, glutathione and sulfate. Minor conjugation mechanisms in

**Table 9.3 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 A. P. Kulkarni and E. Hodgson, Comparative toxicology, in *Introduction to Biochemical Toxicology*. E. Hodgson and F. E. Guthrie, eds., New York: Elsevier, 1980, p. 115.

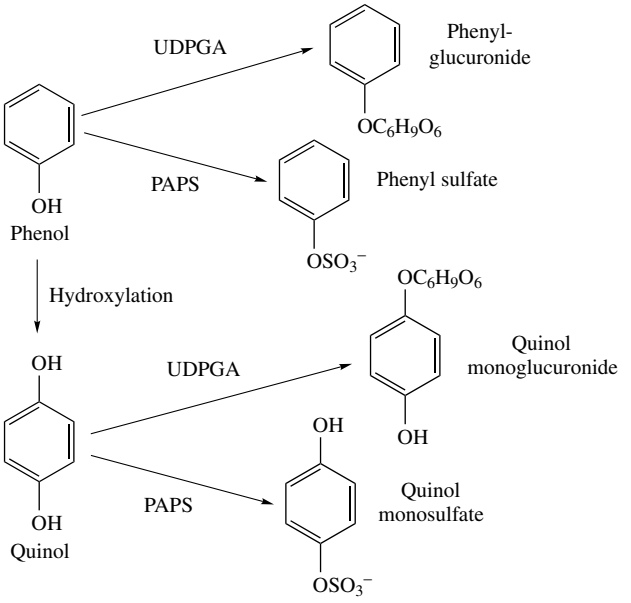
mammals include acetylation and methylation pathways. In some species of birds and reptiles, ornithine conjugation replaces glycine conjugation; in plants, bacteria, and insects, conjugation with glucose instead of glucuronic acid results in the formation of glucosides. In addition to these predominant reactions, certain other conjugative processes are found involving specific compounds in only a few species. These reactions include conjugation with phosphate, taurine, *N*-acetyl-glucosamine, ribose, glycyltaurine, serine, arginine, and formic acid.

From the standpoint of evolution, similarity might be expected between humans and other primate species as opposed to the nonprimates. This phylogenic relationship is obvious 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. New World monkeys, however, 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 human, and their importance decreases with increasing evolutionary divergence from humans. When the relative importance of metabolic pathways is considered, one of the simplest cases of an enzyme-related species difference in the disposition of a substrate undergoing only one conjugative reaction is the acetylation of 4-aminohippuric acid. In the rat, guinea pig, and rabbit, the major biliary metabolite is 4-aminohippuric acid; the cat excretes nearly equal amounts of free acid and its acetyl derivative; and the hen excretes mainly the unchanged compound. In the dog, 4-aminohippuric acid is also passed into the bile unchanged because this species is unable to acetylate aromatic amino groups.

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 glucuronyl-transferase 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 (Figure 9.5). 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.

Certain unusual conjugation mechanisms have been uncovered during comparative investigations, but this may be a reflection of inadequate data on other species. Future




| Species | Percent of 24-hr Excretion as Glucuronide | | Percent of 24-hr Excretion as Sulfate | |
|---|---|---|---|---|
| | *Phenol* | *Quinol* | *Phenol* | *Quinol* |
| Pig | 100 | 0 | 0 | 0 |
| Indian fruit bat | 90 | 0 | 10 | 0 |
| Rhesus monkey | 35 | 0 | 65 | 0 |
| Cat | 0 | 0 | 87 | 13 |
| Human | 23 | 7 | 71 | 0 |
| Squirrel monkey | 70 | 19 | 10 | 0 |
| Rat-tail monkey | 65 | 21 | 14 | 0 |
| Guinea pig | 78 | 5 | 17 | 0 |
| Hamster | 50 | 25 | 25 | 0 |
| Rat | 25 | 7 | 68 | 0 |
| Ferret | 41 | 0 | 32 | 28 |
| Rabbit | 46 | 0 | 45 | 9 |
| Gerbil | 15 | 0 | 69 | 15 |

**Figure 9.5** Species variation in the metabolic conversion of phenol in vivo.

investigations may demonstrate a wider distribution. A few species of birds and reptiles use ornithine for the conjugation of aromatic acids rather than glycine, as do mammals. For example, the turkey, goose, duck, and hen excrete ornithuric acid as the major metabolite of benzoic acid, whereas pigeons and doves excrete it exclusively as hippuric acid.

Taurine conjugation with bile acids, phenylacetic acid, and indolylacetic acid seems to be a minor process in most species, but in the pigeon and ferret, it occurs extensively. Other infrequently reported conjugations include serine conjugation of xanthurenic acid in rats; excretion of quinaldic acid as quinaldylglycyltaurine and quinaldylglycylglycine in the urine of the cat, but not of the rat or rabbit; and conversion of furfural to furylacrylic acid in the dog and rabbit, but not in the rat, hen, or human. The dog and

human but not the guinea pig, hamster, rabbit, or rat excrete the carcinogen 2-naphthyl hydroxylamine as a metabolite of 2-naphthylamine, which, as a result, has carcinogenic activity in the bladder of humans and dogs.

***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 (Table 9.4), whereas qualitative differences, such as the apparent total lack of parathion oxidation by lobster hepatopancreas microsomes, are seldom observed. Although the amount of P450 or the activity of NADPH-cytochrome P450 reductase seems to be related to the oxidation of certain substrates, this explanation is not always satisfactory

**Table 9.4 Species Variation in Hepatic Microsomal Oxidation of Xenobiotics In vitro**

| Substrate Oxidation | Rabbit | Rat | Mouse | Guinea Pig | Hamster | Chicken | Trout | Frog |
|---|---|---|---|---|---|---|---|---|
| Coumarin 7-hydroxylase[a] | 0.86 | 0.00 | 0.00 | 0.45 | — | — | — | — |
| Biphenyl 4-hydroxylase[b] | 3.00 | 1.50 | 5.70 | 1.40 | 3.80 | 1.70 | 0.22 | 1.15 |
| Biphenyl-2-hydroxylase[b] | 0.00 | 0.00 | 2.20 | 0.00 | 1.80 | 0.00 | 0.00 | 1.15 |
| 2-Methoxybiphenyl demethylase[a] | 5.20 | 1.80 | 3.40 | 2.20 | 2.30 | 2.00 | 0.60 | 0.40 |
| 4-Methoxybiphenyl demethylase[a] | 8.00 | 3.0 | 3.20 | 2.30 | 2.30 | 1.70 | 0.40 | 0.90 |
| *p*-Nitroanisole *O*-demethylase[b] | 2.13 | 0.32 | 1.35 | — | — | 0.76 | — | — |
| 2-Ethoxybiphenyl demethylase[a] | 5.30 | 1.60 | 1.40 | 2.10 | 2.50 | 1.70 | 0.60 | 0.40 |
| 4-Ethoxybiphenyl demethylase[a] | 7.80 | 2.80 | 1.80 | 2.30 | 1.80 | 1.50 | 0.40 | 0.90 |
| Ethylmorphine *N*-Demethylase[b] | 4.0 | 11.60 | 13.20 | 5.40 | — | — | — | — |
| Aldrin epoxidase[b] | 0.34 | 0.45 | 3.35 | — | — | 0.46 | 0.006 | — |
| Parathion desulfurase[b] | 2.11 | 4.19 | 5.23 | 8.92 | 7.75 | — | — | — |

*Source*: Modified from A. P. Kulkarni and E. Hodgson, Comparative toxicology, in *Introduction to Biochemical Toxicology*, E. Hodgson and F. E. Guthrie eds., New York: Elsevier, 1980, p. 120.

[a] nmol/mg/h.

[b] nmol/mg/min.

because the absolute amount of cytochrome P450 is not necessarily the rate-limiting characteristic. It is clear that there are multiple forms of P450 isozymes in each species, and that these forms differ from one species to another. Presumably both quantitative and qualitative variation in xenobiotic metabolism depends on the particular isoforms expressed and the extent of this expression.

Reductive reactions, like oxidation, 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.

Blood and various organs of humans and other animals contain esterases capable of acetylsalicylic acid hydrolysis. A comparative study has shown that the liver is the most active tissue in all animal species studied except for the guinea pig, in which the kidney is more than twice as active as the liver. Human liver is least active; the enzyme in guinea pig liver is the most active. The relatively low toxicity of some of the new synthetic pyrethroid insecticides appears to be related to the ability of mammals to hydrolyze their carboxyester linkages. Thus mouse liver microsomes catalyzing (+)-*trans*-resmethrin hydrolysis are more than 30-fold more active than insect microsomal preparations. The relative rates of hydrolysis of this substrate in enzyme preparations from various species are mouse >> milkweed bug >> cockroach >> cabbage looper > housefly.

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 amidases. 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.

Hepatic microsomes of several animal species possess UDP glucuronyltransferase activity and with *p*-nitrophenol as a substrate, a 12-fold difference in activity due to species variation is evident. Phospholipase-A activates the enzyme and results of activation experiments indicate that the amount of constraint on the activity of this enzyme is variable in different animal species.

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.

### 9.4.2 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 P450 enzymes 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 glutathione *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 (see Figure 18.4) involving enzymes different from those involved in photosynthesis.

### 9.4.3 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 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. The frequency of genetic polymorphisms is arbitrarily defined as having a population frequency of greater than 1%. 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 be the result of gene polymorphisms. In cases involving insecticide selection pressure, resistant populations may arise as a result of direct mutations of insecticide-metabolizing enzymes and/or insecticide target sites that are passed on to succeeding generations.

***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 LD50 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 more than 200 species of insects and mites, and resistance of up to several 100-fold 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 recently 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 two 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 2D6 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% to 90% of these populations being rapid acetylators, whereas 40% to 60% of African 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 xenobiotic metabolizing enzymes 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 trimethylamines, 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 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 arylhydrocarbon 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, $Ah^b$ 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 depend 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 glucuronosyl-transferase. 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 P450s have not been studied extensively in different strains of the same vertebrate. The only thorough investigations, those of the Ah Locus, which controls aryl hydrocarbon hydroxylase induction, have shown that in addition to quantitative differences in the amount of P450 after induction in different strains of mice, there may also be a qualitative difference in the P450 isozymes induced (see Section 9.5.2).

## 9.5 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. Some estimation of the number of chemicals in use in the United States are given in Table 9.5. Because it bears directly on the problem of toxicity-related interaction among different 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. 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.
- Compound A, given after exposure to an inducer of the activating enzymes, would appear 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.
- Compound B, given after exposure to an inducer of the detoxifying enzymes, would appear less toxic.

In addition to the previously mentioned cases, the toxicity of the inhibitor or inducer, as well as the time dependence of the effect, must also be considered because, as

**Table 9.5 Some 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.

mentioned, many xenbiotics that are initially enzyme inhibitors ultimately become inducers.

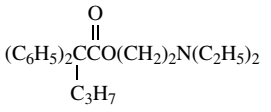
### 9.5.1 Inhibition

As previously indicated, inhibition of xenobiotic-metabolizing enzymes can cause either an increase or a decrease in toxicity. Several well-known inhibitors of such enzymes are shown in Figure 9.6 and are discussed in this section. Inhibitory effects can be demonstrated in a number of ways at different organizational levels.

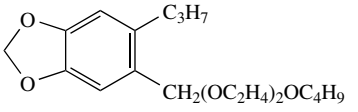
#### *Types of Inhibition: Experimental Demonstration*

*In vivo Symptoms*. The measurement of the effect of an inhibitor on the duration of action of a drug in vivo is the most 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.

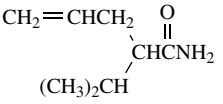
Previously the most used and most reliable of these tests involved the measurement of effects on the hexobarbital sleeping time and the zoxazolamine paralysis time. Both of these drugs are fairly rapidly deactivated by the hepatic microsomal monooxygenase



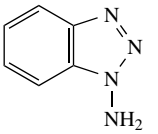
SKF-525A [P450]
2-(Diethylamino)ethyl-
2,2-diphenylpentanoate



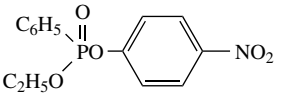
Piperonyl Butoxide [P450]
3,4-Methylenedioxy-6-propylbenzyl
n-butyl diethyleneglycol ether



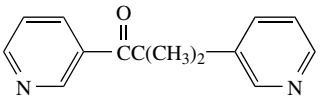
Allylisopropylacetamide
[P450]



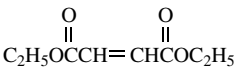
1-Aminobenzotriazole (1-ABT)
[P450]



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



Metyrapone
[P450]



Diethyl maleate
[glutathione S-transferase]

Disulfiram (Antabuse)
[aldehyde dehydrogenase]

**Figure 9.6** Some common inhibitors of xenobiotic-metabolizing enzymes.

system; thus, inhibitors of this system prolong their action. For example, treatment of mice with chloramphenicol 0.5 to 1.0 hour 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 (Figure 9.6), 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 hour before the narcotic is given. As a consequence of the availability of single expressed isoforms for direct studies of inhibitory mechanisms, these methods are now used much less often.

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 P450 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 P450 inhibitor, and the carcinogens, freons 112 and 113.

*Distribution and Blood Levels.* Treatment of an animal with an inhibitor of foreign compound 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 hour 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 hour 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 among 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) 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 re-suspension. 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 P450, such as SKF-525A, piperonyl butoxide, and other methylenedioxphenyl compounds, and amphetamine and its derivatives, can be readily investigated in this way. This is 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 cytochrome P450 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 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.

Although the kinetics of inhibition of xenobiotic-metabolizing enzymes 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:

- The P450 system, a particulate enzyme system, has been investigated many times, but using methods developed for single soluble enzymes. As a result Lineweaver-Burke or other reciprocal plots are frequently curvilinear, and the same reaction may appear to have quite a different characteristics 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.
- Xenobiotic-metabolizing enzymes commonly exist in multiple forms (e.g., glutathione *S*-transferases and P450s). These isozymes 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 $K_m$ but not in $V_{\max}$. 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*-nitronanisole by aminopyrine, aldrin epoxidation by dihydroaldrin, and *N*-demethylation of aminopyrine 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 P450 and to be competitive inhibitors of the *O*-demethylation of *p*-nitronanisole.

*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-Burke 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 9.6), 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 or the disruption of the enzyme structure is involved. In 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 formation of a reactive intermediate that then interacts with the enzyme. An excellent example of this type of inhibition is the effect of the insecticide synergist piperonyl butoxide (Figure 9.6) on hepatic microsomal monooxygenase activity. This methylenedioxyphenyl compound can form a stable inhibitory complex that blocks CO binding to P450 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 carbine formed spontaneously by elimination of water following hydroxylation of the methylene carbon by the cytochrome (see Figure 7.8 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, and *p*-nitroanisole among 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. 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 xenobiotic-metabolizing enzymes, hence the designation "suicide substrates." The drug allylisopropylacetamide (Figure 9.6), as well as other allyl compounds, has long been known to cause the breakdown of P450 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 (see Figure 8.2). Much information has accumulated since the mid-1970s on the mode of action of the hepatotoxicant carbon tetrachloride, which effects a number of irreversible changes in both liver proteins and lipids, such changes being generated by reactive intermediates formed during its metabolism (Figure 8.3).

The less specific disruptors of protein structure, such as urea, detergents, strong acids, and so on, are probably of significance only in vitro experiments.

***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 sequentially than would be expected from a consideration of the toxicities of the compounds given alone. Some toxicologists have used the term synergism for cases that fit this definition, but only when one compound is toxic alone whereas the other has little or no intrinsic toxicity. For example, the nontoxic synergist, piperonyl butoxide is often included in pesticide formulations because of its ability to significantly increase the toxicity of the active pesticide ingredient by inhibiting its detoxication in the target species.

The term potentiation is then reserved for those cases where both compounds have appreciable intrinsic toxicity, such as in the case of malathion and EPN. Malathion has a low mammalian toxicity due primarily to its rapid hydrolysis by a carboxylesterase. EPN (Figure 9.6) another organophosphate insecticide, causes a dramatic increase in malathion toxicity to mammals at dose levels, which, given alone, cause essentially no inhibition of acetylcholinesterase. The increase in toxicity as a result of coadministration of these two toxicants is the result of the ability of EPN, at low concentrations, to inhibit the carboxylesterase responsible for malathion degradation.

Unfortunately, the terms synergist and potentiation have often been used by some toxicologists in precisely the opposite manner. Historically, the term synergist has

been used by pharmacologists to refer to simple additive toxicity and potentiation either as a synonym or for examples of greater than additive toxicity or efficacy. In an attempt to make uniform the use of these terms, it is suggested that insofar as toxic effects are concerned, the terms be used according to the following: *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.*

***Antagonism.*** In toxicology, antagonism may be defined as that situation where 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 9.5.2). Apart 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 P450 isozymes 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 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 where 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.

### 9.5.2 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. Several hundred compounds of diverse chemical structure have been shown to induce monooxygenases and other enzymes. These compounds include drugs, insecticides, polycyclic hydrocarbons, and many others; the only obvious common denominator

is that they are organic and lipophilic. It has also become apparent that although all inducers do not have the same effects, the effects tend to be nonspecific to the extent that any single inducer induces more than one enzymatic activity. Other enzymes often coinduced by P450 inducers include glutathione S-transferase, epoxide hydrolases and others; perhaps as a result of general induction of cellular processes including proliferative responses of endoplasmic reticulum, peroxisomes, and mitochondria.

***Specificity of Monooxygenase Induction.*** The majority of studies involving monooxygenase induction have been conducted in mammals. Mammals have at least 17 distinct CYP families, coding for as many as 50 to 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 7, CYP families 1–4 are the predominant families involved in xenobiotic metabolism. These CYP 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 later in this section.

Inducers of monooxygenase activity fall into four principle classes, exemplified by TCDD (inducer of CYP1A1), phenobarbital (inducer of the CYP2B and 3A families), rifampicin (inducer of CYP3A and 2C families), and ethanol (inducer of 2E1). 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 to 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, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), is effective at 1$\mu$g/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 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 uridine diphosphoglucuronyl transferase may also be 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-16$\alpha$-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), pregnane

compounds (e.g., pregenenolone 16$\alpha$-carbonitrile, PCN), and macrolide antibiotics (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-Ah receptor-dependent mechanism. Peroxisome proliferators, including the drug, clofibrate, and the herbicide synergist tridiphane induce a CYP4A isozyme that catalyzes the $\omega$-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 cytochromes. 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 invertebrate. 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" isozymes, 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 cases of increase in monooxygenase activity there is a true induction involving synthesis of new enzyme, and not the activation of 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 9.7.

Perhaps the best understood example of induction involves induction of the aromatic hydrocarbon receptor (AhR) by compounds such as TCDD and 3-methylcholanthrene. The use of suitable inhibitors of RNA and DNA polymerase activity has shown that inhibitors of RNA synthesis such as actinomycin D and mercapto(pyridethyl)benzimidazole block aryl hydrocarbon hydroxylase induction, whereas hydroxyurea, at levels that completely block the incorporation of thymidine into DNA, has no effect. Thus it appears that the inductive effect is at the level of transcription and that DNA synthesis is not required.

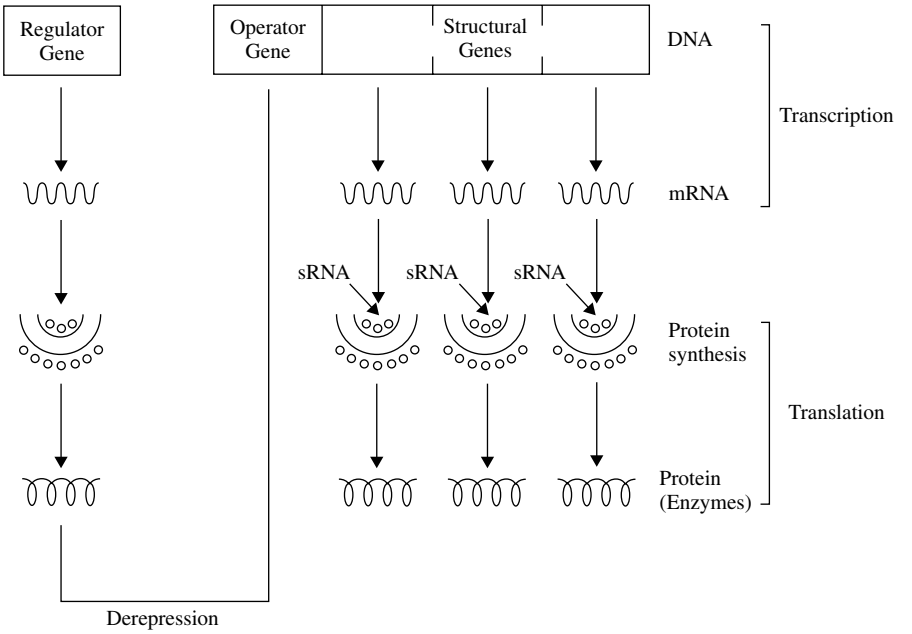



**Figure 9.7** Simplified scheme for gene expression in animals.

These findings imply that compounds that induce xenobiotic-metabolizing enzymes play a role as derepressors of regulator or other genes in a manner analogous to steroid hormones—namely by combining with a cytosolic receptor followed by movement into the nucleus and then derepression 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 9.8). After ligand binding, the receptor translocates to the nucleus where it forms a dimer with another protein known as ARNT. In the nucleus the transformed receptor interacts with specific sequences of DNA known as xenobiotic responsive elements (XREs). Two XREs are located approximately 1000 or more base pairs upstream from the transcriptional start site in the 5′ flanking region of the CYP1A1 gene. A third site is likely to be an inhibitory or suppressor site (Figure 9.9). The protein-DNA interaction that occurs at the XREs is thought to result in a bending of the DNA, which allows for increased transcription followed by increased protein synthesis. Another promoter region is located just upstream from the transcriptional start site. Although several transcription factors may interact with this binding site, including the TATA-binding protein, it has no binding sites for the AhR/Arnt proteins. Transfection experiments indicate that the TATA-binding site is essential for promoter function, while the other sites are less important. This promoter region is silent unless the upstream XREs have been appropriately activated by the AhR and Arnt proteins. The fact that several genes may be responsive to CYP1A inducers is indicative of the fact that similar XREs are found on many Ah-receptor inducible genes.

Although phenobarbital induction has been studied for many years, the mechanism for induction has only recently been established. In bacteria a key feature of phenobarbital induction was demonstrated to involve barbiturate-mediated removal of a

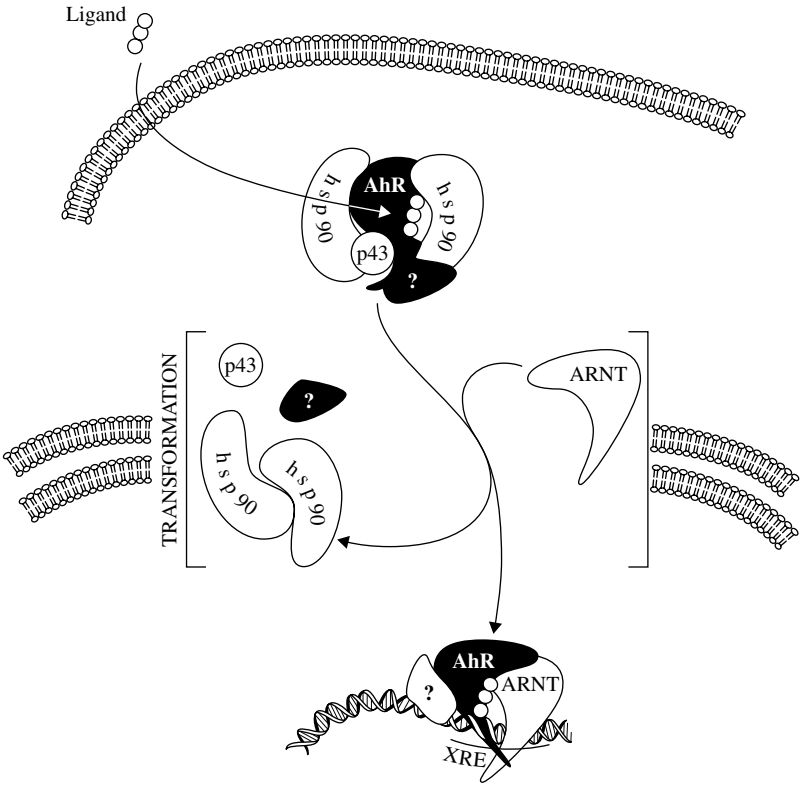



**Figure 9.8** Proposed mechanism for ligand-activated AhR translocation and DNA binding (From J. C. Rowlands and J.-A. Gustafsson, *Crit. Rev. Toxicol.* **27**: 109, 1997.)

repressor protein from a 17-bp promoter regulatory sequence known as the "Barbie box." Although homologous promoter sequences have been observed in several phenobarbital responsive mammalian genes, ample evidence suggests that these sequences are not important in PB-induced transcription of mammalian CYP genes. Rather, in mammalian species the phenobarbital responsive sequences are found far upstream of the start codon.

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 mouse CYP2b10 and CYP2b9 genes. 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. These include a nuclear factor 1 (NF1) binding site that is flanked by two nuclear receptor binding sites, designated NR1 and NR2. Inducibility requires at least one of the NR sites to be present to maintain phenobarbital inducibility. By

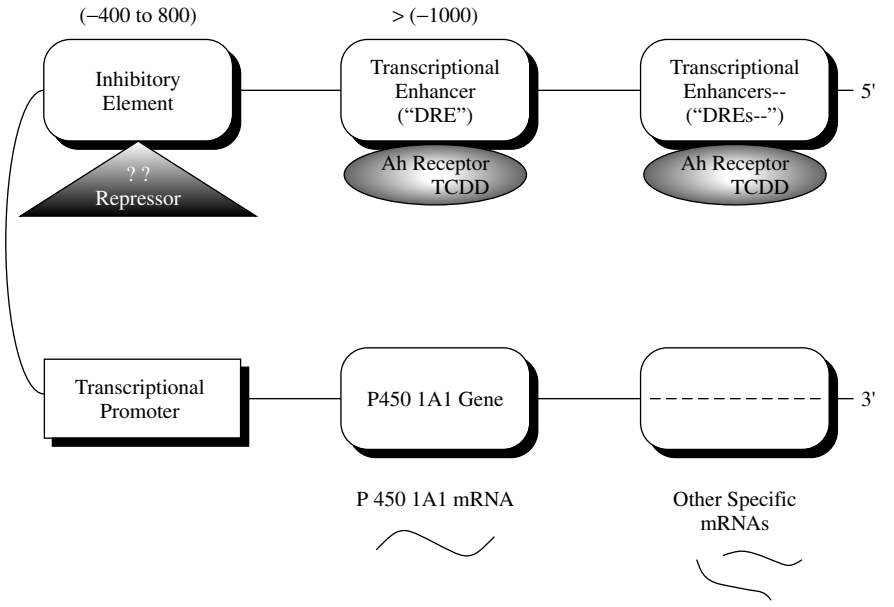



**Figure 9.9** Interaction of the Ah-receptor-ligand complex with the 5′ flanking region of the P450 1A1 gene. Two dioxin responsive elements (DREs) appear to lie approximately 1000 or more base pairs upstream from the 1A1 transcriptional s tart site. These elements appear to be transcriptional enhancers, whereas less direct evidence indicates an inhibitory element ("negative control element") between 400 and 800 bases upstream. The negative control element may inhibit the 1A1 promoted although the conditions for this inhibition are, as yet, undefined. (Adapted from A. B. Okey, *Pharmacol. Ther.* **45**: 241–298, 1990.)

contrast, although the NF1 site is necessary for the maximum phenobarbital response, it is nonessential for the basic phenobarbital response. This conclusion is supported by the fact that in rodent and human CYP2B genes the NR sites are highly conserved while the NF1 site is not.

The key factor that interacts with the PBREM is the orphan nuclear receptor known as a "constitutive active receptor" (CAR). 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 nuclear receptors. Although the CAR-RXR binding does not require treatment with phenobarbital for activity, hence the term "constitutive," inclusion of phenobarbital substantially increases the activity of CYP2B and other PBRE related genes. It is thought that this is due to the displacement of two endogenous inhibitory androstane steroids that bind to the CAR-RXR heterodimer and inhibit its activity in the absence of phenobarbital like ligands. Thus, in the presence of phenobarbital, the binding of the inhibitory androstanes to CAR is abolished and the intrinsic activity of CAR becomes manifest, leading to the activation of PB responsive genes. Recent studies using CAR knockout mice indicate that many drug metabolizing genes are under CAR regulation, including isoforms of CYP2B, CYP3A, NADPH cytochrome P450 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 steroidal chemicals. This particular

group, belonging to the CYP3A subfamily, is well known for the diversity of substrates that it is capable of metabolizing. 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 further known for their ability to induce their own metabolism as well as the metabolism of other CYP3A substrates, resulting in the creation of potentially dangerous drug-drug type interactions. Regulation of the CYP3 family is likely to be primarily through enhanced transcription, although there are also some examples of post-translational regulation. For example, dexamethasone appears to increase CYP3A1 levels by stabilization of the mRNA while erythromycin acts by protein stabilization.

Several recent studies have begun to identify several elements on the 5′ upstream promoter region as well as receptors involved in CYP3 regulation (Figure 9.10). Deletion studies involving 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 of the start of transcription. Subsequent studies demonstrated that for several CYP3A isoforms from different species contained nuclear receptor binding sites that are activated by DEX/PCN but exhibit low activation by rifampicin. Further work identified an 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. XREM contains two nuclear receptor

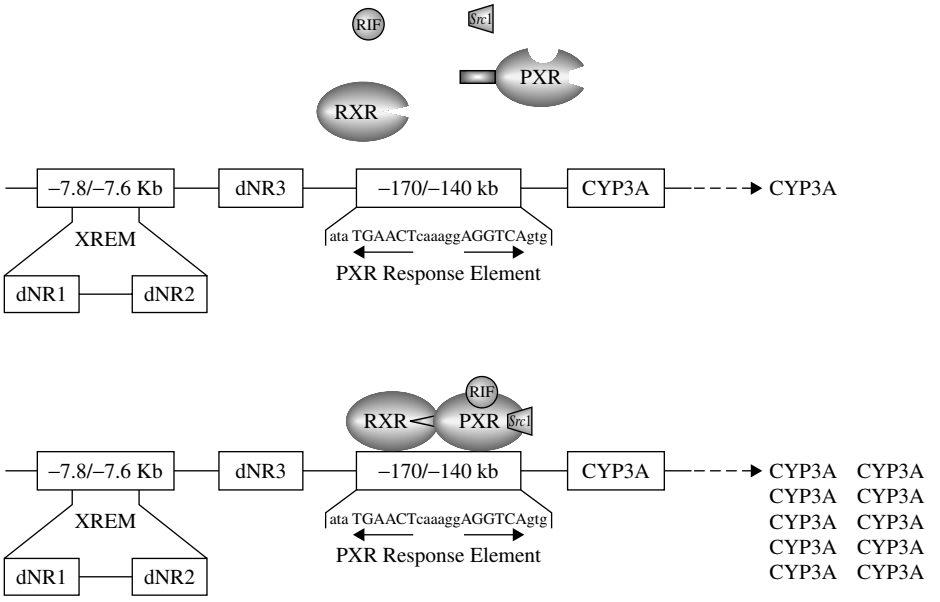



**Figure 9.10** Illustration depicting DNA elements found in CYP3A genes and the activation of the human pregnane X receptor (PXR) by ligand (RIF) and subsequent transcriptional activation of CYP3A4 gene by the PXR/RXR heterodimer. dNR-1–3, nuclear receptors 1, 2, and 3, respectively; PXR, pregnane X receptor; RXR, retinoid X receptor; RIF, rifampicin; SRC-1, steroid receptor co-activator; XREM, xenobiotic responsive enhancer module.

binding sites (dNR1 and dNR2), neither of which is solely responsible for the activity of XREM. An additional nuclear receptor site, dNR3 located several hundred bases downstream of XREM also appears to have some importance in induction.

Recent work has demonstrated that the nuclear orphan receptor, pregnane X receptor (PXR) 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 lungs 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 mice "humanized" with the PXR gene were able to respond to rifampicin induction. These observations suggest that many of the significant differences in CYP3A induction profiles between species may be due to differences in the PXR.

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-$\alpha$ PPAR$\alpha$ was first cloned in 1990. PPAR$\alpha$ knockout mice exposed to chemicals that normally induce CYP4A as well as peroxisome proliferation do not exhibit these characteristics, demonstrating the essential nature of PPAR$\alpha$ for these responses. Like PXR, PPAR$\alpha$, which is constitutively nuclear, also binds to DNA as a PPAR$\alpha$/RXR heterodimer in response to peroxisome proliferating chemicals.

CYP2E1 catalyzes metabolism of several low molecular weight xenobiotics including drugs (e.g., acetaminophen), solvents (e.g., ethanol and 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 post-transcriptional mechanisms.

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 in response to these substrates may be due to protein stabilization (e.g., decreased turnover), as a result of the inhibition of ubiquitin-mediated proteolysis. The ubiquitination process normally tags proteins with a chain of multiple ubiquitin moieties that can be detected as smears at the tops of SDS gels. The ubiquitin tags allow for the selective degradation of associated proteins 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. The same antibody also prevented catalysis of chlorzoxazone. 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. Many other possibilities for mRNA stabilization/destabilization exist within the 5′ and 3′ untranslated regions of the DNA that are being explored.

***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. 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 hours to 17 minutes by treatment of the animal with benzo(a)pyrene 24 hours before the administration of zoxazolamine.

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 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*-deethylation, 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 Xenobiotic-Metabolizing Enzymes Other Than Monooxygenases.*** Although less well studied, xenobiotic-metabolizing enzymes other than those of the P450 system are also known to be induced, frequently by the same inducers that induce the oxidases. These include glutathione *S*-transferases, epoxide hydrolase, and UDP glucuronyltransferase. The selective induction of one pathway over another can greatly affect the metabolism of a xenobiotic.

### 9.5.3 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 P450 combined with methylenedioxyphenyl compounds in an inhibitory complex cannot interact with CO, the cytochrome P450 titer, as determined by the method of Omura and Sato (dependent on 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].

## 9.6 ENVIRONMENTAL EFFECTS

Because light, temperature, and other in vitro effects on xenobiotic metabolizing enzymes 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.

*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 hypothysectomy or adrenalectomy. There appear to be two basic types of temperature effects on toxicity: either an 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.

*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.

*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. Cytochrome P450 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.

*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.

*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 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 $\geq$ 5000 ft, the lethality of digitalis or strychnine to mice is decreased, whereas that of *D*-amphetamine is increased.

*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.

## 9.7 GENERAL 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. Despite the complexity, summary statements of considerable importance can be abstracted:

- Phase I metabolism generally introduces a functional group into a xenobiotic, which enables conjugation to an endogenous metabolite to occur during phase II metabolism.
- 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 excretable.
- 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.
- 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.
- Because both qualitative and quantitative differences exist among species, strains, individual organs, and cell types, a particular toxicant may have different effects in different circumstances.
- Because exogenous chemicals can be inducers and/or inhibitors of the xenobiotic-metabolizing enzymes 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.
- 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.
- It has become increasingly clear that most enzymes involved in xenobiotic metabolism occur as several isozymes, which coexist within the same individual and, frequently, within the same subcellular organelle. An understanding of the biochemistry and molecular genetics of these isozymes may lead to an understanding of the variation among species, individuals, organs, sexes, developmental stages, and so on.

## SUGGESTED READING

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

Banerjee, A., T. A. Kocarek, and R. F. Novak. Identification of a ubiquitination-target/substrate-interaction domain of cytochrome P-450 (CYP) 2E1. *Drug Metabol. Disp.* **28**: 118–124, 2000.

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

Denison, M. S., and J. P. Whitlock, Jr. Minireview: Xenobiotic-inducible transcription of cytochrome P450 genes. *J. Biol. Chem.* **270**: 18175–18178, 1995.

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

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

Hodgson, E., and J. A. Goldstein. Metabolism of toxicants: Phase I reactions and pharmacogenetics. In *Introduction to Biochemical Toxicology*, 3rd ed., E. Hodgson, R. C. Smart, eds., New York: Wiley, 2001.

Kakizaki, S., Y. Yamamoto, A. Ueda, R. Moore, T. Sueyoshi, and M. Negishi. 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.

Lieber, C. S. Cytochrome P-4502E1: Its physiological and pathological role. *Physiol. Rev.* **77**: 517–544, 1997.

Lin, J. H., and A. Y. H. Lu. Interindividual variability in inhibition and induction of cytochrome P450 enzymes. *An. Rev. Pharmacol. Toxicol.* **41**: 535–567, 2001.

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. Disp.* **23**: 154–158, 1995.

Okey A. B. Enzyme induction in the cytochrome P-450 system. *Pharmac. Ther.* **45**: 241–298, 1990.

Ronis, M. J. J., and H. C. Cunny. Physiological factors affecting xenobiotic metabolism. In *Introduction to Biochemical Toxicology*, 3rd ed., E. Hodgson, and R. C. Smart, eds. New York: Wiley, 2001.

Rose, R. L., and E. Hodgson. Adaptation to toxicants. In *Introduction to Biochemical Toxicology*, 3rd ed. E. Hodgson, and R. C. Smart eds. New York: Wiley, 2001.

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

Smith, C. A. D., G. Smith, and C. R. Wolf. Genetic polymorphisms in xenobiotic metabolism. *Eur. J. Cancer* **30A**: 1935–1941, 1994.

Whitlock, J. P., Jr. Induction of cytochrome P4501A1. *An. Rev. Pharmacol. Toxicol.* **39**: 103–125, 1999.