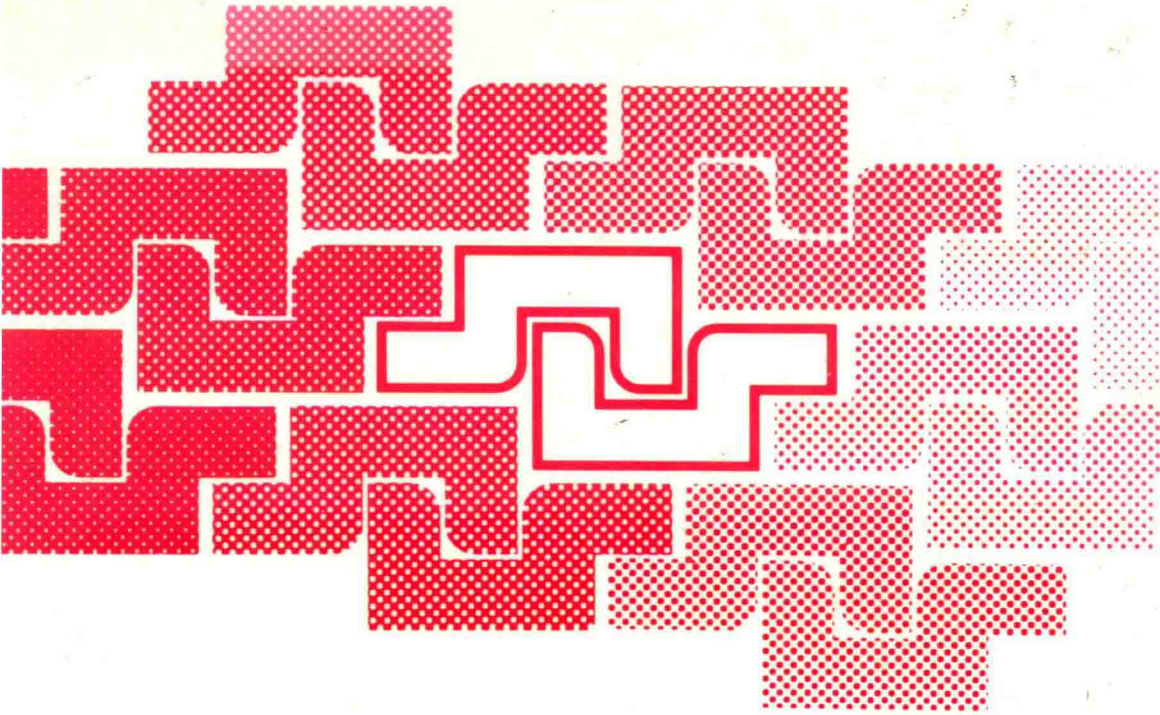

Molecular and Applied Aspects of Oxidative Drug Metabolizing Enzymes

Edited by
Emel Arinç
John B. Schenkman and
Ernest Hodgson

NATO ASI Series

Series A: Life Sciences Vol. 303

Molecular and Applied Aspects of Oxidative Drug Metabolizing Enzymes

Edited by

Emel Arinç

Middle East Technical University
Ankara, Turkey

John B. Schenkman

University of Connecticut Health Center
Farmington, Connecticut

and

Ernest Hodgson

North Carolina State University
Raleigh, North Carolina

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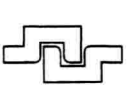

Series A: Life Sciences

PREFACE

The NATO Advanced Study Institute of "Molecular and Applied Aspects of Oxidative Drug Metabolizing Enzymes" was held in Tekirova, Antalya, Turkey, from August 31 to September 11, 1997. This Institute was the third of a series of the NATO ASIs on a similar topic relating to the enzymes of oxidative metabolism of xenobiotics. The first NATO ASI in this series, entitled "Molecular Aspects of Monooxygenases and Bioactivation of Toxic Compounds" (NATO ASI Series A: Life Sciences, Vol. 202), was held in Çesme, Izmir, Turkey, in 1989. The Institute dealt with the potential dangers of drugs, pesticides, pollutants, and carcinogens in the environment resulting from the enzymes of xenobiotic metabolism. The second NATO ASI was entitled "Molecular Aspects of Oxidative Drug Metabolizing Enzymes: Their Significance in Environmental Toxicology, Chemical Carcinogenesis, and Health" (NATO ASI Series H: Cell Biology, Vol. 90). This Institute was held in Kusadasi, Aydin, Turkey, from June 20 to July 2, 1993, and updated and extended the coverage of the first Institute to aquatic species, and delved deeper into the subject of genotoxicity and carcinogenicity. In this third Institute, greater emphasis was put on the human enzymes of oxidative xenobiotic biotransformation, including the flavin-containing monooxygenases and particularly those of the cytochrome P450 family. Considerable effort was made to make the participants aware of the potential dangers of polymorphisms in the enzymes of xenobiotic metabolism, and several of the lecturers addressed this issue.

Today, we are at a crossroads on the information highway. The area of molecular biology has expanded exponentially, with many investigators using new tools to develop methods and techniques for delving into the genetic material of the cell. Others are turning to molecular biology as a means to approach older questions from a different angle: at the level of transcription and translation. Another group of investigators has begun to make use of these methods to study the enzymes of xenobiotic metabolism using the newer tools for the generation of and perturbation of the enzymes. In order to understand newer developments in the field of the oxidative xenobiotic metabolizing enzymes, it is necessary to be well grounded in the methods and procedures currently being used in the studies. In this Institute, efforts were made to put the subject matter into perspective by giving the participants an overview of the various routes that a compound foreign to the body may take after gaining entrance until it is eliminated as a metabolite. Our goal was to provide a ready source of up-to-date information for the student and expert in the field alike, containing sufficient background information for the former to follow the subject matter and

the latest information on the subject to satisfy the latter. Hopefully, you, the reader, will find that we have reached this goal.

Emel Arinç
John B. Schenkman
Ernest Hodgson

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Enzymes of Metabolism

John B. Schenkman

Department of Pharmacology
University of Connecticut Health Center
MC-1505
Farmington, Connecticut 06030

1. ROLE OF BIOTRANSFORMATION IN ELIMINATION OF XENOBIOTICS

Xenobiotics are chemicals that are taken up into the body from exogenous sources, either ingested with the food we eat or imbibed with the water we drink, but may also be contained in the air we breathe and absorbed through our skin and lungs. Most of these chemicals are lipophilic, *i.e.*, they have a greater solubility in lipid than in aqueous media. As a result they pose a potential problem in elimination, because the peritubular cells lining the renal tubules all have cell membranes composed of phospholipid and proteins. As the glomerular filtrate passes through the renal tubules, solutes contained in it are either selectively removed along with the water or become concentrated. Compounds with a sufficiently high lipid/water partition coefficient are absorbed down the concentration gradient, dissolving into the cell membranes. From there they pass through the cells back into the body. In this simplistic model the only elimination of the compound would be that present in the excreted water, the urine and this would be at the concentration present in the plasma and glomerular filtrate. Since the fraction of filtered body water eliminated daily is about 0.8% of the total ($k_e = 0.008\ day^{-1}$), the half-life of such a compound, *e.g.*, hexobarbital (see Table 1), would be in excess of 87 days,

$$T_{\frac{1}{2}} = \frac{0.693}{k_e}$$

and the agent would retain its pharmacological or toxicological properties. For example, a lipophilic hypnotic like hexobarbital would have a duration in the body of about 1.6 years. Actually, the half-life of hexobarbital is a short 3.7 hrs, the result of active metabolism. Table 1 shows the relationship between the partition coefficients of a series of barbiturates

Molecular and Applied Aspects of Oxidative Drug Metabolizing Enzymes,
edited by Arinç *et al.* Kluwer Academic / Plenum Publishers, New York, 1999.

Table 1. Parameters influencing duration of barbiturate action

Drug	Class	Partition coef. τ(o/w)	Metabolism (nmol/min)	% unchanged in urine
Hexobarbital	ultrashort	7.62	23.4	0
Amobarbital	intermediate	4.85	13.1	0
Phenobarbital	long	1.03	7.4	25-30
Barbital	long	0.15	0.15	70-90

Modified from Jansson, et al. (1972) Arch. Biochem. Biophys., *151*,391.

and their rates of metabolism. Those agents with a high partition coefficient have a shorter duration of action and a smaller proportion of the drug is found unchanged in the urine. Although there appears to be a faster rate of metabolism with increased lipophilicity, no direct correlation exists. Enzymes of xenobiotic metabolism are found throughout the cell, in the cytosol and in the particulate fractions of homogenates of most tissues, with highest levels generally residing in the liver.

2. ROUTES OF XENOBIOTIC METABOLISM

2.1. Phase I and Phase II Metabolism

Perhaps the most important concept to grasp is that pathways of xenobiotic metabolism are similar to the other pathways of intermediary metabolism in living organisms, lipid metabolism, carbohydrate metabolism, protein metabolism and nucleic acid metabolism, in that there are two legs to the pathway. These two legs are a synthetic branch and a degradative branch. Like other synthetic routes of intermediary metabolism, *e.g.*, glycogen formation or nucleic acid formation, that of xenobiotic conjugation is energy consuming. Unlike the degradative pathways of lipid, carbohydrate and protein metabolism, but like nucleic acid degradation, catabolic pathways of xenobiotic metabolism are generally not energy yielding. The two branches have been called Phase I and Phase II, indicating a relationship between them (Figure 1). Lipophilic drugs and chemicals enter the body and are biotransformed into inactive, more polar, readily excretable metabolites (Phase I) by the process of unmasking existing functional groups or by the creation of new functional groups. These may also be conjugated with more polar biochemicals of endogenous origin (Phase II), generally rendering the metabolites inactive and more readily excreted. In many instances inactive agents, called prodrugs, may be administered to individuals; Such chemicals may be eliminated directly, or may be conjugated by Phase II enzymes and eliminated, or may be activated by Phase I drug metabolizing enzymes to the pharmacologically active agent. However, in many instances inert chemicals may also be activated to metabolites that have toxic, carcinogenic, mutagenic, or teratogenic potential. Table 2 outlines some of the most frequently utilized pathways of xenobiotic metabolism, both those of the Phase I and the

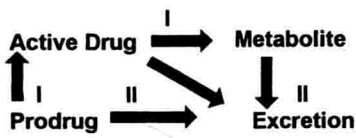


Figure 1. Relationship between Phase I and Phase II metabolic pathways.

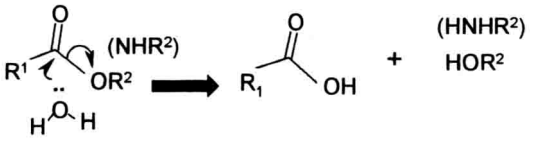
Table 2. Outline of pathways of xenobiotic biotransformation

A. Phase I Reactions
1. Hydrolytic Enzymes
a) amidases
b) esterases
c) epoxide hydrolases
2. Reductive Enzymes
a) azo reductases
b) disulfide reductase
c) aldo-keto reductases
d) nitro reductases
e) reductive dehalogenation
3. Oxidative Enzymes
a) flavin-containing monooxygenases
b) amine oxidases
c) alcohol and aldehyde dehydrogenases
d) cytochrome P450 monooxygenases
B. Phase II Enzymes
1. UDP-glucuronyl transferases
2. Glutathione transferases
3. Glycine N-acetyl transferases
4. Sulfotransferases
5. Acetyl CoA transferases

Phase II routes. In the first two Phase I pathways, hydrolytic and reductive enzymes function to unmask existing functional groups on xenobiotics. The third group of enzymes function to form new functional groups on xenobiotic molecules. The Phase II enzymes use components from carbohydrate metabolism, protein metabolism fat metabolism and nucleic acid metabolism for complexation of xenobiotic molecules.

2.2. Phase I Reactions: Unmasking or Generating New Functional Groups

2.2.1. Hydrolytic Reactions. The hydrolytic reactions all involve the nucleophilic attack of water at an electrophilic carbon atom with the cleavage of an oxygen or nitrogen bond to the carbonyl carbon and its replacement by a water hydroxyl group. The reaction can be described by the equation:

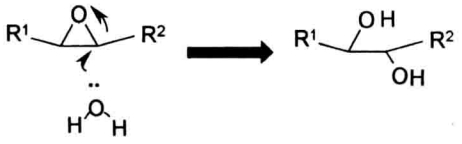


(1)

An ester, such as acetylcholine or aspirin, will be converted to a carboxylic acid (R^1) plus an alcohol (R^2). In contrast, an amide, like procainamide, will be converted to its R^1-carboxylic acid and its R^2-amine. There exists a very large number of amidases and esterases, and they are found in just about every tissue, cellular compartment and also in the plasma. An early review of these enzymes describes many of the properties.[1] The kinetic properties of the enzymes will depend upon the length of both the acyl residue and

the alkyl residue.[2] Amidases generally will also hydrolyze esters and esterases will usually also hydrolyze some amides, and both often will also hydrolyze thioesters.[3] In addition, some esterases exist (A-esterases which can hydrolyze phosphate esters.[4] Hepatic endoplasmic reticulum itself has at least eight specifically different amidase/esterases.[3]

The epoxide hydrolases, which are discussed in a later chapter, differ, since they catalyze an SN2 attack by the nucleophilic water at a side opposite from a strained epoxide ring. This results in stereospecific formation of a vicinal diol in the trans configuration. Steric hindrance will influence the site of attack by the epoxide hydrolases, *i.e,* at R^1 or at R^2. The epoxide hydrolase is located mainly in the endoplasmic reticulum of cells, but cytosolic and mitochondrial forms have also been described.[5] The endoplasmic reticulum enzyme is inducible, responding to challenge by xenobiotics.[6,7]



(2)

2.2.2. Reductive Reactions. 2.2.2.1. Azo Reductase. Reductive reactions include two that are reductive cleavages and three that are direct reductions. Azo group reductions to amino compounds and disulfide reductions to thiols are similar in that they involve reductive cleavage (Figure 2), thereby unmasking amino and thiol groups. The azo group reductive cleavage mainly involves microsomal enzymes, NADPH-cytochrome P450 reductase and cytochrome P450.[8] However, large number of enzymes appear to be capable of catalyzing and contributing to the *in* vivo azo reduction of compounds, from microsomal NADPH-cytochrome P450 reductase and cytochrome P450, to cytosolic proteins like xanthine oxidase and DT-diaphorase. The reactions probably take place in oxygen-poor regions of cells and tissue, as demonstration of the microsomal activity requires anaerobic conditions.[9] Amaranth azo reductase activity is elevated by inducers of cytochrome P450, and carbon monoxide inhibits this P450-mediated azo reductase activity.[10] However, activity can be stimulated by addition of FMN or FAD, and such enhanced azoreductase activity is not CO sensitive.[9] Suggestions

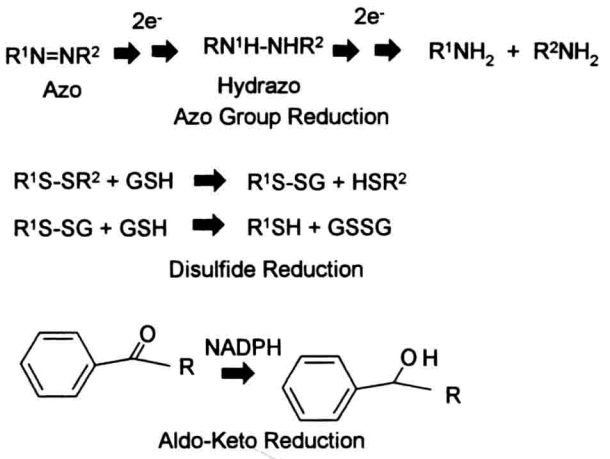


Figure 2. Reductive reactions.

have been made that an azo anion radical is an intermediate in the reduction and this is the oxygen sensitive species. Hydrazo metabolites (2 electron reduced) are also produced, and may be the result of disproportionations between azo anions.

2.2.2.2. Thioltransferase. Reductive cleavage of disulfides and thiosulfate esters appears to be the work of thioltransferases (glutathione:disulfide oxidoreductase) that use glutathione (GSH) as a co-reactant, reducing a disulfide to a thiol (R^2SH) and mixed disulfide with GSH (Figure 2). The resultant mixed disulfide then reacts with a second GSH to form oxidized glutathione and the thiol (R^1SH) of the second half of the disulfide.[11] In this manner drugs like disulfiram are inactivated. The reaction proceeds in two steps and results in formation of oxidized glutathione, the regeneration of which requires NADPH. Mixed small molecule disulfides, protein disulfides and mixed protein small molecule disulfides are all substrates of the different thioltransferases, also called glutaredoxins. Low molecular weight, cytosolic thioltransferases have been purified,[12] and at least one membranous thiol-disulfide exchange enzyme has been isolated which has also been called an insulin thioltransferase. These latter enzymes are thought to function in the repair of protein thiols to their natured state after oxidation by thiols and thiosulfates.

2.2.2.3. Aldo-Keto Reductases. Aldehyde- and ketone-containing chemicals are widely distributed in nature and many have diverse effects in the body. They are metabolized *in vivo* by a family of functional group-specific enzymes that recognize both endogenous and exogenous aldehydes and ketones.[13] Although the physiological roles of these enzymes remains to be established, many are thought to have developed as a means to detoxify reactive aldehydes. Substrates include many aromatic aldehydes and ketones (Figure 2), which are reduced by NADPH-dependent enzymes. Examples of xenobiotic substrates include the anticancer anthracyclines daunorubicin and doxyrubicin. The aldehyde sidechains of these are reduced by aldo-keto reductases. Similarly, the anti-clotting agent warfarin is a substrate for aldo-keto reductases. The enzymes are found in the cytosol of many tissue cells in the body. There is a superfamily of aldo-keto reductases (AKR), and a new nomenclature has just been devised for them.[14] The enzymes are monomeric proteins of 30–40 kDa that bind NADPH and have an extremely wide range of aliphatic and aromatic substrates. At least 39 different genes have been identified in rat, and these may be divided into seven different families, based upon amino acid sequence.[14] At least seven different forms have been identified in man and a similar number have been found in rat. The highest levels are found in liver and in kidney cortex, but enzymes are found in just about every tissue.[15] The enzymes have an interesting mechanism involving first binding of NADPH, followed by an isomerization of the enzyme. The enzyme is then able to accept a substrate and reduce it. Release of the reduction product is followed by isomerization of the enzyme again, and release of the oxidized pyridine nucleotide.[15]

2.2.2.4. Nitro Reductases. Among the reductive reactions that occur in the body are nitro group reductions. These are carried out by a very diverse group of enzymes in the body, including xanthine oxidase, several reductases, including NADPH-cytochrome P450 reductase, cytosolic dicumarol-sensitive NAD(P)H:quinone reductase, and cytochrome P450. The reaction can be viewed as a series of 1-electron transfers to the nitro group, yielding a nitro anion radical (Figure 3), which has been detected by EPR spectroscopy, then another 1 electron reduction to the nitroso intermediate. A third and a 4^{th} -electron reduce the metabolite to a hydronitroxide anion and then to hydroxylamine, respectively. A final two electrons form the 6-electron reduced amino compound.[16] In 1968, Gillette's

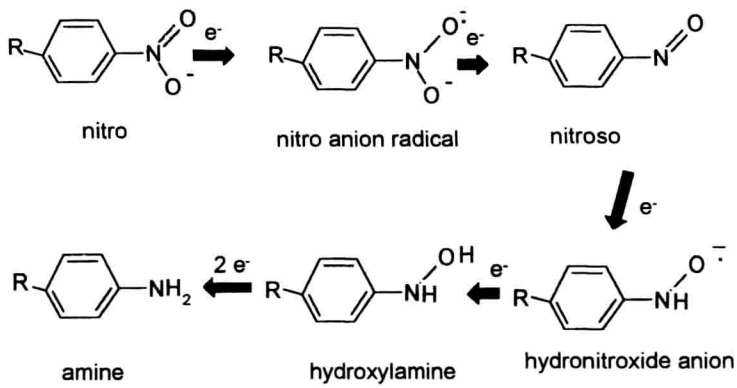


Figure 3. Nitro group reduction.

group isolated the nitroso and the hydroxylamine intermediates of nitrobenzene during formation of aminobenzoate from nitrobenzenzoate. A number of nitro compounds are drugs, or agricultural reagents such as parathion. Recently two additional cytosolic hepatic nitroreductases have been identified, one an NADH-dependent, dicumarol-insensitive enzyme, and the other a pyridine-nucleotide non-specific, dicoumarol-sensitive enzyme (quinone reductase; DT-diaphorase).[17] In fact, it was postulated that oxygen and the nitro group compete for binding to cytochrome P450. Early studies showed that with some compounds (not all) carbon monoxide was actually inhibitory of nitroreductase activity. As with azo reductase activity, it is only possible to carry out nitro group reduction under conditions of strict anaerobiosis, and it is suggested that the rate-limiting, oxygen sensitive step is formation of the nitro anion radical, and that other steps may not all be oxygen sensitive. This would also mean that, since reduced metabolites are found, they are probably produced in oxygen poor regions in the body tissues. Presently, reductive activation of nitrogen compounds to metabolites that bind to cellular DNA is being studied. Indications are that many such reactive intermediates are produced in the gut by nitro reductases of gut bacteria. Studies are also in progress[18] which identify oxygen poor regions of tissue by demonstration of nitro reductase activities (binding to tissue of labeled nitroimidizoles). To the extent that a nitro chemical may be metabolized by a form of cytochrome P450, nitroreductase activity will be elevated by compounds that induce that form of cytochrome P450. Drugs such as chloramphenicol and clonazepam are substrates of nitro reductases, based upon metabolites excreted in the urine. Intestinal microorganism also contain nitroreductases, and these organisms may play a role both in detoxifying xenobiotics, and in susceptibility of their hosts to toxic and carcinogenic effects of these agents.

2.2.2.5. Reductive Dehalogenation. It has been known since the 1940's that the mammalian system has the ability to cleave the carbon halogen bond, releasing halides. Halogenated anesthetics such as chloroform and halothane were once thought to be inert in the body. However, subsequent studies revealed a small proportion of these underwent dehalogenation. For example, the gaseous anesthetic halothane is dehalogenated, with some trifluoroacetic acid appearing in the urine. The reaction is catalyzed by cytochrome P450. The reaction (Figure 4) proceeds stepwise initially releasing bromide.[19] Two one-electron transfers to the halothane results in removal of bromide and chloride. In the absence of sufficient levels of oxygen reductive metabolites react with macromolecules such

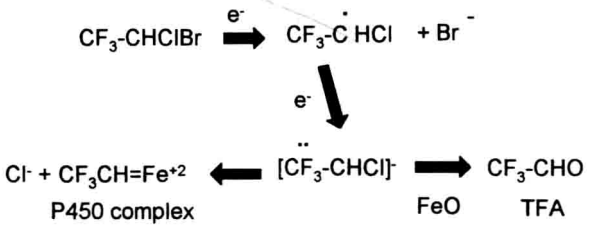


Figure 4. Reductive dehalogenation of Halothane.

as membrane phospholipids, or with cytochrome P450 (Figure 4), in the latter case forming a stable, reduced, inactive, spectrophotometrically visible complex.[19,20] Not all isozymes of cytochrome P450 form the spectrophotometrically observable complex with halothane metabolites, indicating non-reactive forms either do not metabolize this halocarbon or produce different metabolites. In the presence of oxygen the reaction is inhibited due to competition of oxygen for electrons. Carbon monoxide can also inhibit the reaction in microsomes, by stabilizing the reduced hemoprotein. At low oxygen tensions, trifluoracetaldehyde (TFA) can form after elimination of chloride (Figure 4) which then is oxidized by other enzymes in the body to the trifluoroacetic acid found in the urine. Further, studies using cytochrome $P450_{cam}$ have shown a 2-electron reduction, a 1:1 stoichiometry of NADH consumption and substrate consumption, yielding $ClCH_2CF_3$. With haloethanes the two electron transfer yielded haloalkenes, due to concomitant reduction and elimination of two vicinal halogens.[21] With $CFCl_3$ elimination of a chloride and formation of a proposed carbene marked the 2-electron transfer. This intermediate then releases a second chloride and undergoes hydrolysis to carbon monoxide, HF and HCl. Studies on the reductive dehalogenations of haloalkanes have indicated that the order of decreasing activity is I>Br>Cl>F. The reverse order appears to hold for halogenated anilines.[22] Formation of p-aminophenol from the 4-haloanilines had greatest V_{max} values with fluorine and lowest values with iodine.[22] This dehalogenation was suggested to be "oxidative dehalogenation", i.e., the result of attack by active oxygen. A likely mechanism might involve ionization of the halogen after formation of the imine, followed by binding of the active oxygen atom to the C4 carbon, yielding the quinoneimine. This would yield the aminophenol after subsequent reduction.

2.2.3. Oxidative Enzymes. Oxidative enzymes catalyze reactions that result in the appearance of new functional groups containing oxygen. A very wide number of diverse oxidative enzyme families are involved in the metabolism of drugs and other xenobiotics.

2.2.3.1. Flavin-Containing Monooxygenase. Flavin-containing monooxygenase (FMO) is a multigene family of enzymes that metabolize drugs and chemicals with nucleophilic heteroatom groups such as sulfur, nitrogen, phosphorus, or selenium[23] to their respective oxides. Attack does not occur on carbon atoms. Primary, secondary and tertiary amines are substrates of the flavin-containing monooxygenases (Table 3) and products of nitrogen attacks are amine oxides, hydroxylamines and oximes (Figure 5). With sulfur-containing substrates sulfoxides and sulfones are produced. Reaction with phosphines also occurs, as with phonophos or triphenylphosphine.[24] The gene family has almost as wide a range of substrates as the cytochrome P450 gene family. As with the cytochrome P450 enzymes, there appears to be a gender-specific expression of FMOs in rodents, and develop-

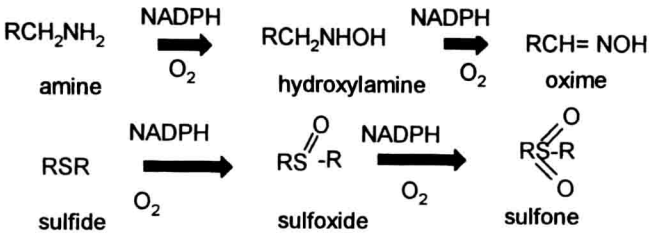


Figure 5. Substrate oxidation by flavin-containing monooxygenase.

mental changes in expression.[25] Human fetal liver expresses FMO1, while in adult liver FMO3 is the predominant form and a small amount of FMO4 is detected. FMO1 is, however, produced in the adult human kidney.[23] Unlike the cytochrome P450 enzymes, however, the enzymes are constitutively expressed and do not appear to be inducible by xenobiotics. The members of this family are membrane proteins, located in the endoplasmic reticulum of tissue, particularly in the liver and kidney and are markedly thermally labile. Five gene families have been identified in rabbit (FMO1-5) with amino acid sequences 50–58% identical between families.[25] Mammalian orthologous forms in another species are assigned based upon sequence identity of greater than 80% with the orthologous rabbit gene product.[23] It appears that each gene family has a single gene. Some forms are tissue specific. The enzymes exist in cells as a stable activated complex, formed by interaction with NADPH and oxygen.

Flavin monooxygenases have as a prosthetic a single FAD molecule. The enzyme binds NADPH (Figure 6) which is oxidized by the FAD prosthetic group. The $FADH_2$ then reacts with molecular oxygen at the 4α-position of the flavin. The activated oxygen form, the hydroperoxy flavin enzyme, is a stable entity in the absence of substrate, but which, in its presence, reacts with the substrate at a nucleophilic center releasing water, the oxidized substrate and the NADP. As noted by Beaty and Ballou,[26] the system is tightly coupled.

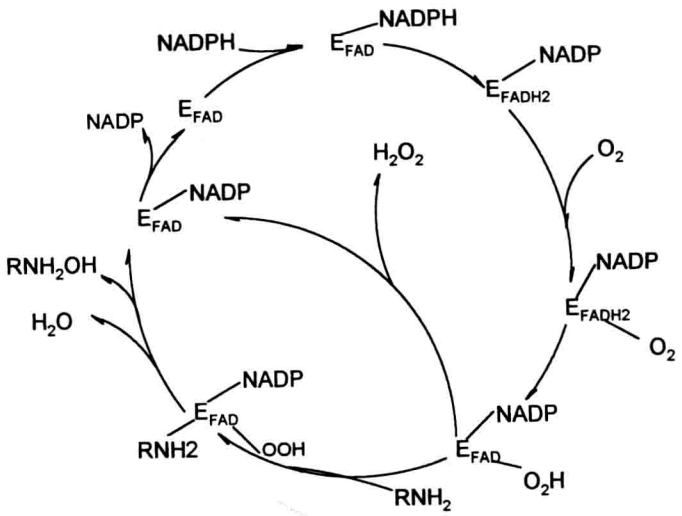


Figure 6. The flavin-containing monooxygenase cycle.