Enzymatic Oxidation of Xenobiotic Chemicals

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I. INTRODUCTION

The concept has been with us for some time that the mammalian body contains systems that transform chemicals that are not of endogenous origin. The term "xenobiotic" will be used to describe such materials in this review. For consideration of the early work in the area, the reader is directed to the classic monographs of Williams.[1,2] According to Williams,[2] Rouelle first isolated a hippuric acid (*N*-benzoylglycine) from the urine of cattle in 1784. In 1842, Keller[3] demonstrated that this had been formed by the conjugation of ingested benzoic acid with glycine. In 1867, Schultzen and Naunyn[4] demonstrated the oxidation of benzene to urinary phenol in dogs and men. Many other fundamental reactions of xenobiotic metabolism were discovered in Germany during the remainder of the 19th century. In 1879, Schmeideberg and Meyer characterized an excreted glucuronide, and studies in the laboratories of Baumann[5] and Jaffe[6] indicated the formation of mercapturic acids, which we now know arise from the degradation of glutathione conjugates. The concept that biotransformation of such xenobiotics can result in the production of electrophilic products and be detrimental has been with us for more than 40 years.[7] Over the years, the chemical and pharmaceutical industries have been able to prepare more and more compounds that may be ingested, and the field of xenobiotic biotransformation has grown considerably. New technologies have been applied to the study of the enzymes involved in the transformation of xenobiotics almost as soon as they became available, and the growing complexity of some of the research areas has led to an increase in the number of scientists involved.

The literature of the enzymes has been reviewed a number of times. The two-volume series of Jakoby[8] was a landmark that included reviews on many of the enzymes. A partial list of more recent reviews, monographs, and lead articles dealing with individual enzymes would include: cytochrome P-450,[9-13] epoxide hydrolase,[14-16] glutathione *S*-transferase,[17-19] UDP-glucuronosyl transferase,[20-23] *N*-acetyltransferase,[24-27] flavin-containing monooxygenase,[28,29] prostaglandin synthase,[30-32] quinone reductase,[33-35] alcohol dehydrogenase,[36,37] aldehyde and ketone dehydrogenases,[37-39] *N*-methyl transferase,[40-43] *O*-methyl transferase,[42] sulfotransferase,[44-46] cysteine conjugate β-lyase,[47-49] and monoamine oxidase.[50]

These are some of the major enzymes involved in the biotransformation of xenobiotics. The list is not totally inclusive, depending upon how one views the field of xenobiotic biochemistry. For instance, ingestion of certain chemicals can lead to stress related to the increased production of partially reduced species of oxygen, and enzymes such as superoxide dismutase, catalase, and glutathione peroxidase could be considered.[51] Transport proteins such as those in the P-170 family appear to be very important in the resistance of cancer cells to therapeutic treatment.[52-54] The enzymes involved in the biosynthesis of glutathione and the degradation of glutathione conjugates have been studied in part because of their relevance to questions about the toxicity of xenobiotics.[55,56] Metallothionein can serve in a protective role in complexing metals and electrophiles.[57] The enzymatic repair of DNA damage inflicted by UV light and xenobiotic chemicals is yet another important area.[58]

Obviously, comprehensive discussion of all of the enzymes that could be considered related to the metabolism of xenobiotics is impossible in the scope of a single review such as this. The decision has been made here to limit this discussion to essentially seven enzymes, all of which can oxidize xenobiotic chemicals. One of these is the prokaryotic *Pseudomonas oleovarans* ω-hydroxylase and the others — cytochrome P-450, prostaglandin H synthase, lignin peroxidase, flavin-containing monooxygenase, monoamine oxidase, and dopamine β-hydroxylase — are eukaryotic enzymes (cytochrome P-450 enzymes, as well as counterparts to some of the other enzymes, are also found in bacteria). Even though several of these enzymes have definite roles in the metabolism of endogenous compounds, much has been learned about them from studies with other chemicals and many parallels can be seen in oxidation mechanisms. Entire books have been written about some of these enzymes and it is not the purpose of this article to recapitulate all of the information. Several key aspects regarding each enzyme are treated, including the nature of protein multiplicity, the catalytic mechanism, and what the author feels are some of the key needs regarding gaps in the knowledge. An attempt is made to point out the similarities of some of these seemingly unrelated enzymes with respect to their catalytic specificity, and some speculation is made concerning potential roles and the significance of these enzymes.

II. GENERAL CONCEPTS

In several of the enzyme systems that will be dealt with, what actually exists is a family of related enzymes that have the same general function. The term "isozyme" will be avoided in referring to families of proteins that catalyze the same general type of reaction. Strictly speaking, "isozymes" refers to groups of enzymes that catalyze a single reaction of a single substrate — with most of the enzymes under consideration here, the focus of attention is the *different* reactions catalyzed by the various proteins. Within each group of enzymes, the chemistry

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involved in the reactions appears to be rather constant and the differences in catalytic specificity are largely a reflection of the shapes of the binding sites for the substrates (or, more accurately, the enzyme transition states). Ultimately, description of the three-dimensional nature of the binding sites will be necessary for the logical analysis of structure-function relationships.

Many of the xenobiotics are of interest because of their potential toxicity, whether due to effects of a compound itself or a product of biotransformation by one of the enzymes under consideration here. One would like to be able to group these enzymes into "good" and "bad" groups, since activities of many can be modulated by induction and inhibition. However, general conclusions about the positive and negative roles of individual enzymes in health cannot easily be made without provision for exceptions. Several examples will be pointed out later, but whether an enzyme has a beneficial or detrimental effect usually depends upon the xenobiotic that is presented to the organism.

Usually the goal of modulating activities of enzymes (which transform xenobiotics) is to reduce the potential toxicity of chemicals to cells. However, when anticancer therapeutic agents are administered, the goal is selective toxicity to a population of cells.[59-61] These cells have enzymes that protect them from these compounds and, in the situation of drug resistance, these enzymes may be elevated.[52-54] Thus, intervention to *increase* toxicity by manipulation of these enzymes is desirable, and the goal is to selectively achieve inhibition of the protective enzymes in the target cells.

Some of the mammalian enzymes that will be considered here are concentrated in the liver and others are localized in other tissues. Such tissue-specific expression has a number of implications. In some cases where a large amount of the enzyme under consideration is present in the liver, first-pass hepatic clearance may occur and rates of transformation of a xenobiotic may be limited only by the rate of blood flow (after administration by certain routes). Factors related to the production and covalent binding of electrophiles generated by biotransformation have been discussed elsewhere.[62,63] As the half-life of a reactive electrophile decreases, the importance of generation of the electrophile in the target tissue increases. When electrophiles of moderate stability are generated, they can migrate from the liver (or other tissue) to the target site before reacting. How rapidly can unstable electrophiles migrate? Studies with several compounds indicate that the bulk of the electrophiles can be trapped outside of the hepatocytes that generate them (*in vitro*).[64-69] Mutagenic benzo(a)pyrene products (primarily phenol glucuronides) are excreted from perfused liver.[70] Injection (ip) of (+) *anti*-7,8-dihydroxy-7,8-dihydrobenzo(*a*)pyrene ($t_{1/2}$ of 27 min at 25°C in neutral buffer)[71,72] produced a high incidence of lung adenomas in weanling mice.[73] The (−) enantiomer of the compound has a much shorter $t_{1/2}$ in neutral buffer (38 s at 25°C)[71] and, for whatever reason, is not really tumorigenic,[73,74] although even with the short $t_{1/2}$ it can enter cells and produce mutations.[75,76] Indeed, aflatoxin-8,9-epoxide (with a $t_{1/2}$ of <10 s in water) has been found to enter bacterial cells and produce a potent biological response as a result of DNA alkylation.[77]

What do changes in the levels and activities of xenobiotic-metabolizing enzymes mean in terms of *in vivo* physiological effects for humans? In the case of drug metabolism, many examples are known where the elevation or inhibition of an enzyme can have dramatic effects, particularly when another drug is administered. For instance, administration of the cytochrome P-450 ($P\text{-}450_{NF}$, IIIA4) inducers rifampicin and barbituates to humans increases rates of oxidation and clearance of drugs such as quinidine[78,79] and 17α-ethynylestradiol[80-82] — in the latter case, therapeutic ineffectiveness results. A deficiency in perhexiline hydroxylation (absence of $P\text{-}450_{DB}$, IID6) can result in neuropathy due to accumulation of the parent drug and exaggerated pharmacological response.[83]

Alterations of enzyme levels can also have effects on the metabolism of endogenous substances, at least in experimental animals. For instance, treatment of mice with 2,3,7,8-tetrachloro-*p*-dioxin or phenobarbital increases the activity of a UDP-glucuronosyl transferase toward thyroxine and thyroid hypertrophy can result.[84]

The relationship between levels of xenobiotic-metabolizing enzymes and cancer risk is hypothetical (for a review of roles for cytochrome P-450 see Reference 85). In animal models, some evidence for roles of enzyme inducers as co-carcinogens or chemopreventative agents exists, but in humans causal relationships, although attractive, have not been proven.

Why do mammals have relatively large amounts of enzymes that function in xenobiotic metabolism? Two major schools of thought exist. The first is that endogenous compounds are the "true" substrates for these enzymes. Thus, many of the enzymes under consideration can be shown to exhibit *in vitro* activity toward compounds normally found in mammals, e.g., cytochrome P-450 (fatty acids, fat-soluble vitamins, steroids, eicosanoids), glutathione *S*-transferase (leukotrienes, steroids, hydroperoxides), epoxide hydrolase (leukotrienes, lipid epoxides), monoamine oxidase (biogenic amines), methylases (histamine, indoles, catechols), UDP-glucuronosyl transferase (bilirubin, steroids), and sulfotransferases (steroids). Clearly, the roles of certain individual enzymes in physiological processes cannot be denied (cytochromes $P\text{-}450_{SCC}$ [XIA1], $P\text{-}450_{17\alpha}$ [XVIIA1], $P\text{-}450_{C\text{-}21}$ [XXIA1], $P\text{-}450_{11\beta}$ [XIB1], $P\text{-}450_{7\alpha}$, $P\text{-}450_{aromatase}$ [XIXA1], leukotriene A4 hydrolase, leukotriene A4:glutathione *S*-transferase, monoamine oxidases A and B, bilirubin UDP-glucuronyl transferase).[86-88] Most of these enzymes show little activity toward any other than their accepted substrates, however. Many of the enzymes that are active toward xenobiotics also use endogenous substrates, often with great regio- and stereo-selectivity (e.g., rat cytochrome $P\text{-}450_{PB\text{-}B}$ [IIB1]).[89] However, little physiological relevance can often

be attached to some of the transformations (other than a clearance mechanism), regardless of how selective they are. The low K_m values for some of the reactions with endogenous substrates should not necessarily be construed as evidence that the processes are important — many key enzymes in intermediary metabolism have relatively high K_m values and the view can be taken that regulation is more easily achieved with a higher K_m, particularly if small amounts of substrate are encountered.[90] Further, in the design of an enzyme, a lowered K_D results in an increased transition state energy peak and no increase in enzyme efficiency (alternatively, one can view the substrate competing with the transition state in the active site).

The other major school of thought is that organisms have these enzymes as part of an overall protective mechanism. This view has been espoused by Jakoby[91] and by Nebert and Gonzalez.[11] Many natural products, particularly those derived from plants, are potentially toxic and may actually be consumed at high levels (for review see Reference 92). Thus, a series of different enzymes within each multi-gene or super-gene family, having a distinct range of catalytic specificity, could provide protection from a wide variety of potential toxicants. As with endogenous substrates, the high degree of regioselectivity often seen (especially with the cytochrome P-450 enzymes) might be largely incidental. An interesting venture would involve identification of the enzymes involved in the metabolism of some of the more noxious toxic principles in common foods.[92] It is of interest to note that humans deficient in cytochrome $P\text{-}450_{DB}$ (IID6) have been reported to have aversions to certain foods (e.g., eggplant).[534] On the other hand, it may be possible that some of the individual enzymes are present to process certain natural products that no longer exist and cannot be identified. If certain enzymes are present for protection, though, how can they have roles in the activation of drugs and carcinogens? Obviously, most of the drugs and carcinogens are of relatively recent origin. The situations in which potentially dangerous electrophiles are generated could be considered mistakes that are not common enough to exert selective pressure. It should also be pointed out that one would not suspect genetic pressure against tumor induction, since cancers usually occur after peak reproductive age.

There is probably some validity in both schools of thought when we consider the enzymes of xenobiotic metabolism as a whole. The point should be made that individual animals and humans can exist apparently quite well without several of the enzymes under consideration, so long as they do not encounter certain xenobiotics that will be detrimental (e.g., see References 13 and 93). Another point of interest is the recent demonstration of the presence of the alkaloids codeine and morphine in mammals.[94] These compounds, which may be important in pain suppression, have long been known to be substrates for some of the enzymes under consideration here (e.g., cytochrome P-450, UDP-glucuronosyl transferase). It is possible that small amounts of other alkaloids of this type will be found *in vivo* and that the enzymes we consider under the heading "xenobiotic metabolizing" may have important roles in regulation of their levels. Further, cytochrome P-450 has recently been found to hydroxylate the cyclic peptide cyclosporin, an immunosuppresive agent of fungal origin;[95,96] conceivably, other highly active peptides of endogenous origin may prove to be substrates for cytochromes P-450 and other xenobiotic-metabolizing enzymes.

Finally, before starting the discussion of individual enzymes, the point should be made that each group of enzymes (multigene or super-gene family) is complex with respect to its catalytic specificity and regulation. The complexity is often accentuated when the enzymes in several experimental animals and humans are all considered. In the future, a challenge will be deriving detailed information about each protein while keeping all of our knowledge in perspective. No longer is it possible for an individual to work on details of all aspects of some of these enzymes, and keeping abreast of the literature has become a formidable task. Nevertheless, important questions remain to be answered and exciting new approaches are now available.

III. CYTOCHROME P-450

A. General Features

Cytochrome P-450 enzymes (EC 1.14.14.1) are defined by their absorption spectra. (The Enzyme Commission[97] uses the term "unspecific monooxygenase" to describe all of the enzymes in this group. In a sense, the term "cytochrome" is a misnomer for these enzymes in that electrons are not transferred onto another acceptor (other than O_2). Since the term "cytochrome" has been used to desribe these enzymes in essentially every paper written about them, it will also be applied here, if for no other reason, for indexing purposes. However, some appear classified under specific reactions as well. The characteristic absorption maximum seen near 450 nm for the Fe^{2+}-CO complex is characteristic of the axial thiolate ligation provided by a protein cysteinyl residue.[98,99] The molecular weights of all cytochrome P-450 enzymes characterized to date fall in the range of 45,000 to 60,000. More than 100 primary sequences have now been elucidated, primarily with the use of recombinant DNA techniques.[100] The sequences show some regions of similarity, particularly in the area of the cysteine thiolate ligand for the heme. Other comparisons among the sequences can be made,[101] although the functional significance of these regions is not very clear at this time. The reactions catalyzed by cytochrome P-450 enzymes are typically mixed-function oxidations:

$$S + NAD(P)H + O_2 \rightarrow SO + NAD(P)^+ + H_2O$$

where S is the substrate and SO is the (oxidized) product. In many cases, the product shows rearrangement or there may be variants on the mechanism such that the reaction may not be

so obvious. Also, Ullrich and co-workers[102,103] have identified two cytochrome P-450 enzymes that do not function in the typical mode of utilizing electrons and molecular oxygen; these enzymes utilize oxygenated substrates and achieve specific rearrangements to form thromboxanes and prostacyclins. Nevertheless, the enzyme reactions can be rationalized in terms of chemistry similar to the other reactions. In addition, the fungal enzyme chloroperoxidase (EC 1.11.1.10, chloride peroxidase) can be considered a cytochrome P-450 by a spectral definition.[104,105] Although chloroperoxidase displays the unusual property of oxidizing chloride ions, it has also been shown to carry out many typical cytochrome P-450 reactions and has many properties that resemble cytochrome P-450 more than other peroxidases.[106-111]

Within each mammalian species there are probably more than 20 cytochrome P-450 enzymes expressed, and much effort has been put into the enzymology. With the availability of sequence comparisons, the cytochrome P-450 enzymes have been classified into groups of a so-called "gene superfamily".[100,112] Primary structures with >80% sequence conservation are grouped into a subfamily, those with 50 to 80% conservation lie in the same family, and those with <50% conservation are only included in the overall superfamily. With the studies on enzyme purification being carried out in many different laboratories, a series of different trivial names developed for some of the cytochrome P-450 enzymes — the reader is referred to references that compare these individual proteins.[10,12] Efforts to categorize the cytochrome P-450 enzymes on the basis of primary sequences have been useful.[100,112] However, three points should be borne in mind: (1) in many cases, cDNAs have been isolated and sequenced, but the proteins are unknown — thus, no information regarding function is available; (2) in some of the more complex gene families, both cDNA sequences and functional proteins are available, but uncertainty still exists concerning matches between cDNAs and individual enzymes with distinct catalytic activities; (3) the gene classification covers all animal species, but the orthologous (related) proteins expressed in different animals may have quite different catalytic properties. Several examples will be mentioned later.

Two major systems exist for electron transport to the cytochrome P-450 enzymes. In bacteria and mitochondria, electrons are passed through a flavoprotein reductase and then an iron-sulfur protein of the ferredoxin-type to the cytochrome P-450. With the eukaryotic microsomal cytochrome P-450 enzymes, electrons from NADPH are delivered via a flavoprotein (NADPH-cytochrome P-450 reductase, EC 1.6.2.4, NADPH-ferrihemoprotein reductase), directly to the cytochrome P-450. The flavoprotein contains one molecule each of FMN and FAD. Recently, Fulco and co-workers have characterized a *Bacillus megaterium* enzyme that contains such a reductase and cytochrome P-450 linked together, as domains of a single protein,[113,114] in the same general manner as a chimeric fusion protein created by Murakami et al.[115] As we will see later, it is possible in some instances to replace these electron transport chains (and O_2) by surrogate oxygen donors, such as iodosylbenzene and hydroperoxides, and some inferences about the catalytic mechanism of cytochrome P-450 have been made from such studies.[116] Although hydroperoxides can be found in cells, it is generally agreed that the physiological relevance of hydroperoxide-supported cytochrome P-450 oxidations is nil.

Obviously, there are many facets of cytochrome P-450 research that could be discussed. For recent reviews on the enzymology,[12] structure,[117] regulation,[11,13] and relevance[12] of the cytochrome P-450 enzymes, the reader is referred elsewhere.

B. Endogenous Substrates

Endogenous cytochrome P-450 substrates include fatty acids, steroids, eicosanoids, and fat-soluble vitamins.[86] In some cases the significance of the reactions is quite clear. However, in other cases no clear metabolic role for a particular reaction is apparent. As pointed out in the general discussion about roles of enzymes involved in biotransformation of xenobiotics, the possibility exists that the reactions observed do not have any physiological significance. In this reaction, I briefly present two areas that are usually not considered in such discussions.

Recently, morphine has been detected in toad skin, beef brain and adrenal gland, and several other mammalian tissues.[94,118,119] The precursors codeine and thebaine have also been found in tissues of animals that were not exposed to related materials.[120] Rat tissues can convert the precursor reticuline to salutaridine,[121] and rat liver cytochrome P-450 enzymes catalyze the *O*-demethylation of thebaine and codeine to morphine.[122] The specific cytochrome P-450 has not been identified. One can hypothesize that some cytochrome P-450 enzymes exist for the express purpose of generating small quantities of pain-suppressing alkaloids. It might be possible that other alkaloids exist with highly specific roles.

Another possibility for cytochrome P-450 function that can be considered is that of peptide metabolism. Recently, the oxidation of the cyclic peptide cyclosporin A has been shown to be mediated by human liver cytochrome $P\text{-}450_{NF}$ (IIIA4) or a very closely related enzyme.[95,96] At least three products (of side chain oxidation) appear to be formed. These reactions appear to be significant in that conditions that elevate cytochrome $P\text{-}450_{NF}$[93] decrease the immunosuppressive effects of cyclosporin and *in vivo* inhibition of cytochrome $P\text{-}450_{NF}$ enhances the pharmacological effects.[123-125] Cyclosporin A has a molecular weight of 1201 and appears to be the largest cytochrome P-450 substrate known to date. This is a rather hydrophobic compound; however, larger peptides might possess hydrophobic regions that could occupy the substrate binding site of a cytochrome P-450. What is not clear is how cyclosporin A interacts with the binding site of cytochrome $P\text{-}450_{NF}$, which can show considerable regio- and stereospecificity in

the oxidation of much smaller molecules.[126-128] Only a portion of the peptide might be inserted. Oxidation does not appear to play a major role in the metabolism of peptides used to date for therapeutic use, but important transformations might have been overlooked.

C. Extramicrosomal Cytochrome P-450

For many years, the primary location of cytochrome P-450 in the liver and most tissues has been recognized to be the endoplasmic reticulum. However, cytochrome P-450 enzymes are also found in other locations and have been the source of considerable interest.

In the mid-1970s, numerous papers appeared describing nuclear cytochrome P-450s.[129-133] One reason for the interest in nuclear cytochrome P-450 was the feeling that reactive metabolites (of pro-carcinogens) generated in the region of the endoplasmic reticulum would be too unstable to migrate to chromatin in the nucleus to bind DNA. One of the problems associated with the study of nuclear cytochrome P-450 is that the endoplasmic reticulum is continuous with the nuclear membrane and homogenization of cells breaks off fragments of endoplasmic reticulum with nuclei, so that estimating the degree of microsomal contamination in nuclear preparations is difficult. Although one report argues for the localization of cytochrome P-450 in the nucleolus of *isolated* nuclear preparations,[134] all immunohistochemical localization studies with intact sections have shown staining for cytochrome P-450 enzymes *only* in the outer nuclear membrane.[135] This view of nuclear localization also finds support in the fractionation study of Fahl et al.[136] To summarize the many papers on nuclear cytochrome P-450, it appears that some cytochrome P-450 is present in the nuclear membrane because of its continuous nature with the endoplasmic reticulum. A number of lines of investigation indicate that many electrophilic metabolites are capable of migrating throughout and out of cells rapidly and can readily alkylate nuclear DNA (for a review see Reference 63).

More recently, the subject of mitochondrial cytochrome P-450 has attracted interest. For many years, the mitochondrial location of cytochrome P-450$_{SCC}$ (the cholesterol side-chain cleavage enzyme, XIA1) and cytochrome P-450$_{11\beta}$ (XIB1) has been recognized (in steroidogenic tissues).[87] In addition, the cytochrome P-450 vitamin D_3 1α-, 24-, and 25-hydroxylase activities are localized in kidney mitochondria.[137-140] The liver is known to have ferredoxin-type proteins,[141,142] and such iron-sulfur proteins are the electron donors to cytochrome P-450 in bacteria and mitochondria. Cholesterol 25-hydroxylation is also known to occur in mitochondria.[143] However, the metabolism of xenobiotics in mitochondria and the presence there of cytochrome P-450 enzymes related to those in the endoplasmic reticulum until recently received little consideration.

Niranjan et al.[144] prepared rat liver mitoplasts that had <1% contamination with microsomal markers and found 25% of the specific content of cytochrome P-450 found in microsomes (nmol cytochrome P-450 per milligram protein). The level of cytochrome P-450 in these mitoplasts was induced by treatment of rats with phenobarbital or 3-methylcholanthrene, classic inducers of microsomal cytochrome P-450, and such treatment shifted the wavelength maxima of the ferrous-CO complexes of the microsomes. The mitoplast preparations were shown to oxidize benzo(*a*)pyrene, *N,N*-dimethylnitrosamine, and aflatoxin B_1; the catalytic activities could be supported by tricarboxylic acid cycle intermediates, evidence that the reactions could not be attributed to microsomal contamination. In an extension of this work, Niranjan et al.[145] showed that the polycyclic hydrocarbon-induced protein reacted with a polyclonal antibody raised against rat liver microsomal cytochrome P-450$_{\beta NF\text{-}B}$ (P-450c, IA1) and had the same apparent monomeric M_r (54,000). *In vitro* translation experiments indicated that a precursor protein of 57,000 Da was apparently being synthesized and cleaved to give the mitochondrial product. The mitochondrial protein and rat liver microsomal cytochrome P-450$_{\beta NF\text{-}B}$ had the same molecular weight and similar peptide maps,[146] but differed in pI (as judged by isoelectric focusing). Although polyclonal antibodies raised against rat microsomal cytochrome P-450$_{\beta NF\text{-}B}$ recognized this mitochondrial protein (termed cytochrome P-450$_{mt\text{-}2}$), a monoclonal antibody (the 1-7-1 clone of Park et al.[147]) recognized microsomal cytochrome P-450$_{\beta NF\text{-}B}$, but not mitochondrial cytochrome P-450$_{mt\text{-}2}$. Unfortunately, attempts to determine the N-terminal amino acid sequence of cytochrome P-450$_{mt\text{-}2}$ were unsuccessful, possibly because of posttranslational modification.[146] Another mitochondrial protein, cytochrome P-450$_{mt\text{-}1}$, was also purified from livers of rats treated with the inducer β-naphthoflavone (which has inducing properties similar to 3-methylcholanthrene). This protein had an apparent monomeric M_r of 52,000 and is clearly distinct from cytochrome P-450$_{mt\text{-}1}$ (its relationship to cytochrome P-450$_{ISF\text{-}G}$ [IA2] was not explored). Both cytochrome P-450$_{mt\text{-}1}$ and cytochrome P-450$_{mt\text{-}2}$ could be coupled with an electron transport system consisting of beef adrenal ferredoxin and ferredoxin reductase to reconstitute catalytic activity, but microsomal cytochrome P-450$_{\beta NF\text{-}B}$ could not. Both cytochrome P-450$_{mt\text{-}1}$ and cytochrome P-450$_{mt\text{-}2}$ were effective in catalyzing cholesterol 25- and 26-hydroxylation and vitamin D 25-hydroxylation, but microsomal cytochrome P-450$_{\beta NF\text{-}B}$ was not. However, when coupled with (microsomal) NADPH-cytochrome P-450 reductase, the two mitochondrial cytochrome P-450 enzymes were only one tenth as effective as microsomal cytochrome P-450$_{\beta NF\text{-}B}$ in catalyzing the oxidation of benzo(*a*)pyrene or 7,12-dimethylbenz(*a*)anthracene.

Honkakoski et al.[148] compared DBA/2N mouse liver mitochondrial and microsomal coumarin 7-hydroxylases, utilizing the purified microsomal enzyme and antibodies raised to it. In these mice, the specific mitochondrial coumarin 7-hydroxylase activity is 55% of that in the microsomes. The mitochondrial and microsomal cytochrome P-450 enzymes were equivalent,

as judged by apparent monomeric M_r and immunochemical and catalytic properties. However, only the mitochondrial cytochrome P-450 could efficiently utilize electrons from a ferredoxin/ferredoxin reductase system.

All of these results raise interesting questions about the identity of the mitochondrial proteins. It is especially intriguing that the monoclonal antibody did not react with cytochrome P-$450_{mt\text{-}2}$ in the study of Niranjan et al.,[145] since M_rs of the proteins were very similar. The size of the so-called CYP1A gene family is limited in rats and mice and there are not really many possibilities unless some type of alternate reading or processing were to occur. The interesting possibility has been raised by Honkakoski et al.[148] that some posttranslational modification related to transport into the mitochondria may change the electron transfer interactions of cytochrome P-450 (i.e., from recognizing NADPH-cytochrome P-450 reductase to the ferredoxin). Such a posttranslational modification might also explain the loss of reactivity of cytochrome P-$450_{mt\text{-}2}$ with monoclonal antibody 1-7-1 (while retaining reactivity with the polyclonal antibody). If such posttranslational modifications exist in these proteins, their characterization could prove very useful in understanding general features of electron transport to cytochrome P-450 enzymes.

D. Catalytic Mechanism

A general mechanism for microsomal cytochrome P-450 catalysis is depicted in Figure 1. Mechanisms have been reviewed in more detail elsewhere.[9,149-151] Much earlier discussion of oxidation mechanisms focused on mobile oxidizing species,[152-155] but now it is clear that the reactions are best understood in terms of hypervalent Fe-O complexes and nonconcerted reactions.

The scheme for the entire reaction is reviewed briefly. The bacterial cytochrome P-450_{cam} (CI) has been more amenable to analysis[156,157] and has served as a generally useful model for the microsomal enzymes, although the pathway of electron import differs and there are some other features that may be peculiar to the system. Substrate binding (step 1) is thought to be very rapid.[149,158,159] Binding of substrate to cytochrome P-450 has the effect of changing the iron spin-state (d electrons) from low spin to high spin in some cases, but not in others[160] (and some cytochrome P-450 enzymes already exist in the high-spin state without the addition of substrate).[161,162] Cytochrome P-450 reduction (step 1) tends to be more rapid after the substrate is bound.[149,158,163] This faster rate of reduction is not, however, generally correlated with the iron spin-state;[163] furthermore, the oxidation-reduction potential for the reduction (~ -300 mV vs. E_h) is not correlated to the spin-state with most of the mammalian cytochrome P-450s.[161,164,165]

Binding of molecular oxygen to ferrous cytochrome P-450 (step 3) is thought to be rapid[149,158] and the unstable [$Fe^{II}O_2$] complex can be observed transiently.[166-168] Evidence for a role

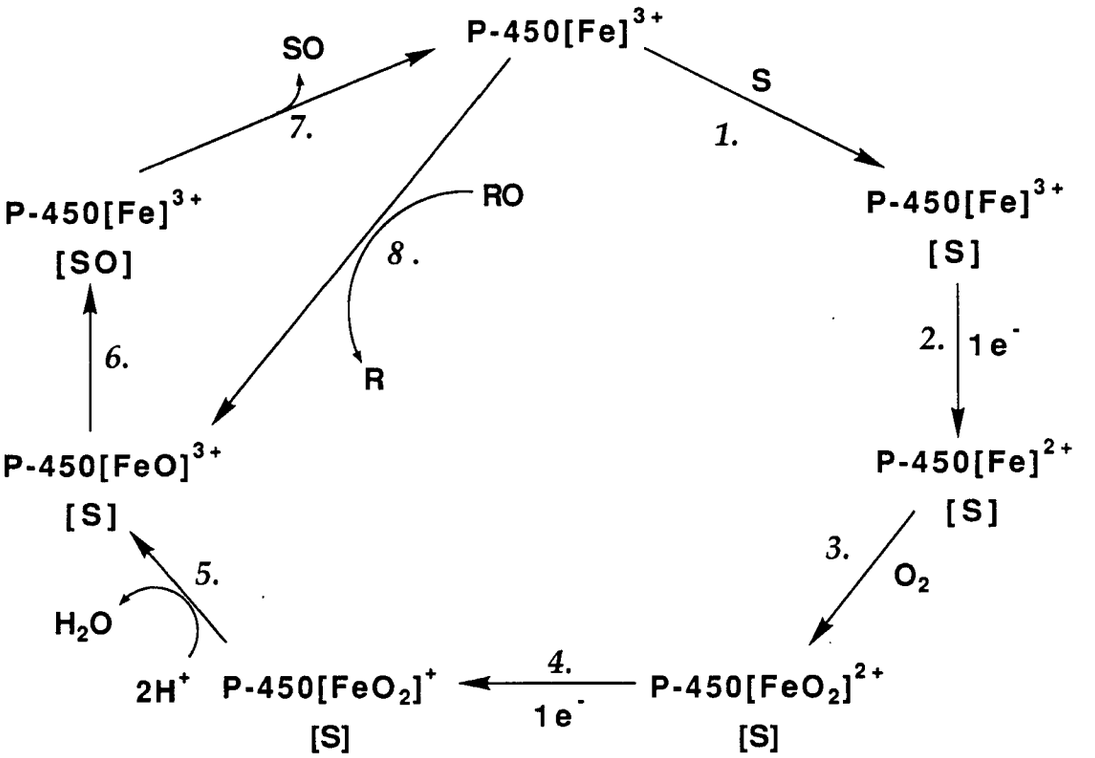

FIGURE 1. Catalytic mechanisms for cytochrome P-450 function.

of cytochrome b_5 in the stimulation of cytochrome P-450 reactions was first provided by Hildebrandt and Estabrook.[169] Perhaps the most compelling evidence for a role for cytochrome b_5 comes from studies in which certain cytochrome P-450-catalyzed reactions can be strongly inhibited by anti-cytochrome b_5 in rat[170] and human[127,171] liver microsomes. The mechanism is now generally thought to involve electron transfer from ferrous cytochrome b_5 in step 4, for the oxidation-reduction potentials for the two couples are both estimated to be near 0 mV (vs. E_h).[161] Pompon[172] has proposed that the potential for reduction of the $(Fe\text{-}O_2)^{2+}$ complex may be sensitive to the subtle influences of different apoprotein structures and substrates and that in some cases step 4 may be reversible, with ferric cytochrome b_5 serving as an oxidant. This hypothesis may explain why some cytochrome P-450-associated catalytic activities are stimulated by cytochrome b_5 while others are inhibited.

Information concerning the intermediates related to steps 5 and 6 is mainly available from model systems. A main function of the cysteinyl thiolate ligand is thought to be assistance in the heterolytic cleavage of the O-O bond to yield the $(FeO)^{3+}$ complex,[173] although possibilities for additional roles have also been considered.[159] Step 6 is expanded below in Figure 2, where the general mechanism of oxidation involves initial abstraction of a hydrogen atom or an electron (π or nonbonded), followed by rapid oxygen rebound to the incipient (and very short-lived) radical (radical recombination). Aspects of step 6 are considered in more detail later.

In the final step (7) of Figure 1, substrate binding completes the reaction. Rough calculations[159] and the results of kinetic deuterium isotope effect studies[174] suggest that this is generally a relatively rapid process, except perhaps in certain cases where the affinity of the product is greater than that of the substrate and a sequential series of steps on the same substrate is performed (cytochrome $P\text{-}450_{SCC}$ [XIA1], cytochrome $P\text{-}450_{aromatase}$ [XIXA1]).[175]

What limits rates of cytochrome P-450-catalyzed reactions? The evidence would suggest that different reactions (catalyzed by different cytochrome P-450 enzymes) may have different rate-limiting steps.[149] Rates of some microsomal reactions, but not others, can be increased when more exogenous NADPH-cytochrome P-450 reductase is added.[176,177] Furthermore, rates of iodosylbenzene-supported reactions are often faster than the same ones supported by NADPH-cytochrome P-450 reductase.[159,178] These observations indicate that reduction must be limiting in some cases. In some situations, rather large kinetic deuterium isotope effects are seen, even in noncompetitive experiments,[174,179] and indicate that C-H bond breaking must limit rates; furthermore, the dependence of rates of *N*-demethylation upon the ease of aminium radical formation likewise argues for nonbonded electron abstraction.[159,180] Thus, various steps may contribute to rate limitation in different situations.

In 1983, we wrote a review describing the catalytic mechanism of cytochrome P-450 and made two major proposals:[9] (1) cytochrome P-450 reactions can be viewed in the light that the formal $(FeO)^{3+}$ species acts a sort of electrode to abstract either electrons or hydrogen atoms, and the second phase of each reaction can be understood in terms of an oxygen rebound or radical recombination event; and (2) cytochrome P-450 reactions can be understood in terms of a small number of categories of related reactions, which include (a) carbon hydroxylation, (b) heteroatom oxygenation, (c) heteroatom release (dealkylation), (d) epoxidation, (e) oxidative group migration, and (f) suicidal inactivation of cytochrome P-450 via porphyrin *N*-alkylation (Figure 2). Since that time, the catalytic mechanism of cytochrome P-450 has been reviewed by Ortiz de Montellano,[150] and implications of work with model metalloporphyrins to cytochrome P-450 have been discussed by McMurry and Groves.[116] Some implications of biomimetic models to cytochrome P-450 are also discussed later in this article. At this point, new information arising from experiments with the enzymes will be discussed.

1. Carbon Hydroxylation

The view that hydrogen atom abstraction occurs and is followed by oxygen rebound continues to be accepted. Although corrections were not made for kinetic isotope effects, the work of Shapiro et al.[181] with microsomes provides evidence that extensive scrambling of the labeled hydrogens on a methyl carbon occurs during hydroxylation. In similar studies, White et al.[182] found extensive scrambling of the methylene stereochemistry in the benzylic hydroxylation of ethylbenzene by rabbit cytochrome P-450 2 (IIB4) — about one third of the alcohol showed a configuration opposite that of the original hydrocarbon substrate. These results are consistent with a mechanism in which an intermediate with trigonal character is formed, followed by oxygen rebound. Ortiz de Montellano and Stearns[183] examined the hydroxylation of a series of cyclopropyl hydrocarbons and only observed a small amount of rearranged product in one case (bicyclo[2.1.0]pentane). These studies were interpreted to mean that the radical pair ($C^{\bullet}/Fe^{IV}OH$) must collapse (with stereochemical specificity) at a rate $>10^9\ s^{-1}$, although caveats should be considered regarding constraints in the active site that may prevent the ring opening of radicals derived from some substrates. The time constant for the rotation of an unhindered methyl group is on the order of $10^{10}\ s^{-1}$,[184] and extensive rehybridization of the radical must occur to explain the results of Shapiro et al.[181] and White et al.[182] Moreover, ring expansion has been observed in the case of aminium radicals (*vide infra*).

Another interesting set of experiments was carried out by Stearns and Ortiz de Montellano on the oxidation of strained hydrocarbons.[185] Quadricyclane and certain other compounds have unusually low potentials ($E_{1/2}$) for 1-electron oxidation (~0.94 vs. SCE), and different products are diagnostic of pathways involving abstraction of a hydrogen atom or electron.

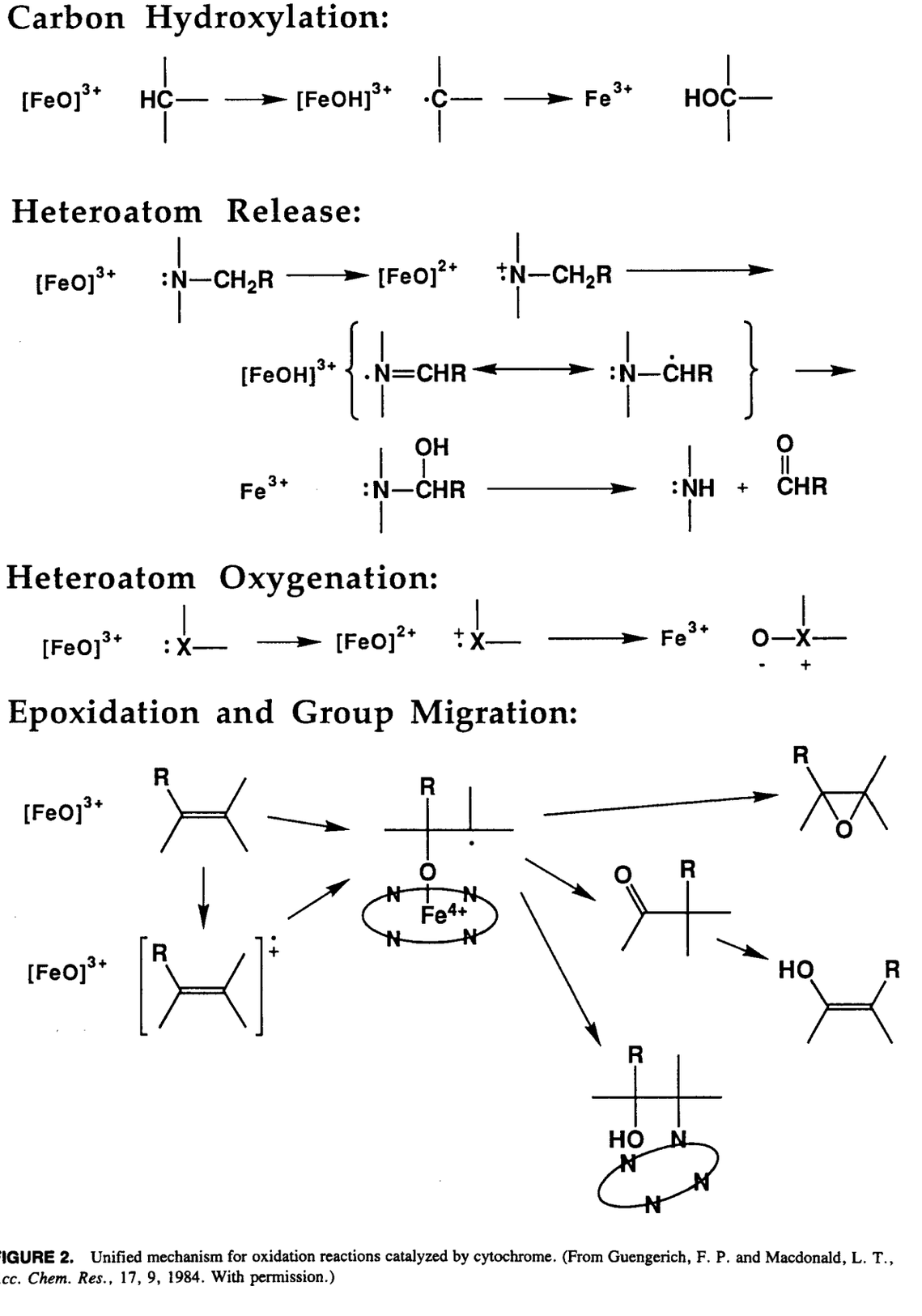

FIGURE 2. Unified mechanism for oxidation reactions catalyzed by cytochrome. (From Guengerich, F. P. and Macdonald, L. T., *Acc. Chem. Res.*, 17, 9, 1984. With permission.)

Products diagnostic of 1-electron oxidation were found. These and other considerations regarding heteroatom release (*vide infra*) suggest that cytochrome P-450 enzymes are quite proficient at abstracting an electron from a substrate with a low oxidation potential, even at carbon. As the potential of a substrate increases, hydrogen atom abstraction becomes a dominant mode of oxidation. Of course, one should bear in mind that the effective free energy difference to consider is a function of the interatomic distance between the reaction centers (site of oxidation on the substrate and the $(FeO)^{3+}$ complex).

One other point of relevance to carbon hydroxylation should be noted. It has now been demonstrated that cytochrome P-450 enzymes oxidize some alkanes to olefins without the intermediacy of alcohols. Such reactions have been observed with the substrates testosterone[186] and valproic acid.[187] A similar situation may occur in the formation of dehydrowarfarin, although control experiments with the tertiary alcohol have not been carried out.[188] The chemistry can be understood in the same general terms as other reactions but, instead of oxygen rebound, abstraction of a second hydrogen atom occurs (whether as a hydrogen atom or an electron and proton) (Figure 3).

With valproic acid and testosterone, there is partitioning between hydroxylation and desaturation, with the tendency toward the former. What the factors are that influence the extent of this reaction is not yet clear.

2. Heteroatom Release

A large body of evidence supports the view that heteroatom release with amines (*N*-dealkylation) involves sequential 1-electron transfer reactions (Figure 4): Evidence for this view includes the incorporation of ^{18}O (from $^{18}O_2$) into the carbonyl function,[189-192] arguments against *N*-oxide intermediates in *N*-dealkylation,[193] dependence of the rate on the amine oxidation potential,[159,180] cycloalkyl ring opening,[194-196] low kinetic deuterium isotope effects,[197,198] and the loss of alkyl radicals from 1,4-dihydropyridines.[199] In cytochrome P-450 reactions sup-

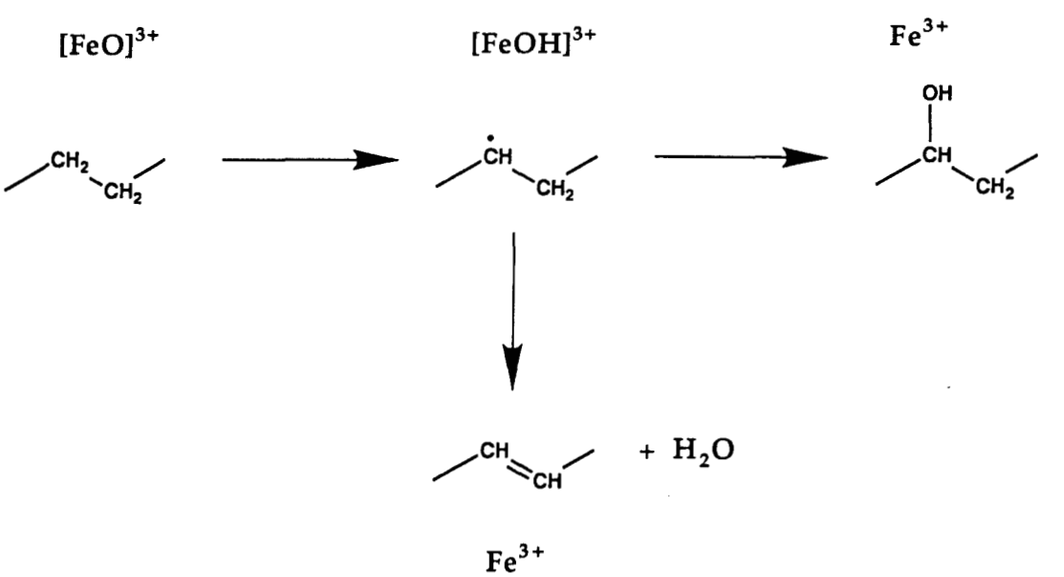

FIGURE 3. Desaturation of alkanes by cytochrome P-450.

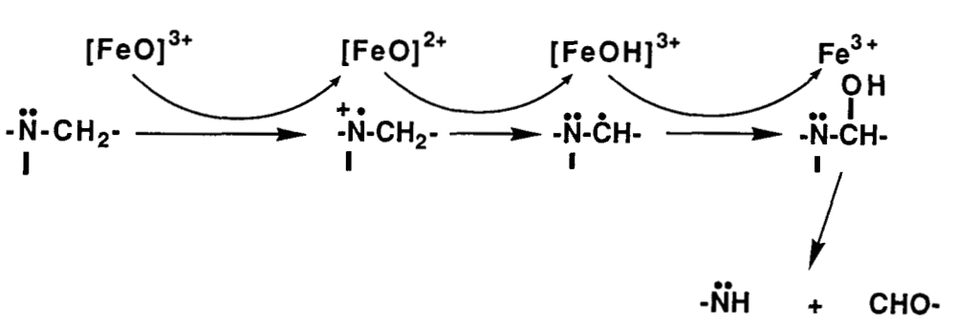

FIGURE 4. Postulated scheme for *N*-dealkylation reactions catalyzed by cytochrome P-450 enzymes.

ported with alkyl hydroperoxides,[200,201] mechanisms can involve free radicals, probably due to the homolytic scission of the alkyl hydroperoxide (Figure 5).

In such a mechanism, oxygen rebound does not occur and the oxygen in the carbonyl will be derived from H_2O, which is not the case in the situation of the cytochrome P-450 reaction supported by NADPH-cytochrome P-450 reductase.

As heteroatoms with higher $E_{1/2}$ values are encountered, cytochrome P-450 has a tendency to resort to hydrogen abstraction mechanisms as a viable alternative. Of course, steric constraints allow such a choice only when the substrate is of such a size and shape to fit in more than one way into the catalytic site. Thus, the view of sequential 1-electron transfer is restricted largely to nitrogen and sulfur compounds and some ethers.[195] In some cases, sulfur dealkylation and oxygenation are both seen, and the extensive studies of Oae and Iyanagi have provided evidence that the same mechanistic formulations as invoked for nitrogen compounds are probably correct.[201a-e]

Desaturation of alkanes appears to be a relatively minor pathway, but in the case of dihydropyridines such dehydrogenation is a predominant reaction.[199] In contrast to the oxidation of pyridine nucleotides by classic dehydrogenases, such dehydrogenations appear to be driven by single electron transfer processes, as judged by several lines of investigation.[126,202,203] Augusto et al.[199] demonstrated the release of 4-alkyl groups as radicals, and both the release of the proton and the alkyl group at the 4-position are described by low kinetic deuterium isotope effects.[202-204]

A considerable body of literature now supports the view that the intrinsic kinetic deuterium isotope effects accompanying cytochrome P-450 reactions that proceed via hydrogen abstraction are large (6 to 13), while the loss of hydrogen in a reaction in which the formation of an aminium (or related heteroatomic) radical should only proceed with a low isotope effect (~2).[116,150,197,198] The low kinetic deuterium isotope effects observed for dehydrogenation of 1,4-dihydropyridines provides evidence for a role of aminium radicals.[202] The dehydrogenation of these dihydropyridines is somewhat unusual in that it resembles a peroxidase reaction (which is also true of alkane desaturation, although peroxidases do not carry these out). The evidence suggests that hydroxylation per se does not occur.[126,199,202,203] Thus, the overall process consists of the net removal of two electrons and one proton, regardless of the concertedness of the transfers. Several possibilities exist (Figure 6) — the low intrinsic kinetic deuterium isotope effects ($\leq$2.9)[202] argue that C-4 hydrogen abstraction is not occurring, for the precedent with alkanes is that this step should display a high isotope effect.

A sequential 1-electron/proton/1-electron pathway is preferred, which does have precedent in model studies.[205-207] The oxidation potential for removal of an electron from the nitrogen is certainly within the range of cytochrome P-450.[205] As we will see later, this is the same formal mechanism whereby peroxidases carry out amine dealkylation. However, peroxidases do this with rather high kinetic deuterium isotope effects. The reason for the difference may be that a specific base exists in cytochrome P-450 to facilitate the removal of the α-proton. Although the loss of the proton in the 9-position of the *N*-methyl acridan aminium radical is rapid,[208] in the oxidation of a dihydropyridine by ferricyanide, the loss of the C-4 proton can be rate limiting.[206] Further, Nelsen and Ippoliti[209] present evidence that the lifetime of an aminium radical bearing an α-proton can be greatly increased when the C-H bond happens to be held near the modal plane of the $R_3N^{\cdot}_{+}$ moiety. Thus, some of the existing dogma concerning the facility of rearrangement of aminium radicals to α-amino radicals may be too presumptuous. For instance, in solution a free amine would facilitate the α-deprotonation process. Unfortunately, the known amino acid sequences of cytochrome P-450 enzymes are not helpful in that the residues near the oxygen-binding face of the mammalian enzymes are not known (and amine dealkylation has not been demonstrated in the bacterial cytochrome $P\text{-}450_{cam}$ for which the structure is known),[210] but it seems unlikely that a basic residue would be in the appropriate position for a direct intercept in each of the various cytochrome P-450 enzymes, which all show low kinetic isotope effects for *N*-dealkylation and are thought to proceed via a similar mechanism.[197,202] A likely alternative is that the $(FeO)^{2+}$ entity left after the initial

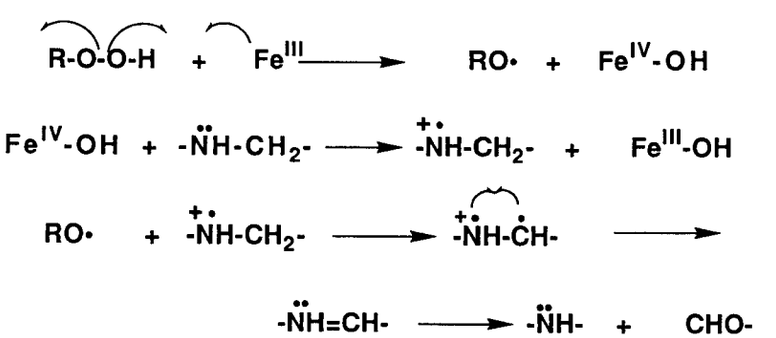

FIGURE 5. Postulated mechanism of hydroperoxide-supported *N*-dealkylation catalyzed by cytochrome P-450.

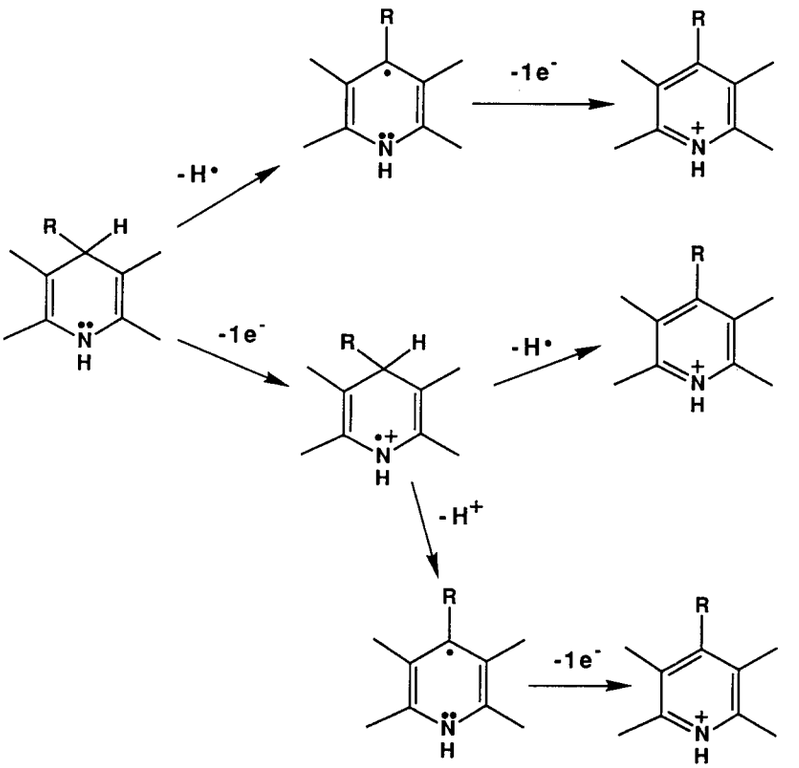

FIGURE 6. Potential routes for dehydrogenation of 1,4-dihydropyridines by cytochrome P-450.

1-electron abstraction is acting as the base, as it would be in a close position, even in the case of the dihydropyridines (Figure 7).

Why, then, does peroxidase Compound II not act in a similar mode? The difference may lie in the view that the heme pocket of many peroxidases, or at least horseradish peroxidase, is very closed and that substrates cannot approach the FeO moiety.[111,211] Thus, electron transfer is thought to occur via the heme edge, and the FeO moiety could not act as a specific base in the catalysis of amine dealkylation (Figure 8). Proton abstraction could become rate limiting (assuming that electron transfer is rapid).

An alternative explanation is that in cytochrome P-450 the $E_{1/2}$ of the analogous Compound I species (FeO) is much lower than in horseradish peroxidase, giving rise to rate-limiting proton abstraction. Initial 1-electron oxidation appears to be rate limiting in the *N*-demethylation of substituted *N,N*-dimethylanilines by cytochrome P-450_{PB-B} (IIB1), but the effective $E_{1/2}$ of the enzyme appears to be as high or higher than horseradish peroxidase,[159] although the peroxidase-catalyzed *N*-demethylations are faster than those catalyzed by cytochrome P-450 enzymes.[197] However, further investigations into the mechanism of the peroxidase-catalyzed reaction are warranted. Recently, Born and Hadley[212] have reported that the oxidation of nifedipine, which differs from other model compounds studied only in the presence of an *o*-nitro group on the phenyl ring, exhibits a high ^{D}V, but a low $^{D}(V/K)$, in rat liver microsomes, which the authors interpret as evidence for a hydrogen atom abstraction process. This result (^{D}V) and its interpretation would appear to be inconsistent with work done with other compounds,[202] for the influence of the nitro group on the (benzylic) 4-carbon would seem to tend to destabilize any radical. The apparent isotope effect seen in the dehydrogenation of the *N*-phenyl, 4-ethyl compound remains to be explained.[203]

In another study, a series of different cyclopropyl-substituted heteroatoms were prepared for examination as mechanism-based inactivators of rat cytochrome P-450_{PB-B}.[195] Rates of cytochrome P-450 heme destruction ($k_{inactivation}$) were related to the $E_{1/2}$ potentials for single-electron oxidation of the compounds (in CH_3CN) in a log-linear manner, consistent with the view that single-electron transfer is involved in the inactivation. It is actually surprising that any oxidation at all is observed in the case of some of the halides and the compounds may simply have little other alternative. Unfortunately, the nature of the inactivated heme products has not been characterized.[196] The presence of a substituent at the 1-position of the cyclopropane ring does not block inactivation, ruling out possibilities involving imines.[194,196] The oxidation of *O*-ethyl cyclopropanone hydrate to ethyl 3-chloropropionate is rationalized by a mechanism involving 1-electron oxidation of the substrate, ring opening, and oxygen rebound at the rearranged methylene radical (Figure 9).[195]

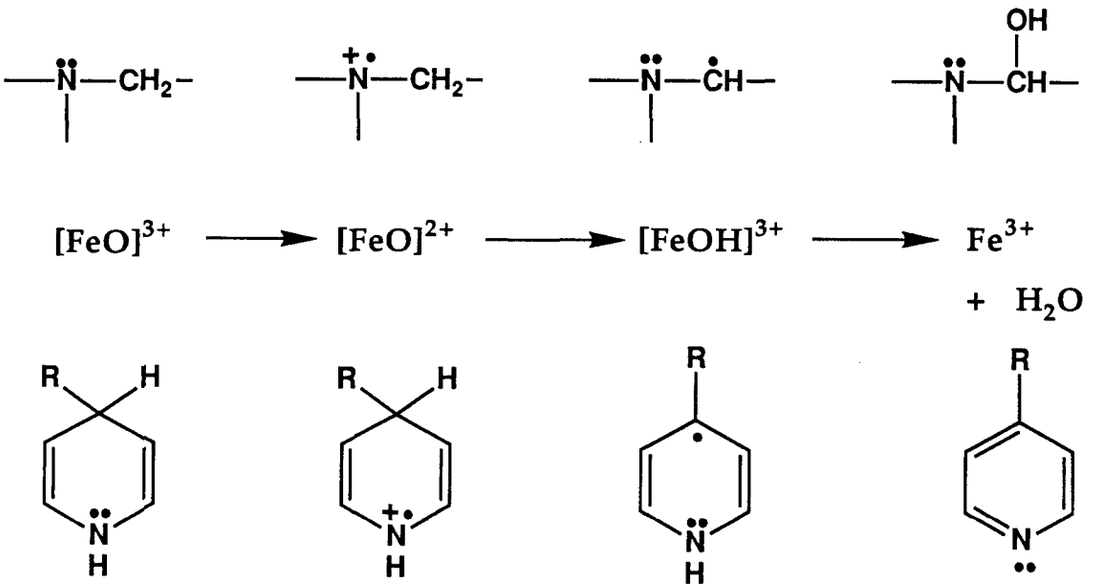

FIGURE 7. Postulated proton abstraction by $(FeO)^{2+}$ in *N*-dealkylation and 1,4-dihydropyridine oxidation by cytochrome P-450.

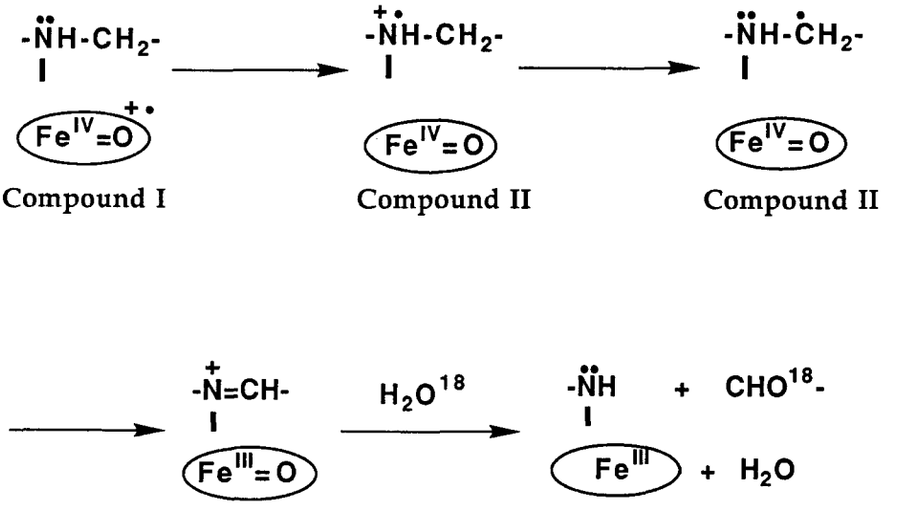

FIGURE 8. Proposed *N*-dealkylation mechanism of horseradish peroxidase.

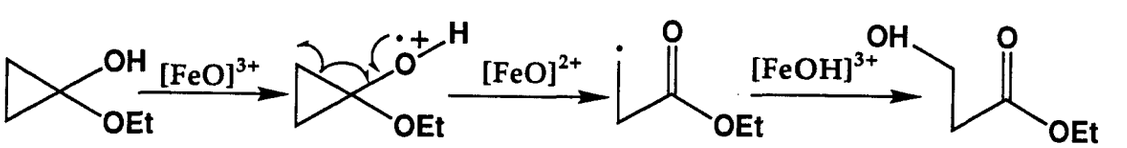

FIGURE 9. Oxidation of *O*-ethyl cyclopropanone hydrate to ethyl 3-chloropropionate by cytochrome $P\text{-}450_{PB\text{-}B}$. (From Guengerich, F. P., Willard, R. J., Shea, J. P., Richards, L. E., and Macdonald, T. L., *J. Am. Chem. Soc.*, 106, 6446, 1984. With permission.)

Further studies with cyclobutylamines have also demonstrated their efficacy as mechanism-based cytochrome P-450 inactivators,[196] as might be expected on the basis of work with monoamine oxidase[213] if similar catalytic mechanisms are involved (i.e., sequential electron transfer). As expected, the cyclobutylamines are not as destructive as the corresponding cyclopropylamines due to slower ring opening of the aminium radicals and competition with other reaction possibilities. As in the case of monoamine oxidase, ring expansion of cyclobutylamines to pyrrolines can be documented, providing more evidence for aminium radical intermediates (Figure 10).

With the extensive evidence now available to support the view that cytochrome P-450 oxidizes amines via a single electron process, an approach was used to estimate an effective $E_{1/2}$ of a cytochrome P-450 enzyme $(FeO)^{3+}$ species.[159] The rates of *N*-demethylation of a series of *para*-substituted *N,N*-dimethylaniline derivatives by cytochrome $P\text{-}450_{PB\text{-}B}$ are known to vary considerably, and Hammett analysis of the results yields $\rho = -0.6$ to -0.75, consistent with the view that an aminium radical is an intermediate.[214] Similar data were fitted to plots of log k_{cat} vs. the measured $E_{1/2}$ of each compound. Analysis of the plots by curve fitting to modified Marcus equations yielded an estimated $E_{1/2}$ of about +1.8 V (vs. SCE) for the $(FeO)^{3+}$ complex.[159] $E_{1/2}$ and the λ (self-exchange energy) were similar regardless of whether the reaction was supported by NADPH-cytochrome P-450 reductase and O_2 or by iodosylbenzene, and a similar mechanism is proposed for both reactions, as in the case of the Hammett analysis.[214] The results provide some insight into the intrinsic ability of cytochrome P-450 enzymes to abstract electrons;[159] however, it should be considered that the $E_{1/2}$ will be a function of the distance between the reactive centers and the dielectric constant of the protein:

$$E_{1/2(\text{apparent})} = E_{1/2(\text{intrinsic})} + E_{(cf)}$$

and

$$E_{(cf)} = +14.4$$

where $E_{(cf)}$ is the electrostatic contribution (correction factor) and is expressed in volts, $r_{1,2}$ is the center-center internuclear distance in the transition state between the nuclei of the interacting spheres (in Å), and D is the static dielectric constant in the enzyme active site. $E_{(cf)}$ can be quite appreciable (0.5 to 1.5 V) when reasonable estimates for $r_{1,2}$ and D are introduced.[159] Electron transfer should be operative over a longer distance than hydrogen atom abstraction, but the distance may vary with different substrates, even within the same cytochrome P-450. Furthermore, both interradial distance and dielectric constant may well vary when different cytochrome P-450 enzymes are compared. Thus, an *intrinsic* $E_{1/2}$ of $(FeO)^{3+}$ protoporphyrin IX may show some alteration in every protein structure.

The final point considered under the subject of heteroatom release is the oxidative cleavage of esters (Figure 11), a reaction that was clearly documented in this laboratory.[174,215] The reaction involves oxidation of the alkyl moiety and is believed to take place via carbon hydroxylation (Figure 12).

The intrinsic kinetic deuterium isotope effect estimated by the method of Northrop[216] is very high (13 to 15), and noncompetitive primary deuterium isotope effects as high as 8 are seen in liver microsomes. These isotope effects are similar to those observed for *O*-dealkylation of ethers by cytochrome P-450.[217,218] The generality of this mode of ester cleavage is yet unknown, but studies with nifedipine derivatives indicate that this can be the predominant means of cleavage of a particular ester *in vivo*.[219]

The kinetic deuterium isotope is not appreciably attenuated in experiments involving inter- and intramolecular competition.[174] These studies can be interpreted to mean that movement of the substrate in and out of the active site is rapid relative to the overall reaction. "Metabolic switching" (isotopically sensitive branching)[220,221] to another site of hydroxylation (Figure 11b) can be observed when the ester is blocked with deuterium (a); however, the reverse switching pattern (b→a) is not observed, even though a large kinetic isotope effect exists.[174] These observations have been related to the relative ratios of the two products and the heights of the transition state barriers within the enzyme.[174,222]

3. Heteroatom Oxygenation

In a previous review,[9] the view was expressed that when aminium radicals are formed, α-protons are rather easily ab-

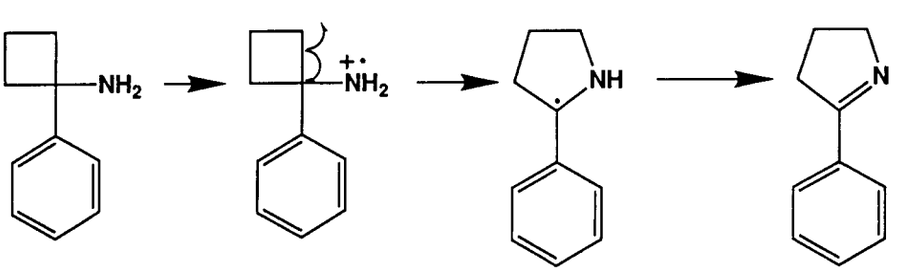

FIGURE 10. Ring expansion of 1-phenylcyclobutylamine rationalized by 1-electron transfer. (From Bondon, A., Macdonald, T. L., Harris, T. M., and Guengerich, F. P., *J. Biol. Chem.*, 264, 910, 1989. With permission.)

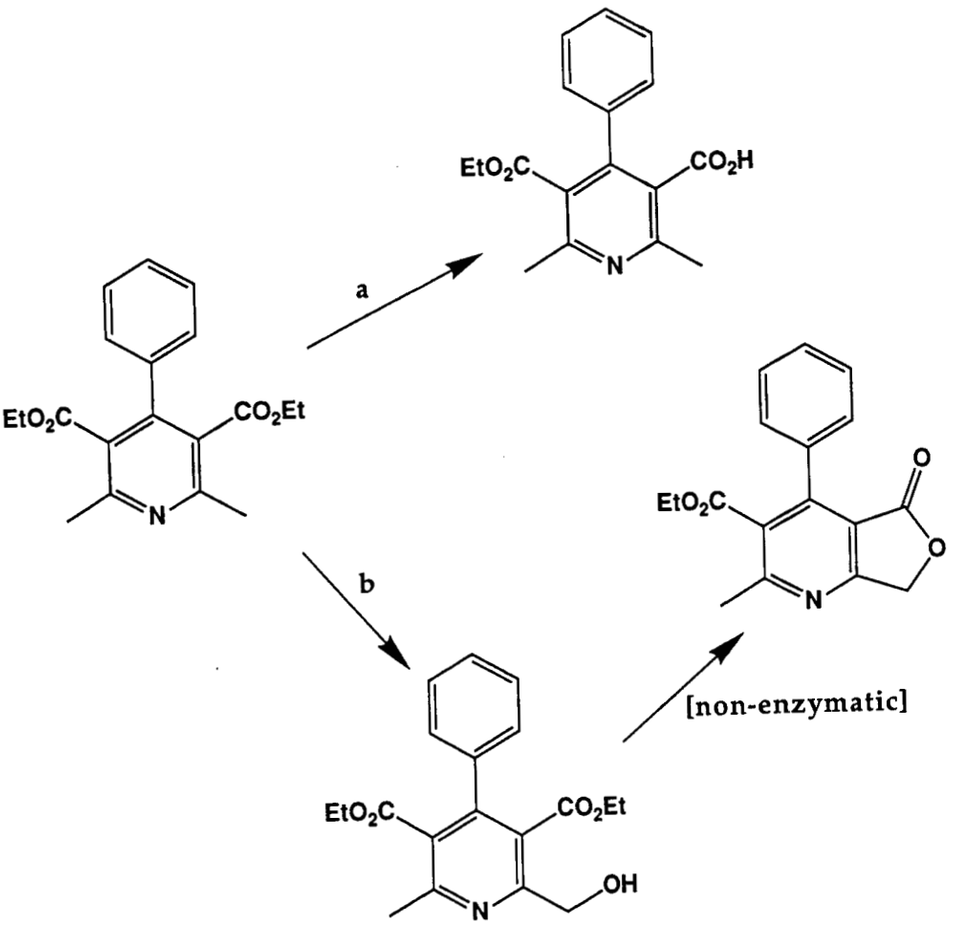

FIGURE 11. Oxidative cleavage of the carboethoxy moiety of 2,6-dimethyl-4-phenyl-3,5-pyridinedicarboxylic acid diethyl ester by cytochrome P-450.[174,215]

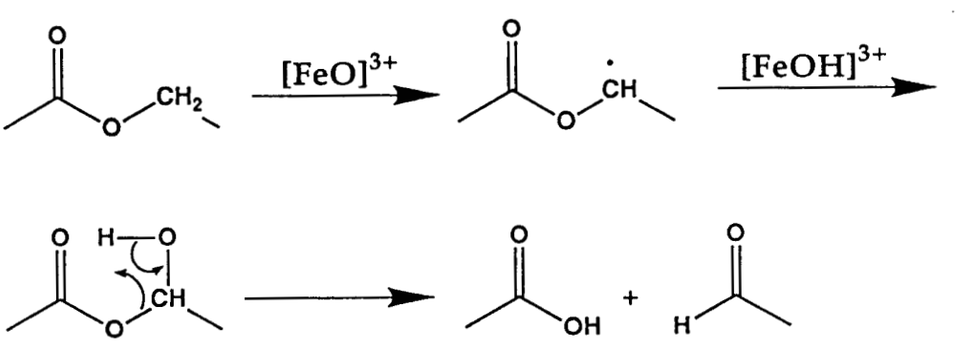

FIGURE 12. Mechanism of cytochrome P-450-catalyzed ester cleavage.[172,213]

stracted and, in general, *N*-oxygenation is not observed when α-hydrogens are available. Thus, at that time it was thought that *N*-oxygenation should only be observed when (1) α-hydrogens are unavailable (arylamines), (2) α-hydrogens are inaccessible because of Bredt's rule,[79] or (3) the aminium radical is stabilized (e.g., by neighbor donation in the case of the oxidation of azo to azoxy groups).[223] The sulfur radical is relatively stable and one would expect *S*-oxygenation to predominate over *S*-dealkylation, as is the general situation. The difference between the radical cytochrome P-450 mechanism

and the heterolytic mechanism of the flavin-containing monooxygenase should be emphasized,[28,29,224] for although in some cases identical reactions are catalyzed, the mechanisms are quite distinct and one would not expect one of the two enzymes to be a reliable guide as to reactions catalyzed by the other.

Recently, some exceptions to the above general rules about the lack of *N*-oxygenation of amines bearing α-protons have been found. Baba et al.[225] reported the conversion of amphetamine derivatives to hydroxylamines, and Bondon et al.[196] found that a cyclobutyl-substituted benzylamine was oxidized to a nitrone via an apparent hydroxylamine intermediate. In addition, Williams et al.[226] clearly identified a role for cytochrome P-450 in the conversion of the pyrrolizidine alkaloid senecionine (a tertiary amine) to its *N*-oxide. It is not particularly clear exactly why aminium radicals should be particularly stable in these molecules. However, of interest are reports that the acidity of an aminium nitrogen proton is not considerably less than that of the α-proton,[227,228] and rebound to the $-\dot{\underset{\cdot\cdot}{N}}-$ may follow (Figure 13). The *N*-oxidation of pyrrolizidine alkaloids is not attributed to Bredt's rule, for this axiom only applies to bridged bicyclic systems (e.g., quinidine), where rings are fused through nonadjacent atoms.[228a]

As discussed under the section dealing with heteroatom release, the α-deprotonation of aminium radicals may not be as predominant as originally thought in all situations.[209] In experiments involving the 1-electron oxidation of amines in solution, excess amine is available to abstract the proton and the tendency to α-deprotonate may be greater than in a situation where an isolated aminium radical is generated. The situation imposed upon aminium radical structures by proteins may be more complex than originally thought and perhaps it is not so surprising that the range of cytochrome P-450-catalyzed reactions with amines is not totally restrictive.

The oxygenation of diethylphenylphosphine by cytochrome P-450 has been reported.[229] This result was to be expected, in light of the low oxidation potential.[9] Indeed, model compounds containing $(FeO)^{2+}$ are capable of carrying out this reaction.[230]

The question of whether cytochrome P-450 enzymes can oxygenate halogen atoms has been elusive. Halogen atoms have oxidation potentials at the level of or above ethers,[9] but several studies suggest that these may be oxidized by cytochrome P-450 enzymes (see Macdonald[231]). Indeed, Burka et al.[232] showed that a cytochrome P-450 could catalyze the exchange of labeled iodobenzene and iodosylbenzene (Figure 14).

In these experiments, labeled iodosylbenzene was trapped in the presence of a large amount of "carrier" iodosylbenzene. However, the instability of haloso compounds in the presence of proteins has made experiments difficult involving cytochrome P-450 supported by NADPH-cytochrome P-450 reductase. Recently, the compound 4-*tert*-butyl-2,6-*bis*[1-

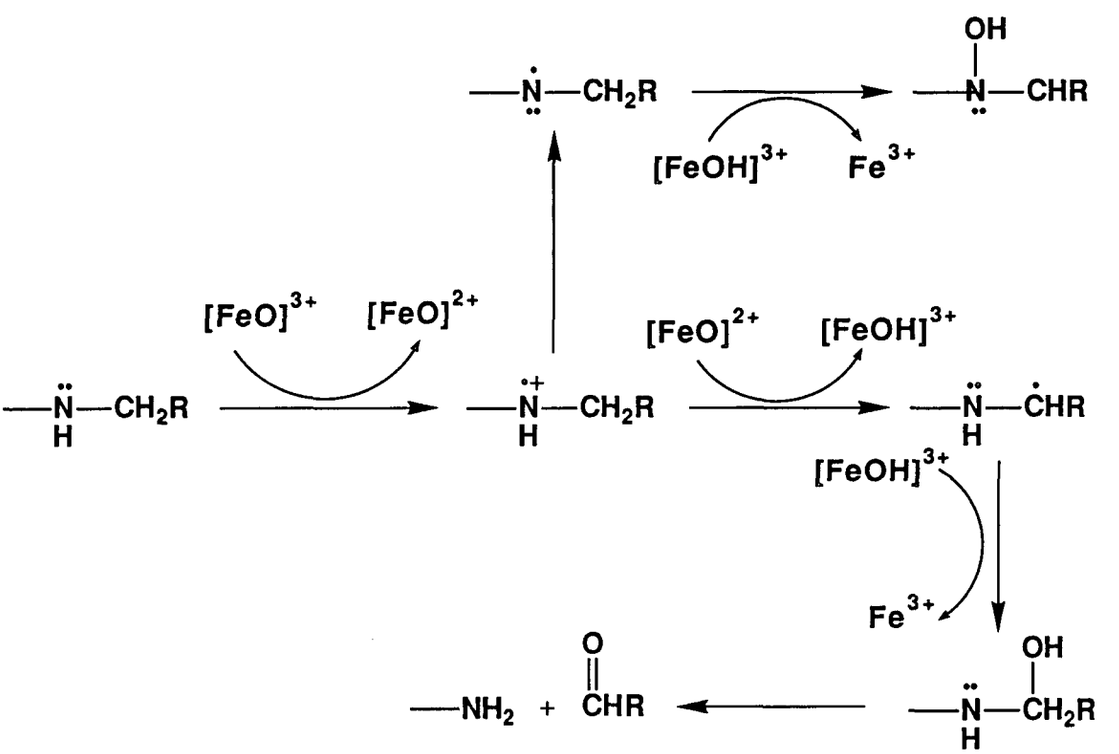

FIGURE 13. Possible mechanisms for hydroxylamine formation and *N*-dealkylation proceeding from an aminium radical.

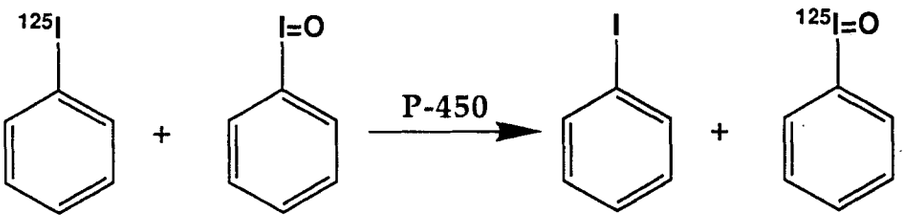

FIGURE 14. Transfer of oxygen from iodosylbenzene to iodobenzene. (From Burka, L. T., Thorsen, A., and Guengerich, F. P., *J. Am. Chem. Soc.*, 102, 7615, 1980. With permission.)

hydroxy-1-(trifluoromethyl)-2,2,2-trifluoroethyl]iodobenzene (RI) has been prepared by Martin and co-workers (Figure 15).[233] The oxidized derivative {10-*tert*-butyl-3,3,6,6-tetrakis(trifluoromethyl)-4,5,6-benzo-1-ioda-7,8-dioxabicyclo[3.3.1]octane}(RIO) is relatively stable and can be recovered from incubations containing protein in reasonable yield. It was subsequently found that cytochrome P-450 could catalyze the oxidation of the iodide to the iodinane when supported by either NADPH-cytochrome P-450 reductase or iodosylbenzene.[535]

Selectivity among several cytochrome P-450 enzymes for catalyzing the reaction was seen, and several model metalloporphyrins also catalyzed the same reaction (supported by iodosylbenzene). Studies with the analogous bromide indicate that its rate of oxidation is at least two orders of magnitude less, a result consistent with its higher oxidation potential.[9,535]

4. Olefin Oxidation

Groves et al.[234] have shown that a vinylic hydrogen of pro-

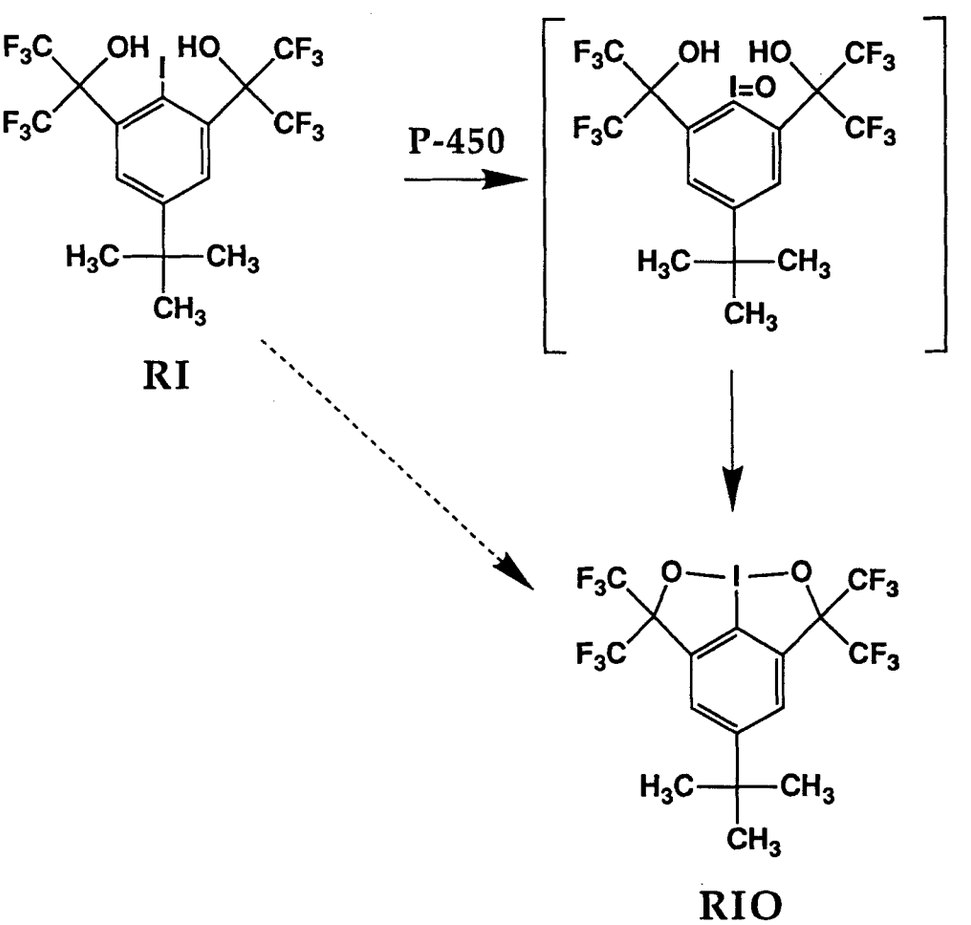

FIGURE 15. Oxidation of an iodo compound to the iodosyl level by cytochrome P-450. (Guengerich, F. P., *J. Biol. Chem.*, 264, 17198, 1989.)

pylene can exchange with the bulk water protons during epoxidation by rabbit cytochrome P-450 2 (IIB4). This unexpected result can be rationalized in terms of Fe-C bond chemistry (*vide infra*) and model studies have focused on possible intermediates (Figure 16). The relevant studies with model metalloporphyrins are discussed later in this review. The exchange reaction is stereospecific and probably requires the presence of a specific base at a requisite position in the protein; moreover, not all olefins show this exchange.

Our original postulates concerning 1,2-group migration (Figure 2) have been substantiated in models (*vide infra*), although further searches for the phenomenon have not been carried out in enzyme systems.[235] The point has been made that the existence of a 1,2-shift *does not* necessarily implicate the existence of an epoxide.[9,235,236] Naphthalene has been examined to determine if a portion of the 1-naphthol formed could be explained by a nonepoxide route. However, the addition of a large amount of purified epoxide hydrolase to the cytochrome P-450 system was able to completely attenuate the formation of 1-naphthol, indicating that in this case the major fate of the putative intermediate is collapse to the epoxide, and the hydride apparently tends not to migrate from an intermediate.[536]

The work on mechanism-based inactivation has been extended to include a number of other reactions, such as the modification of the cytochrome P-450 heme by benzyne and hydrazine products.[237,238] During oxidative catalysis in the absence of catalase, cytochrome P-450 heme can be destroyed rather readily.[239] Products identified in the degradation are maleimides and propentdyopents;[240] however, investigations to date have not indicated that these are formed at significant levels *in vivo* under normal conditions. The same products appear to be formed in microsomes in the presence of iodosylbenzene or cumene hydroperoxide or in a system where lipid peroxidation is taking place.[240]

Although *N*-alkyl porphyrins ("green pigments") are formed *in vivo* and have been shown to have physiological signifi-

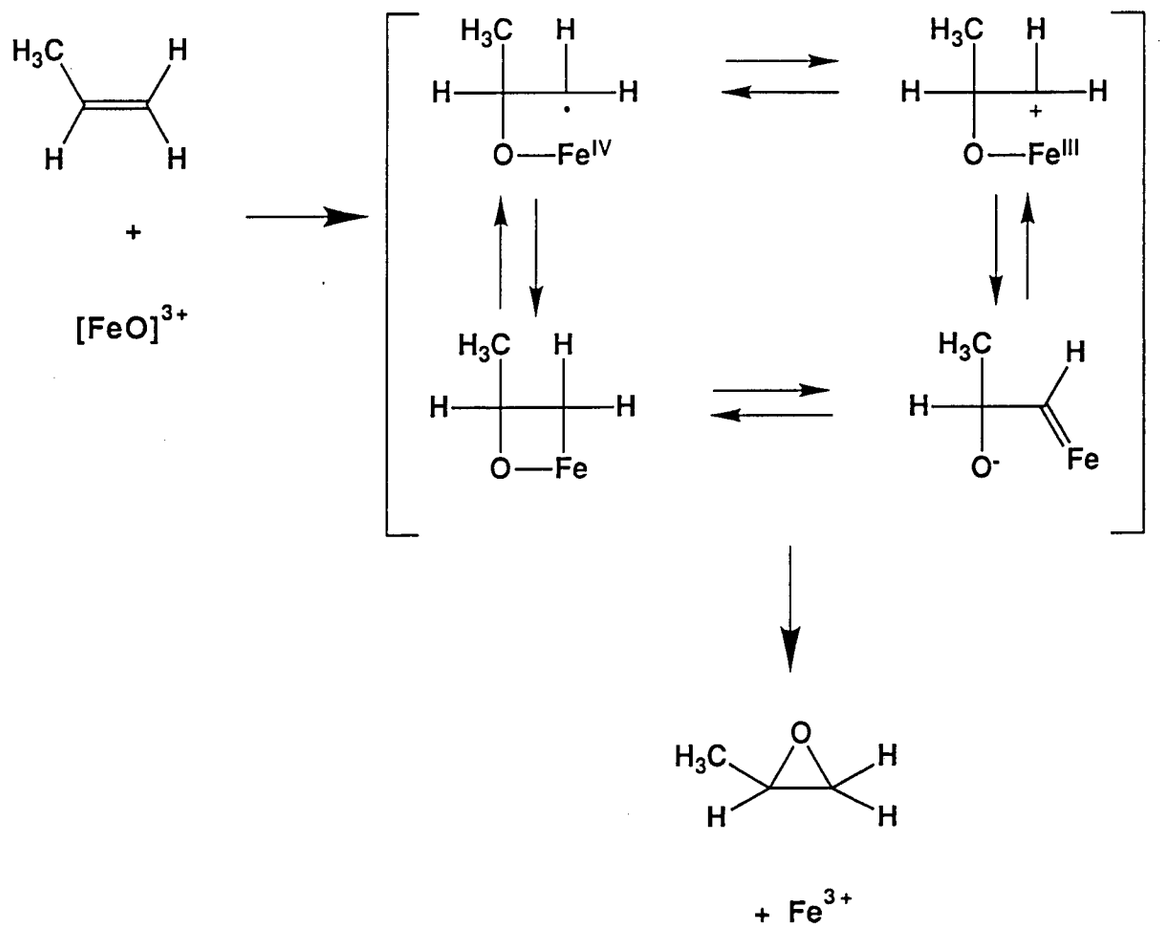

FIGURE 16. Possible mechanism for exchange of vinylic hydrogen with water during cytochrome P-450-catalyzed epoxidation of propylene. (From Groves, J. T., Avaria-Neisser, G. E., Fish, K. M., Imachi, M., and Kuczkowski, R. L., *J. Am. Chem. Soc.*, 108, 3837, 1986. With permission.)

cance,[237] they do not account for the major portion of the heme destroyed *in vitro*.[241-243] The maleimides and propentdyopents also are not formed in sufficient quantities to explain the loss of cytochrome P-450 heme that occurs under oxidative conditions in the absence of substrate.[240,241] In both cases, the bulk of the radioactivity associated with cytochrome P-450 heme becomes irreversibly attached to the apoprotein.[196,241-244] The nature of the interaction is yet uncharacterized, and it seems highly unusual in that such cross-linking can be demonstrated with or without substrates.

E. Active Site

If we are to understand the catalytic specificity of a cytochrome P-450 enzyme at the molecular level, then the amino acid residues that interact with the substrate and the heme prosthetic group must ultimately be known. There are three major approaches that can be used to study the general problem.

The first is actual elucidation of protein structure and the position of the substrate by means of X-ray diffraction or NMR spectroscopy. NMR analysis cannot be used to study the structure of a protein as large as cytochrome P-450 (although limited information could be obtained with isotopically labeled substrates). X-ray diffraction has been used to determine the structure of the soluble bacterial cytochrome $P\text{-}450_{cam}$ (CI) at 1.63 Å resolution.[210] The structure is discussed in the cited reference[117] and may be a reasonable model for the structures of the eukaryotic cytochrome P-450 enzymes, all of which appear to be intrinsic membrane proteins. Recently, Iwamoto et al.[245] have reported the crystallization of bovine adrenocortical mitochondrial cytochrome $P\text{-}450_{SCC}$, the enzyme that cleaves cholesterol. The crystallization of a microsomal cytochrome P-450 protein has not been accomplished. One of the serious problems is potential microheterogeneity. Several of the major liver microsomal cytochrome P-450 proteins are the products of complex multigene families, and it is not easy to be sure that one is dealing with a single protein if the possibility for simultaneous isolation of closely related proteins exists. Of course, the presence of very closely related proteins in a preparation is a serious problem in crystallization. One means of avoiding this problem is with the use of proteins expressed in artificial vectors, but the yields in the systems used to date are probably too low to be practical. Evidence has been presented with two different phenobarbital-inducible cytochrome P-450 enzymes that a major mode of membrane association is via insertion of a N-terminal segment and that the remainder of the cytochrome P-450 is cytosolic.[246,247] This view contrasts with earlier thoughts about the topology of cytochrome P-450, where several hydrophobic segments were assumed to span the membrane.[248] In principle, expression of a truncated cytochrome P-450, devoid of the hydrophobic N-terminal sequence, might be useful in terms of producing a soluble protein that could be crystallized more readily. Work by Ohkawa and co-workers suggests that such an approach might be feasible and that such a truncated protein may even be active.[249] On the other hand, Imai[250] has found that such a derivative of a rabbit cytochrome P-450 is unstable when expressed in yeast. Larson and Porter[251] expressed rabbit cytochrome P-450 3a (IE1) in *Escherichia coli* using a vector that directs transport to the periplasmic space. The enzyme, which was functional catalytically, was sequestered into the inner membrane. However, an expressed cytochrome P-450 3a devoid of the N-terminal 24 amino acids was also sequestered into the inner membrane, suggesting that other elements of this cytochrome P-450 may be involved in membrane association. Thus, strategies for crystallization of microsomal cytochrome P-450 proteins may involve (1) optimization of conditions for proteolytic cleavage of intact protein prior to crystallization or (2) direct attempts to crystallize an intact protein. Either approach is likely to be expensive in terms of the amount of material required.

A second approach to the characterization of catalytic sites involves the identification of involved residues by covalent modification with affinity labels. It is somewhat surprising that relatively little progress has been reported in this area. Frey et al.[252] were able to label rat liver cytochrome P-450 PB-4 (cytochrome $P\text{-}450_{PB\text{-}B}$, IIB1) with the competitive inhibitor 3-(trifluoromethyl)-3(*m*-iodophenyl)diazinine, but further identification of the site of attachment has not been reported. Several groups have modified cytochrome P-450 apopoteins with mechanism-based (''suicide'') inactivators,[196,253-257,537] but in no case has a site of modification been identified. Many substrates and inhibitors of cytochrome P-450 enzymes have very high affinities and the design of useful radiolabeled affinity labels should be possible.

The last major approach involves the comparison of primary amino acid sequences and carrying out substitution with analysis of catalytic function. Kalb and Loper[101] have recently analyzed the primary sequences of known cytochrome P-450 proteins and found that a region of 170 residues (out of a total of about 510) contains most of the similarity across a broad range. This region begins nearly 300 residues into the sequence (from the N-terminus) and can be divided into four domains (A, B, C, and D) joined together with segments of various length. As pointed out by Kalb and Loper,[101] the A, C, and D domains all have counterparts in the structure of bacterial cytochrome $P\text{-}450_{cam}$. The section A-11 through A-27 in cytochrome $P\text{-}450_{cam}$ is known to span the heme distal surface and to contact both the substrate (camphor) and heme. Antibodies raised against the peptide corresponding to residues A-8 through A-19 of cytochrome P-450b ($P\text{-}450_{PB\text{-}B}$, IIB1) react with cytochrome P-450c ($P\text{-}450_{\beta NF\text{-}B}$, IA1), a generally rather dissimilar protein.[258] In the B domain is found a somewhat conserved dodecapeptide,[259] and the D domain contains the highly conserved region that contains the axial thiolate ligand to the heme. This is a useful analysis, although discerning what contributions each domain makes will require more effort.

Sakaki et al.,[260] using a yeast expression system, made constructs involving switches of parts of two related cytochrome P-450 proteins, rat cytochrome P-450c and cytochrome P-450d ($P\text{-}450_{\beta NF\text{-}B}$, IA1 and $P\text{-}450_{ISF\text{-}G}$, IA2). From these experiments, the authors concluded that the middle one third of the coding sequence appears to play a major role in influencing catalytic specificity. Imai has also used yeast vector systems to express rabbit liver cytochrome P-450 sequences, in particular to study the expression of laurate ω-1 hydroxylase activity. The region spanning amino acid residues 211 to 262 was found to be essential for both hydroxylase activity and for binding of the substrate, as judged by the lack of a shift of the Soret spectrum to the high-spin state.[261] Imai[261] also found that the sequence-spanning residues 43 to 210 were also essential for hydroxylase activity. Kronbach et al.[262] have expressed chimeric derivatives of rabbit liver cytochrome P-450 proteins in COS-1 cells and examined progesterone 21-hydroxylase activity. Using a systematic approach of substitution of regions between two cytochromes P-450 (IIC4 and IIC5 gene products), they found that the residues at 113, 115, and 118 were critical to activity. Two substitutions could convert the progesterone 21-hydroxylase cytochrome P-450 (IIC4) to an estradiol 2-hydroxylase, but the complementary substitutions in the estradiol 2-hydroxylase could not confer progesterone 21-hydroxylase activity.

Ishida et al.[263] sequenced cDNA clones of a strain of *Saccharomyces cerevesiae* (SG1) defective in lanosterol 14α demethylation and found that a single substitution was responsible for the loss of catalytic activity of cytochrome $P\text{-}450_{14DM}$ (LI). The single nucleotide changed gly 310 to asp (G310D) and the resulting protein appears to have a distal his ligand to the heme (instead of the water and oxygen normally there in the various parts of the catalytic cycle). In cytochrome $P\text{-}450_{cam}$, thr 252 is located at the distal heme surface.[117,210] Imai and Nakamura[264] replaced the corresponding thr in the rabbit laurate ω-1 hydroxylase (thr 301) with his (T301H). The spectrum of the chimeric protein was not unusual, suggesting that the newly introduced his was not behaving as a heme ligand. However, the modified protein appeared not to catalyze laurate ω-1 hydroxylase activity, or to bind laurate (as judged by lack of perturbation of the heme spectrum). In a further series of experiments,[265] mutations of thr 301 in the same protein were shown to have a variety of effects on the protein (Table 1).

The ser, val, and asn mutants had lower laurate ω-1 hydroxylase activities than did the wild-type (thr) cytochrome P-450. The thr-to-ser mutation (T301S) did not affect caprate ω-1 hydroxylase activity and increased caprate ω-hydroxylase activity twofold. The val and asn derivatives did not hydroxylate caprate, and the ile mutant was devoid of all laurate and caprate hydroxylase activities. The results are surprising in light of the difference of only two methylene groups between the two substrates. A thr-to-val substitution (T301V) decreased both the testosterone 16α-hydroxylase and progesterone 16α-hydroxylase activities of the same protein; the thr-to-ser substitution decreased the testosterone 16α-hydroxylase activity, but did not affect the progesterone 16α-hydroxylase activity. When the affinities of substrates were examined (using perturbation of the heme spectrum), the ser 301 mutant (T301S) had much lower affinities for both substrates (K_S 20 μ*M* for laurate, K_S 90 μ*M* for caprate) than did the wild-type enzyme and the val and ile mutants (K_S 8 μ*M* for both). The asn mutant showed caprate affinity similar to the wild-type enzyme (K_S 6 μ*M*), but reduced affinity for laurate (K_S 29 μ*M*).

Some other accounts of major differences in the catalytic activities of cytochrome P-450 proteins due to small changes have also been reported. Rat liver cytochromes $P\text{-}450_{PB\text{-}B}$ and $P\text{-}450_{PB\text{-}D}$ (P-450b and P-450e, P-450 PB-4 and P-450 PB-5, IIB1 and IIB2, respectively) differ in only 14 residues, but usually show differences in catalytic activity toward most substrates, with cytochrome $P\text{-}450_{PB\text{-}B}$ being more active. It is probably not accurate to say that a major change in catalytic *specificity* exists. The rat cytochrome $P\text{-}450_{\beta NF\text{-}B}$ and mouse

Table 1
Hydroxylase Activities of a Series of Chimeric Cytochrome P-450 Proteins Based Upon Rabbit Laurate ω-1 Hydroxylase

	Hydroxylase activity (nmol product/min/nmol cytochrome P-450)				
Residue at position 301	Laurate[a] (ω-1)	Caprate[a] (ω-1)	Caprate[a] (ω)	Testosterone (16α)	Progesterone (ω-1)
thr[b]	12.8	10.8	2.1	3.4	4.7
ser	4.6	11.2	4.7	1.7	5.2
val	2.5	< 0.1	< 0.1	0.7	0.2
ile	< 0.1	< 0.1	< 0.1		
asn	4.9	< 0.1	< 0.1		

[a] The substrate concentration was 1 m*M*.
[b] Wild-type enzyme.

From Imai, Y. and Nakamura, M., *Biochem. Biophys. Res. Commun.*, 158, 717, 1989. With permission.

P_1-450 primary sequences are 93% identical,[266] yet the rat protein is highly active toward the substrate warfarin (6- and 8-hydroxylation), while the mouse protein is inactive.[267] These two proteins are both termed "IA1" and, although the nomenclature is most logically based on sequence comparisons,[100,112] caveats are needed in the consideration of catalytic activities. In another example of a dramatic change in catalytic activity induced by amino acid substitutions, Yanase et al.[268] reported that a human cytochrome $P\text{-}450_{17\alpha}$ (XVIIA1) mutant devoid of 17α-hydroxylase and 17,20-lyase activities contains a 4-base duplication near the 3′ end of the protein coding region. In the normal cytochrome $P\text{-}450_{17\alpha}$, the C-terminal sequence from the position of the duplication is 29 amino acids long, while in the mutant protein this sequence is 26 amino acids long before an in-frame stop codon is observed. All but 3 of the C-terminal 26 residues in the mutant differ from their normal counterparts, and the difference appears sufficient to inactivate the cytochrome $P\text{-}450_{17\alpha}$. Dramatic changes can also be seen in the mouse cytochrome P_1-450 protein (IA1). Kimura et al.[269] analyzed benzo[*a*]pyrene-resistant mutants of a mouse hepatoma Hepa-1 wild-type cell line. The L118R mutation was found to have a negligible effect on benzo[*a*]pyrene hydroxylase activity, and the R245P mutation led to a two- to threefold decrease in catalytic activity. The presence of *both* mutations in the same protein abolished the catalytic activity. The results suggest the importance of arg 245 and a complementary function for leu 118 in the normal cytochrome P_21-450 function. In addition, the mouse cytochrome P_2-450 sequence represents an allelic variant of the cytochrome P_3-450 (IA2), and a single substitution (I384M) appears to have a dramatic effect in decreasing acetanilide hydroxylation.[270,271]

Suffice it to say that the catalytic specificity of cytochrome P-450 enzymes other than cytochrome $P\text{-}450_{cam}$ cannot be understood in molecular terms at this time. Crystallization and X-ray diffraction studies are probably not impossible, but still in the future. The site-directed mutagenesis studies are exciting, but the results are rather preliminary in terms of providing a general picture of substrate interactions. Describing catalytic sites of the cytochrome P-450 enzymes through affinity labeling and site-directed mutagenesis will require considerable work and interpretation will require time. Clearly, small changes can have dramatic effects on catalytic activity, as exemplified in the cases presented above. The results also suggest some caution in making assumptions about the catalytic activities of what appear to be orthologous proteins on the basis of sequence similarity, particularly when many of the substrates used have no endogenous function and there is no pressure for conservation of function. Even if two cytochrome P-450 proteins are 90% identical in their sequences, there are over 50 substitutions, and we have seen that even a single change can have dramatic and diverse effects.

Another point to be made is that cytochrome P-450 enzymes with quite different structures may catalyze the same reaction.[127] Further, a small change in the structure of a substrate may change the specificity of cytochrome P-450 enzymes for carrying out a reaction, as exemplified in the case of the 7-alkoxyresorufins.[272] Finally, I also point out that some seemingly small changes in *in vitro* conditions may change apparent substrate specificity.[273]

IV. PEROXIDASES

A. General Features and Mechanisms

Peroxidases (collectively grouped under EC 1.11.1.7, peroxidase) are found throughout nature and catalyze the peroxidase-dependent oxidation of chemicals.

$$ROOH + AH_2 \rightarrow ROH + A + H_2O$$

Some of the enzymes are reviewed elsewhere.[274] When the peroxidase bond is cleaved heterolytically, a formal $(FeO)^{3+}$ species results. Such an entity is commonly referred to as Compound I. Peroxidase reactions may be generally described as single electron transfers.

$$\text{Enzyme} + ROOH \rightarrow \text{Compound I} + ROH$$

$$\text{Compound I} + AH_2 \rightarrow \text{Compound II} + AH\cdot$$

$$\text{Compound II} + AH_2 \rightarrow \text{Enzyme} + AH\cdot$$

$$AH\cdot \rightarrow \text{Nonradical products}$$

Some peroxidases have been reported to carry out 2-electron transfer processes as well.

$$\text{Compound I} + AH_2 \rightarrow \text{Enzyme} + A^{275,276}$$

When ROOH has a good leaving group (e.g., peracids), heterolytic cleavage results.

$$Fe^{III} + ROOH \rightarrow (FeO)^{3+} + ROH$$

If the leaving group is poor (pKa >10), homolytic scission is generally observed.

$$Fe^{III} + ROOH \rightarrow (FeOH)^{3+} + RO\cdot$$

Cytochrome P-450 enzymes tend to fall in the latter category when they react with alkyl hydroperoxides, and the chemistry that results can be unique, but not necessarily relevant to the normal mechanism, where $(FeO)^{3+}$ is the entity that abstracts a hydrogen atom or electron (*vide supra*). Apparently, peroxidases have specific groups (histidine?) in the active site to participate in general acid-base catalysis. Such appropriate juxtaposition is exemplified in the structure of yeast cytochrome *c* peroxidase derived using X-ray diffraction.[277] The charge in

the high-valent iron compounds is distributed in different ways. As pointed out later in this review under the subject of model studies, Fe^{V} is not particularly accessible[278] and evidence has been presented that in cytochrome *c* peroxidase and perhaps prostaglandin H synthase, Compound I consists of a porphyrin $Fe^{IV}=O$ species and a protein radical. In model metalloporphyrins and horseradish peroxidase (and perhaps cytochrome P-450?) the equivalent entity exists as a $Fe^{IV}=O$/porphyrin radical cation species.[279] The $E_{1/2}$ for both 1-electron transfer steps is about +0.75 V (vs. SCE).[280] Several lines of evidence suggest that the effective $E_{1/2}$ of the cytochrome P-450 "Compound I-like" $(FeO)^{3+}$ species may be higher than that of horseradish peroxidase.[159] Both cytochrome P-450 and peroxidases start their oxidative reactions by abstracting an electron or hydrogen atom. At that point, the enzymes differ in that with cytochrome P-450 the substrate remains in place until the oxygen is rebounded to the radical, while in the peroxidases the radical is not held, but migrates away from the formal $(FeO)^{2+}$ species and participates in radical reactions.[211,274] These are relatively simple distinctions among the cytochrome P-450s and peroxidases that may explain many of the observations that have been made. With model metalloporphyrins, of course, substrate binding sites are not generally incorporated and the models may mimic the cytochrome P-450s or the peroxidases in different situations.

Horseradish peroxidase, prostaglandin synthase, and lactoperoxidase have been reported to catalyze the sulfoxidation of thiols[281-283] and the hydroxylation of *N*-methylcarbazole involves incorporation of oxygen from molecular oxygen.[284] *N*-Demethylation reactions catalyzed by peroxidases show high kinetic deuterium isotope effects, while the same cytochrome P-450 reactions do not.[197] The peroxidase-supported mechanism appears to involve rate-limiting formation of an imine by a formal hydrogen atom process, whereas in cytochrome P-450 reactions the mechanism involves rapid oxygen rebound to an α-carbon radical site generated by rearrangement of an aminium radical. The peroxidase-catalyzed oxidation has been mentioned above in terms of a sequence of transfers of an electron and a hydrogen atom. However, Bruice[206,285] has characterized the sequential 1-electron oxidation of 1,4-dihydropyridines by ferricyanide as occurring in a sequential 1-electron/proton/1-electron transfer sequence and the base-catalyzed deprotonation step is rate-limiting, yielding a significant kinetic deuterium isotope effect (Figure 17).

It is possible that such a model is applicable for the peroxidases, and the rate-limiting step would be base-catalyzed deprotonation if no specific groups are located near the active site. However, careful kinetic experiments have not yet been done on the dehydrogenation of dihydropyridines by peroxidases. Can the high kinetic deuterium isotope effects seen in peroxidase-catalyzed *N*-demethylation reactions be attributed to a rate-limiting α-H deprotonation? This might seem unlikely in that deprotonation of aminium radicals is generally considered to be rather facile, but the possibility needs to be considered that, for some reason, the rearrangement is not so rapid (see Figure 18). Again, we consider the work of Hammerich and Parker[228] and Bordwell et al.,[227] showing that deprotonation from aminium *nitrogen* can compete with the α-methylene.

As discussed previously, the possibility exists that in cytochrome P-450 reactions the $(FeO)^{2+}$ species acts as a specific base to facilitate α-deprotonation of the aminium radical, but in peroxidases (such as horseradish peroxidase)[211] the heme is buried. The substrate does not have access to the FeO entity and electron abstraction occurs through the heme edge (or even farther away if a transfer system exists); thus, the FeO is also not in a position to act in specific base catalysis.

Hemoglobin and myoglobin have been shown to carry out some peroxidative and oxidative reactions which can be explained with a mechanism involving formation of an $(FeO)^{2+}$ species and a tyrosine radical after reaction with peroxides: molecular oxygen adds to the protein radical to yield a peroxy radical that can epoxidize olefins and perform other reactions.[211] Chloroperoxidase, in addition to its best-known function of oxidizing halide ions, also catalyzes *N*-demethylation, epoxidation, and heteroatom oxygenation; it has a heme thiolate ligand and, on the basis of its spectral and catalytic properties, is more akin to cytochrome P-450 enzymes than to other peroxidases.[211] As Ortiz de Montellano et al.[111] point out, horseradish peroxidase and other peroxidases primarily oxidize substrates at the heme edge (phenyl attachment only occurs at the δ *meso* carbon of horseradish peroxidase during the oxidation of phenylhydrazine), but with chloroperoxidase reaction with the ferryl oxygen is accessible.[104-110,197]

Peroxidases carry out a number of interesting reactions with

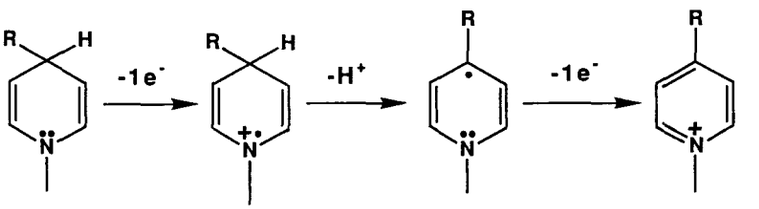

FIGURE 17. Stepwise mechanism for the dehydrogenation of 1,4-dihydropyridines by $K_3Fe(CN)_6$, cytochrome P-450, and possibly peroxidases.[196,199,203,204,282]

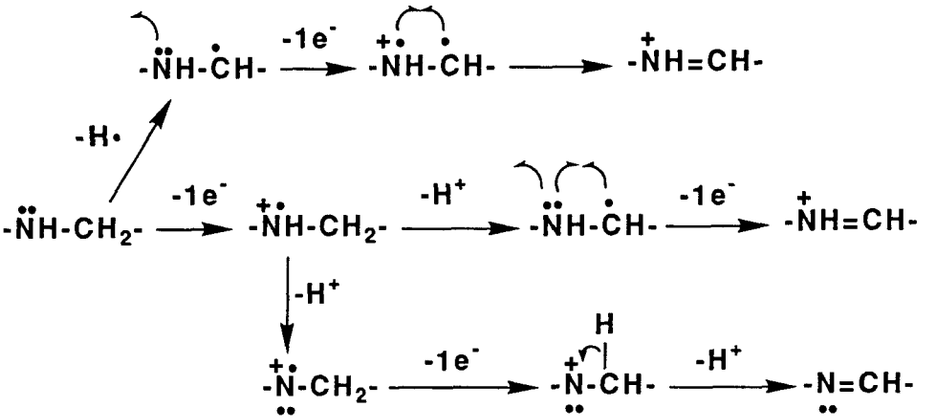

FIGURE 18. Possible mechanisms to be considered for *N*-dealkylation reactions catalyzed by horseradish peroxidase.

xenobiotic chemicals, as well as with endogenous substrates. For instance, the toxicity of benzene metabolites such as catechol and hydroquinone is thought to be enhanced by peroxidases in target tissues.[286] With this background concerning peroxidases, two peroxidases are considered, prostaglandin synthase and lignin peroxidase.

B. Prostaglandin Synthase

Prostaglandin G/H synthase (EC 1.14.99.1, prostaglandin synthase) catalyzes the conversion of arachidonic acid to the cyclic endoperoxide/hydroperoxide prostaglandin G_2, and its subsequent reduction is somewhat complex in that some hydroperoxide is apparently required to "prime" the enzyme, presumably to form Compound I. The enzyme then converts arachidonate to prostaglandin G_2, and this hydroperoxide is used as the source of further oxidizing equivalents (Figure 19).

The enzyme has been purified from sheep seminal vesicle microsomes. The protein appears to be glycosylated and functions as a dimer. Recently, cDNA clones have been isolated and sequenced to deduce the primary amino acid sequence.[287-289] One region of the sequence shows similarity to myeloperoxidase. As indicated above, the enzyme has both cyclooxygenase and peroxidase activities. Although both of these functions utilize the heme (present in each monomer), the cyclooxygenase and peroxidase activities appear to have distinct binding sites for their lipid substrates.[290,291] The heme appears to have two histidine ligands in the low-spin form of the iron — the spin population is temperature dependent.[292] The Compound I and II forms appear to resemble those of other peroxidases (*vide supra*), and the kinetics of interconversion have been described.[293] The regulation of this enzyme is of interest because of the variety of mediators that are produced from the product of the reaction. The drugs aspirin and indomethacin are thought to exert their effects through inhibition of prostaglandin synthase — aspirin appears to act by acetylating the enzyme.

Prostaglandin synthase and other peroxidases are of interest not only with respect to eicosanoids in the body, but also because they are involved in the metabolism of xenobiotic chemicals. The peroxidase function of prostaglandin synthase is rather unselective and Marnett et al.[294] observed that the enzyme would oxidize other chemicals, i.e., when arachidonate was present to generate prostaglandin G_2, the peroxidase could be utilized to oxidize another substrate. The list of substrates includes a number of drugs and carcinogens that have accessible electrons or hydrogen atoms.[32,295] The list of reactions includes the oxidation of aromatic amine carcinogens such as 2-aminofluorene, 2-naphthylamine, and benzidine, activation of the carcinogenic nitrofurans *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide and 2-amino-4-(5-nitro-2-furyl)-thiazole,[296] oxidation of diethylstilbesterol, oxidation of *p*-phenetidine and acetaminophen,[297] the 8-epoxidation of aflatoxin B_1[298] and 7,8-dihydroxy-7,8-dihydrobenzo(*a*)pyrene,[299] and the oxidation of 13-*cis*-retinoic acid to 4-hydroxy-,5,6-epoxy-, and 5,8-oxy-13-*cis*-retinoic acid and all-*trans*-retinoic acid.[300]

The prostaglandin synthase-catalyzed oxidation of drugs and carcinogens may be important in certain situations and a considerable amount of effort has been exerted toward characterization of these processes. Josephy et al.[301] found that prostaglandin synthase and horseradish peroxidase show similar behavior in the oxidation of benzidine. As pointed out above, the radicals generated by peroxidase oxidations can escape the protein and undergo addition and other reactions. Thus, the azo dimer of benzidine is formed;[301] in addition, the free radicals can be detected using EPR techniques (this is *not* the case in cytochrome P-450 reactions). The covalent DNA adducts derived from peroxidase-catalyzed oxidation of aromatic amines have been difficult to characterize. Yamazoe et al.[302] identified the benzidine-derived adducts and concluded that the 2-electron oxidation product, benzidine diimine, is the predominant reactive intermediate involved in DNA binding (in peroxidase-based systems). Of the three adducts isolated,

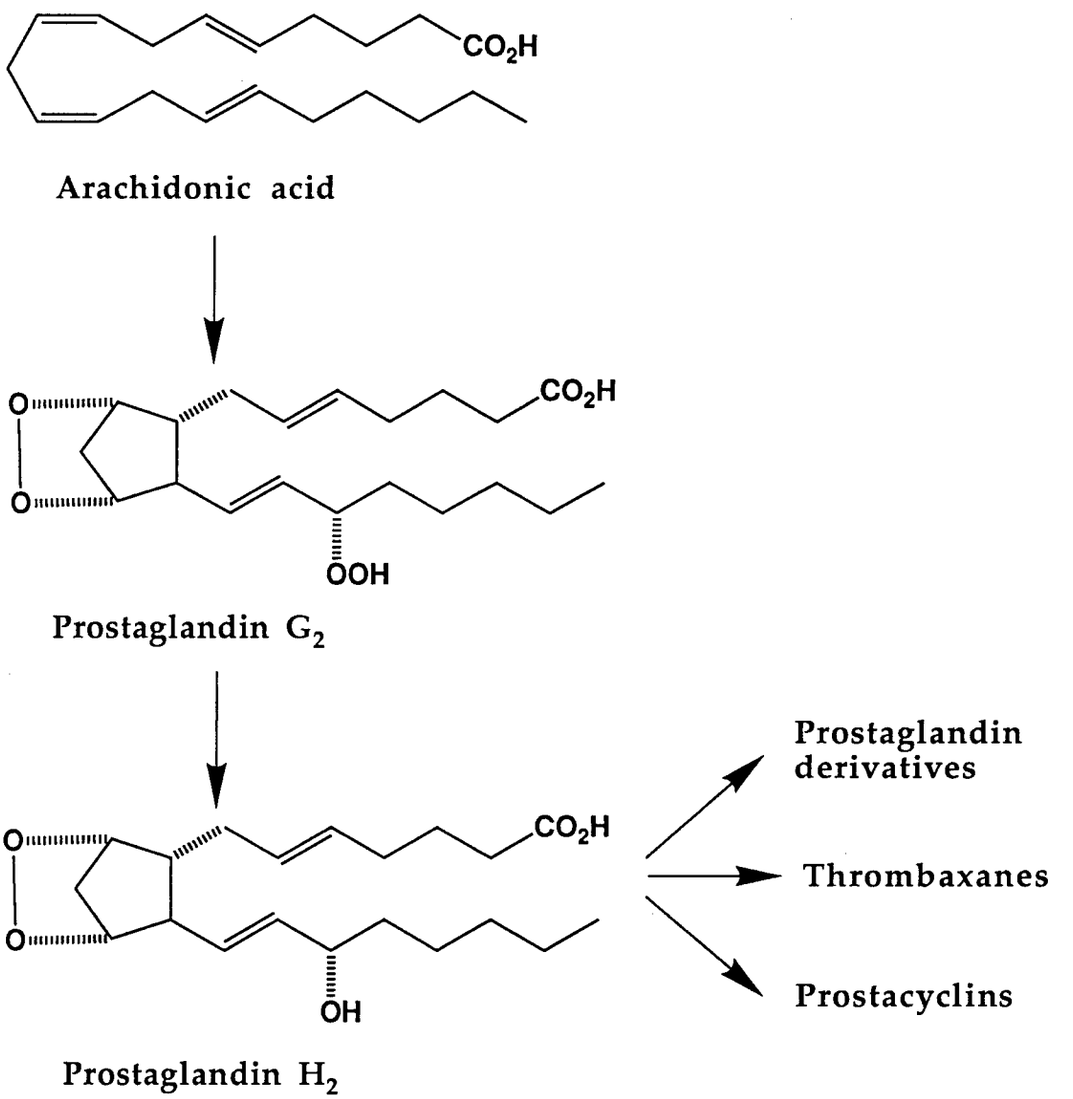

FIGURE 19. Conversion of arachidonic acid to prostaglandins G_2 and H_2 catalyzed by prostaglandin synthase.

two [*N*-deoxyguanosin-8-yl)benzidine and *N*-(deoxyguanosin-N^7-yl)benzidine] could also be derived from cytochrome P-450 reactions. The novel *N*,3-(deoxyguanosin-N^7,C^8-yl)benzidine was formed only in peroxidase incubates and is the result of oxidation of DNA that has been modified by benzidine diimine. A mechanism is suggested (Figure 20).[302]

Prostaglandin synthase does not oxidize benzo(*a*)pyrene (although lignin peroxidase does — *vide infra*), but when 7,8-dihydroxy-7,8-dihydrobenzo(*a*)pyrene is formed by the action of cytochrome P-450 and epoxide hydrolase, either cytochrome P-450 or prostaglandin synthase can catalyze subsequent oxidation to highly tumorigenic 7,8-dihydroxy-7,8-dihydro-9,10-oxobenzo(*a*)pyrene. The mechanism is quite distinct from that of the cytochrome P-450 reaction and a scheme is presented on the basis of work done with a hematin model[299,303] (Figure 21).

In this mechanism, the peroxy radical is used to form the epoxide and the source of the epoxide oxygen is molecular oxygen, as in the case of the cytochrome P-450 reaction. However, this reaction is distinguished from its cytochrome P-450 counterpart not only by the co-factors needed to support the reaction *in vitro*, but also by the observed stereochemistry.[299] In the above peroxyl radical-based reaction, (+) 7,8-dihydroxy-7,8-dihydrobenzo(*a*)pyrene is converted predominantly (80%) to the *anti*-diol epoxide, as judged by analysis of the stereochemistry of the tetraols and DNA-guanosine adducts.[304-310] In contrast, rat liver cytochrome $P\text{-}450_{\beta NF\text{-}B}$ (IA1), the best cytochrome P-450 catalyst of the epoxidation reaction, converts the (+) diol essentially only to the (+) *syn* diol epoxide. More recently, we have found that a rather distinct form of cytochrome P-450, $P\text{-}450_{NF}$ (IIIA4), is the major human cytochrome P-450 catalyzing this reaction.[311] It proceeds

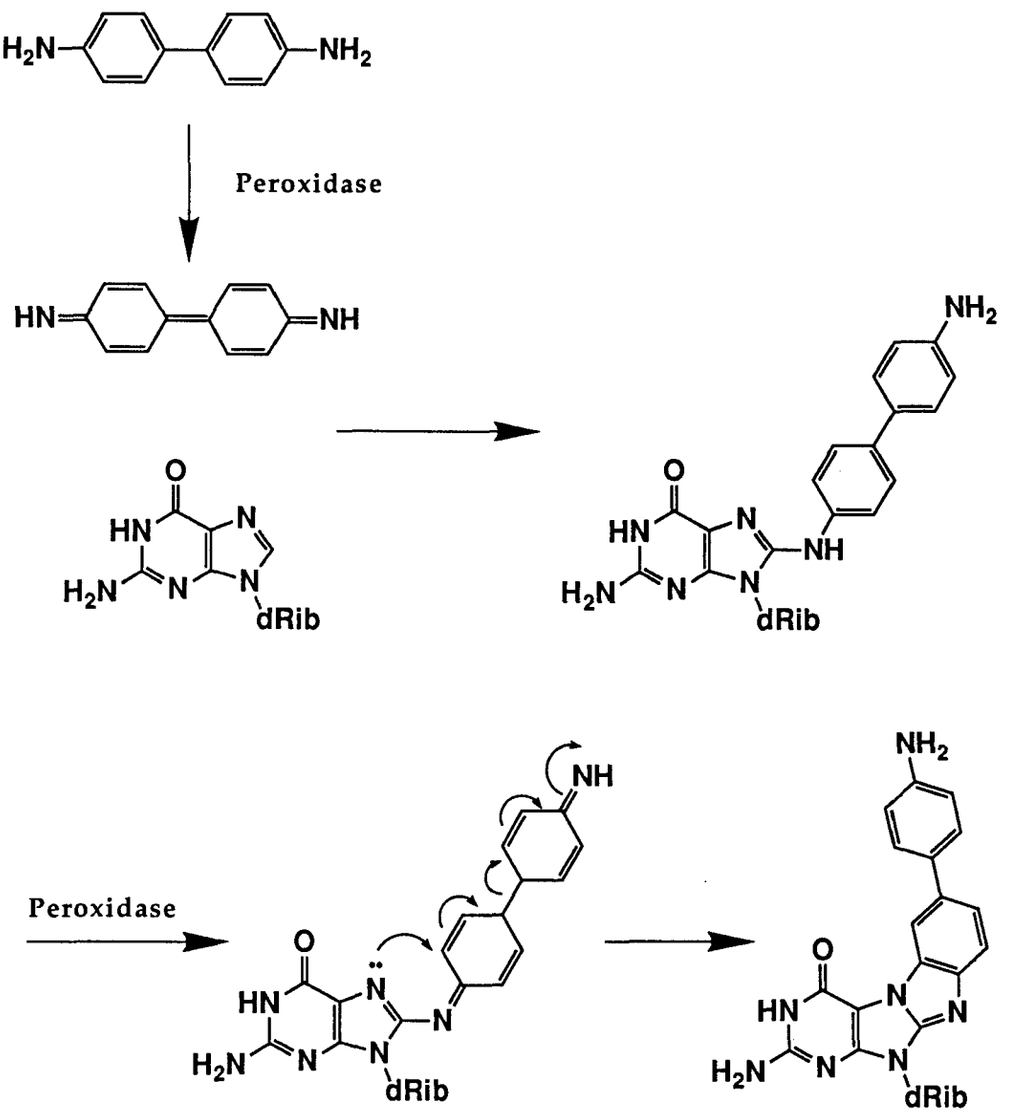

FIGURE 20. Formation of *N*,3-(deoxyguanosin-N^7,C^8-yl) benzidine by peroxidase-catalyzed oxidation of benzidine. (From Yamazoe, Y., Zenser, T. V., Miller, D. W., and Kadlubar, F. F., *Carcinogenesis*, 9, 1635, 1988. With permission.)

with less optical purity, but still forms predominantly (60 to 70%) (+) *syn*-diol epoxide from the (+) diol.[538]

Peroxidase enzymes, including prostaglandin synthase, have been shown to form styrene oxide and styrene-glutathione conjugates in the presence of glutathione.[312,313] Several reactions are possible, depending upon whether oxygen is present or not. The reactions all appear to proceed from the initial oxidation of glutathione to a thinyl radical (Figures 22 and 23). The significance of these reactions under other than *in vitro* conditions is presently unclear.

Of what practical importance is such co-oxidation of drugs and carcinogens? In liver, a major site of metabolism, prostaglandin synthase is not really present. However, in many extrahepatic tissues, cytochrome P-450 levels are very low and the importance of prostaglandin synthase (and other peroxidases) needs to be considered. Another point to be made is that little information is available concerning the variability of levels of prostaglandin synthase among individual humans or the factors that regulate the activity in humans or experimental animals. Rates of prostaglandin synthase-catalyzed epoxidation of aflatoxin B_1 are very low and do not contribute substantially to the tumorigenicity of this hepatocarcinogen.[298]

While studies with enzymes and cellular extracts can be done by manipulation with co-factors and the like, problems arise in establishing the contribution of the peroxidative pathways in intact cells and *in vivo*. Two approaches have been used.

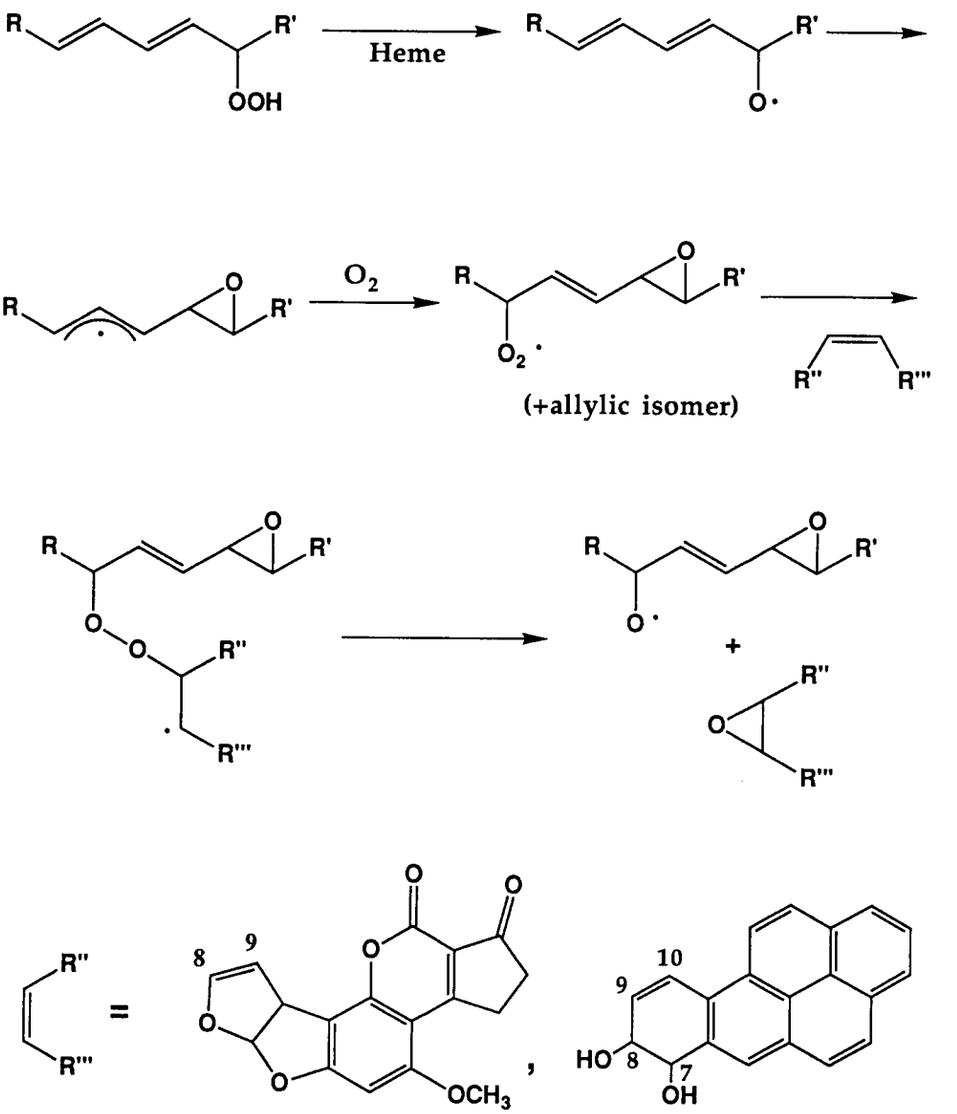

FIGURE 21. Proposed mechanism for the heme- or prostaglandin synthase-catalyzed epoxidation of olefins by hydroperoxides. The structures of aflatoxin B_1 and 7,8-dihydroxy-7,8-dihydrobenzo(*a*)pyrene are shown as representative olefins for epoxidation. (From Dix, T. A., Fontana, R., Panthani, A., and Marnett, L. J., *J. Biol. Chem.*, 260, 5358, 1985. With permission.)

One involves the administration of inhibitors of prostaglandin synthase to animals. Zenser and Cohen reported that dietary aspirin can inhibit nitrofurantoin-induced precancerous lesions and carcinomas of the rat urinary bladder.[314,315] Adriaenssens et al.[316] reported that the prostaglandin synthase inhibitors aspirin and indomethacin did not reduce the level of pulmonary DNA adducts derived from benzo(*a*)pyrene or the number of adenomas. However, Kraus and Eling[32] have pointed out some of the difficulties in interpreting the results of experiments of this type, because other physiological effects may complicate the intended purpose of the inhibitors.

Another approach involves the measurement of nucleic acid adducts specifically derived from the prostaglandin synthase pathway (as opposed to other known enzymatic reactions involving the same substrate). Indeed, the most extensive use of this approach has been made with adducts derived from (+) 7,8-dihydroxy-7,8-dihydrobenzo(*a*)pyrene, making use of the differences in stereochemistry associated with oxidation via the different pathways (*vide supra*).[317]

The substrates that can be activated by prostaglandin synthase include aromatic amines and phenols that can be readily oxidized. Most of these compounds are also cytochrome P-

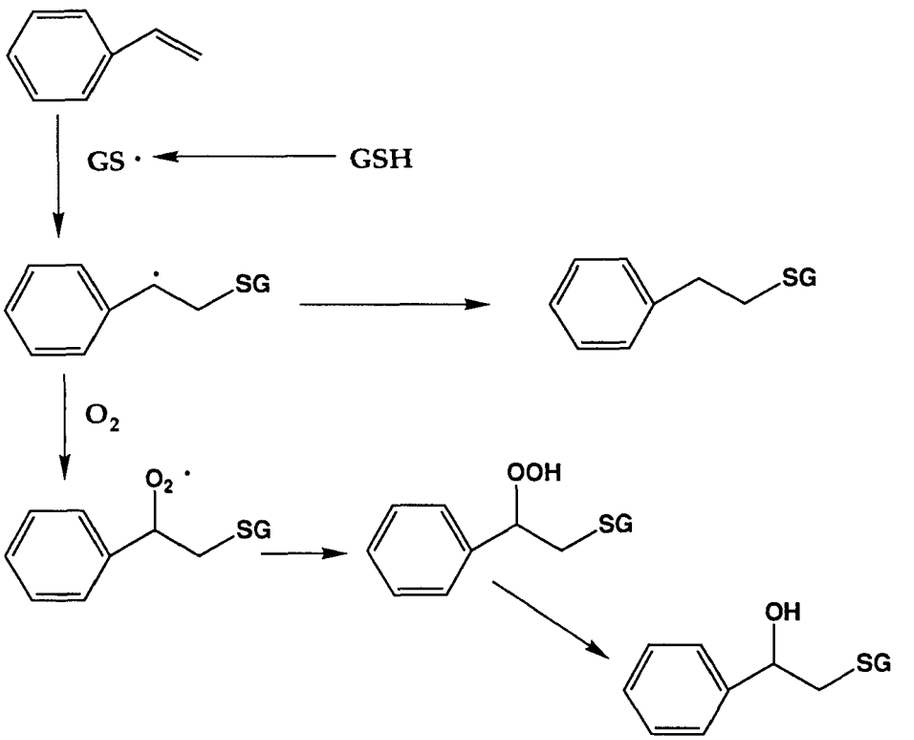

FIGURE 22. Postulated mechanism of peroxidase-catalyzed formation of a glutathione conjugate of styrene.[312] (From Stock. B. H., Schreiber, J., Guenat, C., Mason, R. P., Bend, J. R., and Eling, T. E., *J. Biol. Chem.*, 261, 15915, 1986. With permission.)

450 substrates, but the products and catalytic mechanisms differ.[318,319] Acetaminophen can be oxidized by prostaglandin synthase[276,320-322] and this reaction is the major oxidation process for the compound in some tissues.[323] Many carcinogenic aromatic amines can be oxidized by prostaglandin synthase[32]: purified ram prostaglandin synthase catalyzed the activation (to covalently bound DNA adducts) in the order benzidine >> 2-naphthylamine ~ 2-amino-6-methyldipyrido[1,2-a:3′,2′-d]imidazole (Glu-P-1) > 4-aminobiphenyl > 2-amino-3-methylimiadzo[4,5-f]quinoline (IQ) > 3-amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2).[324] Human bladder and colon microsomes showed a similar order of reactivity in the presence of arachidonic acid. Activity toward benzidine was detected in human bladder epithelium, prostatic epithelium, colonic mucosa, and peripheral lung tissue, but not in placenta, breast, or liver microsomes.[324] Other studies have shown localization of the enzymes in the inner medulla region of the kidney.[323,325]

Another matter of interest is the elucidation of factors involved in the regulation of expression of the enzyme. The work of Flammang et al.[324] showed a 200-fold variation in *in vitro* benzidine activation among human bladder epithelial samples, and these differences may be of relevance to cancer incidence. Wu et al.[326] reported that phorbol esters stimulate *de novo synthesis* of the enzyme and estimated that the $t_{1/2}$ of the enzyme is less than 10 min.

C. Lignin Peroxidase (Ligninase)

Another interesting peroxidase is the enzyme that cleaves lignin, one of the major constituents in wood. As we will see, this enzyme may have considerable usefulness not only in the degradation of wood, but also in the destruction of unwanted chemicals. Lignin is linked by β-aryl ether linkages, which must be broken in degradation.[327] The synthesis of lignin is actually catalyzed by other peroxidases present in plants.[328] The most efficient degradation of lignin is done by the white-rot fungi, and most studies have employed the basidiomycete *Phanerochaete chrysosporium*. There are probably several different ligninase proteins, as judged by chromatographic behavior. A cDNA clone has been isolated and sequenced.[329] Cloning and genomic blotting experiments indicate the presence of several related genes, but correspondence of the sequence to individual proteins has not been ascertained. The proteins are glycosylated.

Resonance Raman spectra indicate that the ferric enzyme is high spin/pentacoordinate at 25°C, but becomes high spin/hexacoordinate at ≤2°C.[330] Ligninase reacts with H_2O_2 and has defined Compound I, II, and III ($Fe^{II}O_2$) forms not dissimilar from other peroxidases, and some kinetic measurements have been made on rates of interconversion of these forms.[331-333] The enzymes appear to operate by sequential electron transfers. The Raman spectra are consistent with a low-spin/hexacoor-

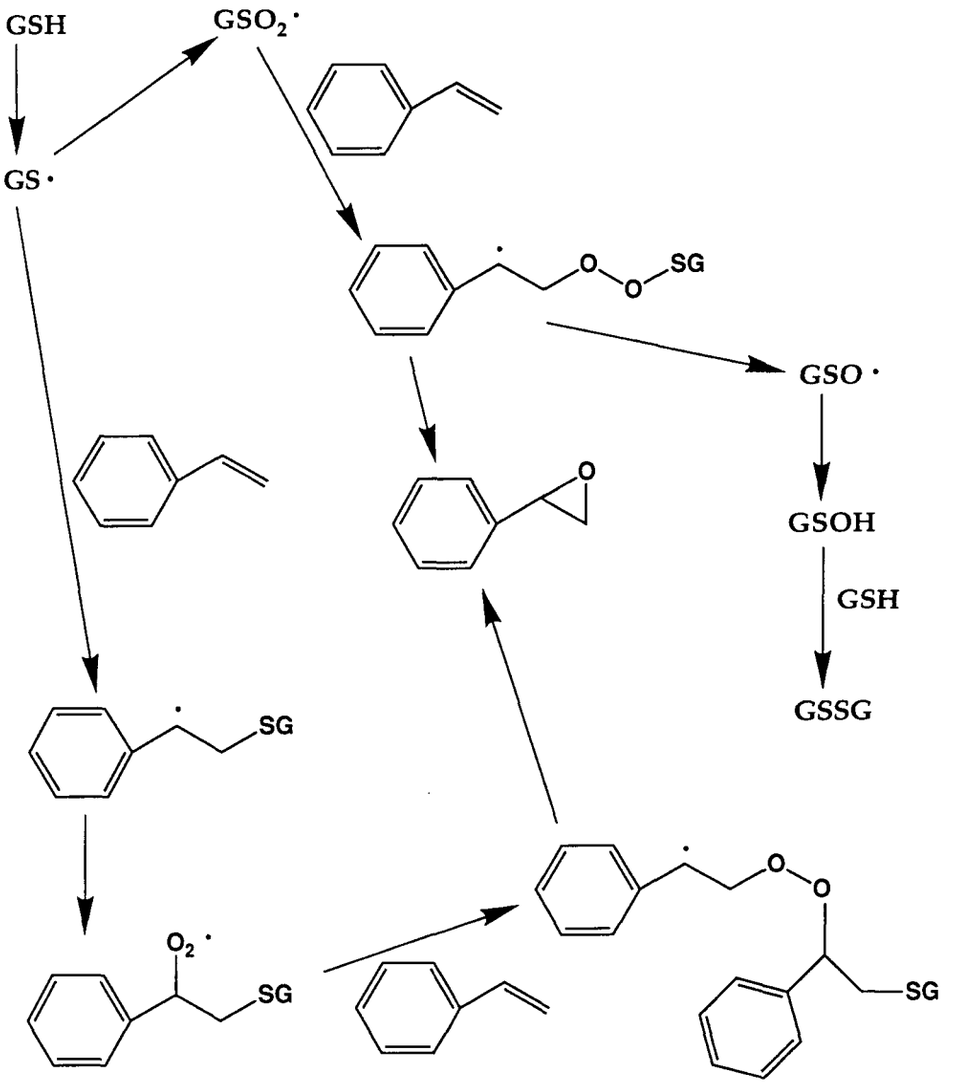

FIGURE 23. Postulated mechanism of formation of styrene-7,8-oxide from styrene in a glutathione- and peroxidase-dependent reaction.[313] (From Ortiz de Montellano, P. R. and Grab, L. A., *Mol. Pharmacol.*, 30, 666, 1986. With permission.)

dinate $Fe^{IV} = O$ structure for lignin peroxidase Compound II.[330] It should be pointed out that the predicted sequence of the protein contains portions that are conserved with turnip, cytochrome *c*, and horseradish peroxidases and are thought to be the sites surrounding the axial and distal histidine ligands.[329]

The reaction mechanism of lignin peroxidase involves oxidation of aromatic substrates to their cation radicals.[334] All subsequent reactions appear to be nonenzymatic and lead to a variety of products. Models for the enzyme include iron tetraphenylporphin/*tert*-butylhydroperoxide (*vide infra*) and tris (phenanthroline) Fe^{III}.[335] Fenton's reagent does not appear to be a satisfactory model.[327]

The reaction of a model cross-link of lignin is shown in Figure 24.[327]

The ring-centered radical can also react, either anaerobically or with molecular oxygen, to form peroxyl radicals and possibly cyclic peroxides.[336-338] Through mechanisms such as those presented in the cited references, lignin is degraded to a variety of low-molecular-weight products. There are some indications that the high-valent states of lignin peroxidase (generated by reaction with H_2O_2) may have higher potentials than the corresponding states of classic peroxidases,[334] and this property may explain the ability of ligninase to carry out some of the oxidations that it does.

Lignin peroxidase can oxidize polynuclear aromatic hydrocarbons such as benzo(*a*)pyrene.[334,339] The products are quinones. The ability of the enzymes to oxidize polycyclic hydrocarbons and halogenated hydrocarbons such as DDT [1,1-

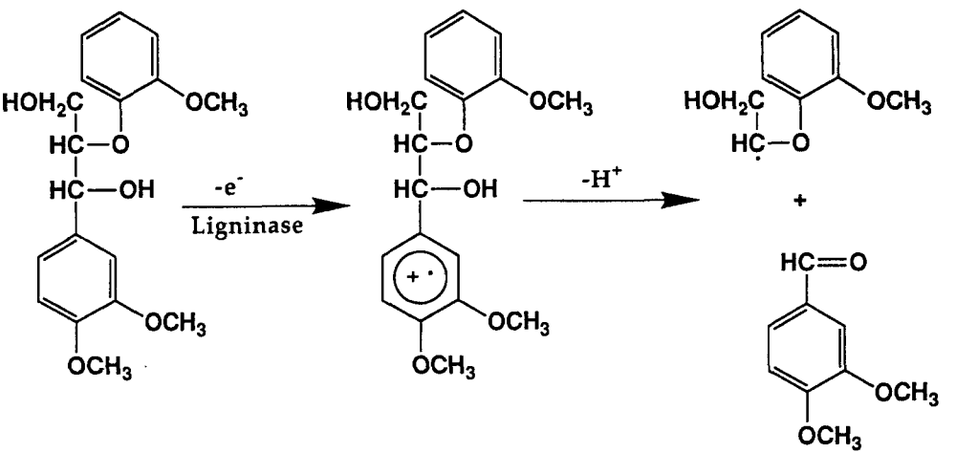

FIGURE 24. Oxidation of a lignin model by lignin peroxidase. (From Stock, B. H., Schreiber, J., Guenat, C., Mason, R. P., Bend, J. R., and Eling, T. E., *J. Biol. Chem.*, 261, 15915, 1986. With permission.)

bis(4-chlorophenyl)-2,2,2-trichloroethane], TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin), and lindane (1,2,3,4,5,6-hexachlorocyclohexane) suggests that the enzyme and/or organism may be of use in the destruction of unwanted chemicals in the environment, as in the case of waste dumps.[340,341]

Ligninase can also function as a haloperoxidase, oxidizing bromide and iodide (but not chloride).[342] The positively charged halogen atoms are incorporated into monochlorodimedone and model olefins and aromatic compounds. The same enzyme also catalyzes the dechlorination of polychlorinated phenols.[343] A mechanism is shown in Figure 25.

It would be of interest to know exactly what the $E_{1/2}$ of ligninase is. The high-valent forms may be stable enough to apply the approach of Hayashi and Yamazaki.[280] Why the or-

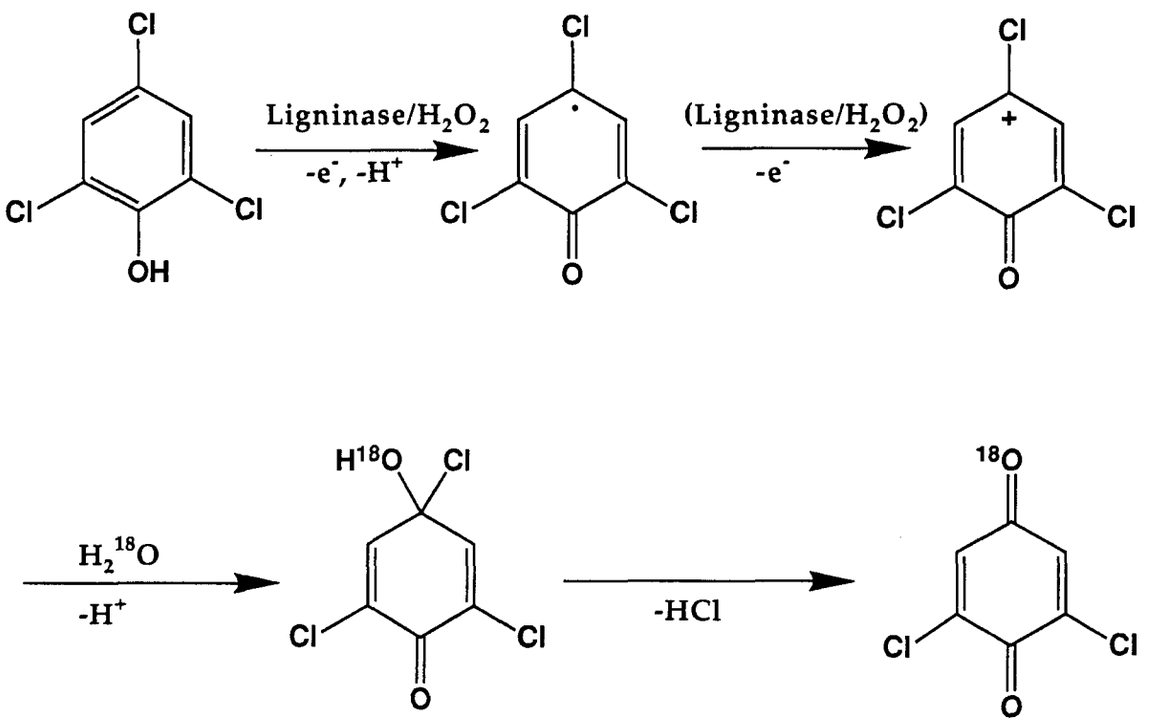

FIGURE 25. Proposed mechanism of dechlorination of a chlorinated phenol by lignin peroxidase. (From Hammel, K. E. and Tardone, P. J., *Biochemistry*, 27, 6563, 1988. With permission.)

ganism contains a complex gene family for the production of these enzymes is unclear, since the enzyme would appear to have a very broad specificity.

V. FLAVOPROTEINS

A. FAD-Containing Monooxygenase

Many amino and sulfur compounds are oxygenated by the flavoprotein usually termed FAD (or flavin)-containing monooxygenase (EC 1.14.13.8, dimethylaniline monooxygenase ([*N*-oxide-forming]), an enzyme first isolated from hog liver by Ziegler and Mitchell.[344] The protein is localized in the endoplasmic reticulum and has a molecular weight of about 56,000, as estimated by electrophoretic mobility. Most other species contain a similar enzyme that shows immunochemical cross-reactivity,[345] and the enzyme has now been purified from rat and mouse liver microsomes.[346,347] The work of Mehendale and co-workers[348] suggested intertissue differences in the enzyme. The enzyme was purified from rabbit[349] and mouse[350] lung microsomes; in each species, the liver and lung enzymes could be distinguished by apparent monomeric molecular weight, catalytic properties, and immunochemical behavior.

The chemical mechanism of catalysis is fairly well accepted (Figure 26). The FAD is reduced by NADPH, and the dihydroflavin reacts with molecular oxygen to form the C-4a hydroperoxide, which has been observed spectrally.[28] Biomimetic model N-5 alkyl C-4a hydroperoxyflavins are known to be able to react directly with amines to transfer oxygen,[351] and this is the accepted mechanism for this enzyme. The reaction cycle is completed by dehydration of the resulting C-4a hydroxyflavin and release of $NADP^+$ from the enzyme. Thus, the overall stoichiometry is that of mixed-function oxidation ($AH_2 + R + O_2 \rightarrow A + RO + H_2O$), as in the case of the cytochrome P-450 enzymes, but the chemistry is quite distinct. The mechanism of oxygen transfer is heterolytic, as opposed to homolytic, and the difference leads to distinct reactions, as we will see later.

Ziegler has reviewed the catalytic specificity of the enzyme in a series of articles.[28,29,224] Substrates for the hog liver enzyme include acyclic and cyclic secondary and tertiary amines, mono- and di-substituted hydrazines, hydroxylamines, thiols, disulfides, sulfides, thiocarbamides, thioamides, mercaptopurines, mercaptopyrimidines, and some other *N*- and *S*-containing compounds.[352] Primary alkyl amines are substrates for the rabbit lung enzyme, but not the hog liver enzyme.[353-355] Only some primary arylamines[356] are substrates for the liver enzyme, and Ziegler et al.[357] have shown that *N*-methylation renders arylamines much better substrates for such *N*-oxygenation. Ballou has reported that iodide, thiocyanate, and borate are oxidized by the hog liver enzyme.[358] In the catalytic mechanism, the rate of flavin dehydration or $NADP^+$ release (following substrate oxidation) is rate limiting and the enzyme appears to accumulate as the C-4a hydroperoxide form.[359-361] Thus, the rates of oxidation of many substrates are very similar (i.e., V_{max} values are very similar). However, it has been pointed out that this conclusion is based largely on results measured on the basis of oxygen uptake; the possibility should be considered that some substrates might catalyze the breakdown of the C-4a hydroperoxide (to H_2O_2?) without accepting an oxygen atom.[28] With the hog liver enzyme, addition of a charged

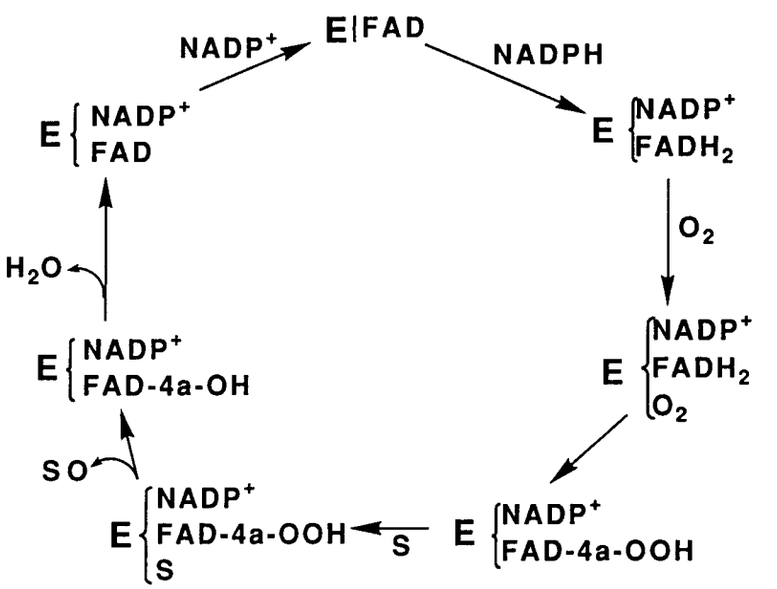

FIGURE 26. Catalytic mechanism of FAD-containing monooxygenase. (From Ziegler, D. M., *Drug Metab. Rev.*, 19, 1, 1988. With permission.)

moiety (anion or cation) to the substrate (in addition to the amine) results in a loss of catalytic activity. The hog liver enzyme is also stimulated *in vitro* by the presence of primary alkylamines such as *n*-octylamine, but the rabbit lung enzyme is not.[349,353] It is possible that the stimulation is due to enhanced rates of (base-catalyzed) decomposition of the C-4a hydroxyflavin, although some alternative mechanisms cannot be discounted.

Although much is known about this enzyme, several major questions still need to be answered. The primary sequence is unknown for any of the flavin-containing monooxygenases. The N-terminus may be blocked,[539] and no cDNA clones have been reported. As pointed out above, there are some differences in the enzyme in different animal species and tissues, and the human enzyme(s) has not been isolated. Obviously, the catalytic properties of the human enzyme are of chief concern in consideration of human health. Of interest in this respect are the recent observations of Smith and co-workers concerning the polymorphic distribution of phenotypic trimethylamine oxidase activity in humans. Several considerations suggest that the lack of oxidation seen in 1 to 2% of humans is more likely due to lack of the flavin-containing monooxygenase and not to an excess of trimethylamine *N*-oxide reduction.[362,363] Obviously, elucidation of the mechanism underlying the apparent deficient expression of the enzyme would be of interest, as would studies on the impaired ability of affected individuals to oxidize drugs known to be metabolized by the enzyme.

The identification of distinct forms of the enzyme in rabbit lung and liver very strongly suggests that a multi-gene family is involved. The question of how many genes/gene-like sequences are in the family arises, as well as how many are expressed. The question of whether or not multiple proteins are expressed in a single tissue still remains. Preliminary studies in the author's own laboratory suggest that more than one form of the enzyme can be isolated from human liver microsomes, as judged by the results of fractionation studies. However, this view must be regarded as tenuous until proteins can be completely purified and more appropriately characterized.

Some questions can also be raised concerning the catalytic mechanism. With the report of oxidation of iodide, thiocyanate, and borate by the C-4a hydroperoxide,[358] consideration can be given to other potential substrates. The chemistry involved in the flavin-catalyzed reaction resembles that of hydroperoxides, which carry out several reactions, including epoxidation of olefins. Other flavoprotein monooxygenases catalyze reactions such as aromatic hydroxylation. However, flavoprotein monooxygenases tend to hydroxylate only those aromatic positions already activated, i.e., ortho to an $-OH$ or $-NH_2$ group.[364,365] The flavin-containing monooxygenase, likewise, has been shown to catalyze the 2-hydroxylation of 1-naphthylamine and the 1-hydroxylation of 2-naphthylamine (Figure 27).[256]

This reaction is not unexpected on the basis of flavin model chemistry, and it may be possible to find cases in which this enzyme will hydroxylate phenols. However, Bruice et al.[366] carried out an extensive study with flavin 4a-hydroperoxide models and could not obtain olefin epoxidation. Thus, it seems unlikely that this enzyme will be capable of epoxidizing olefins. Many sulfur compounds are excellent substrates and a charged group is apparently not critical. Some other atypical substrates may be revealed in the future.

B. Monoamine Oxidase

Monoamine oxidase (EC 1.4.3.4, amine oxidase [flavin-containing]) is a FAD-containing enzyme that oxidizes amines to their corresponding aldehydes:

$$RCH_2NH_2 + O_2 + H_2O \rightarrow RCHO + H_2O_2 + NH_3$$

Most of the known substrates are primary amines, but the tertiary amine 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is also oxidized by the enzyme to the dihydropyridine and pyridine products,[367] the latter of which appears to be responsible for the toxic effects of the MPTP, a drug of abuse[368,369] (Figure 28).

Monoamine oxidase has attracted considerable interest over the years because inhibitors of the enzyme show therapeutic effects in the treatment of clinical depression and Parkinson's disease.[50] The enzyme is found in many tissues and has usually been isolated from the liver and placenta;[372-375] the brain enzyme is of major interest. The subcellular localization is the outer mitochondrial membrane.[376,377] Synthesis of the enzyme can be regulated by glucocorticoids and steroid hormones,[378] although details of the mechanism are not available at the nucleic acid level. The FAD-containing protein is not to be confused with a copper-containing plasma amine oxidase that catalyzes some similar reactions.[379] Many reviews on the FAD-containing monoamine oxidase have appeared over the years and the reader is directed to some of these.[50,380-384]

At least two forms of the enzyme exist in each animal species, the A and B forms. This duplicity has been recognized for 20 years, but only very recently has the basis been defined.[384-387] The two forms differ in their specificity with respect to inhibitors (A: clorgyline, B: deprenyl), substrates (A: serotonin, B: benzylamine), and regulation and developmental expression.[384,388] For more complete compilations of substrates and inhibitors for the individual forms, see the reviews by Tipton[381,383a] and others.[389,390] Over the years a number of possibilities have been raised regarding the basis of the A and B forms:

1. The A and B activities reside in a single protein that has two distinct substrate binding sites.
2. A single protein exists and the different catalytic activities are a function of the position of individual protein molecules in the different microenvironments of the membrane.

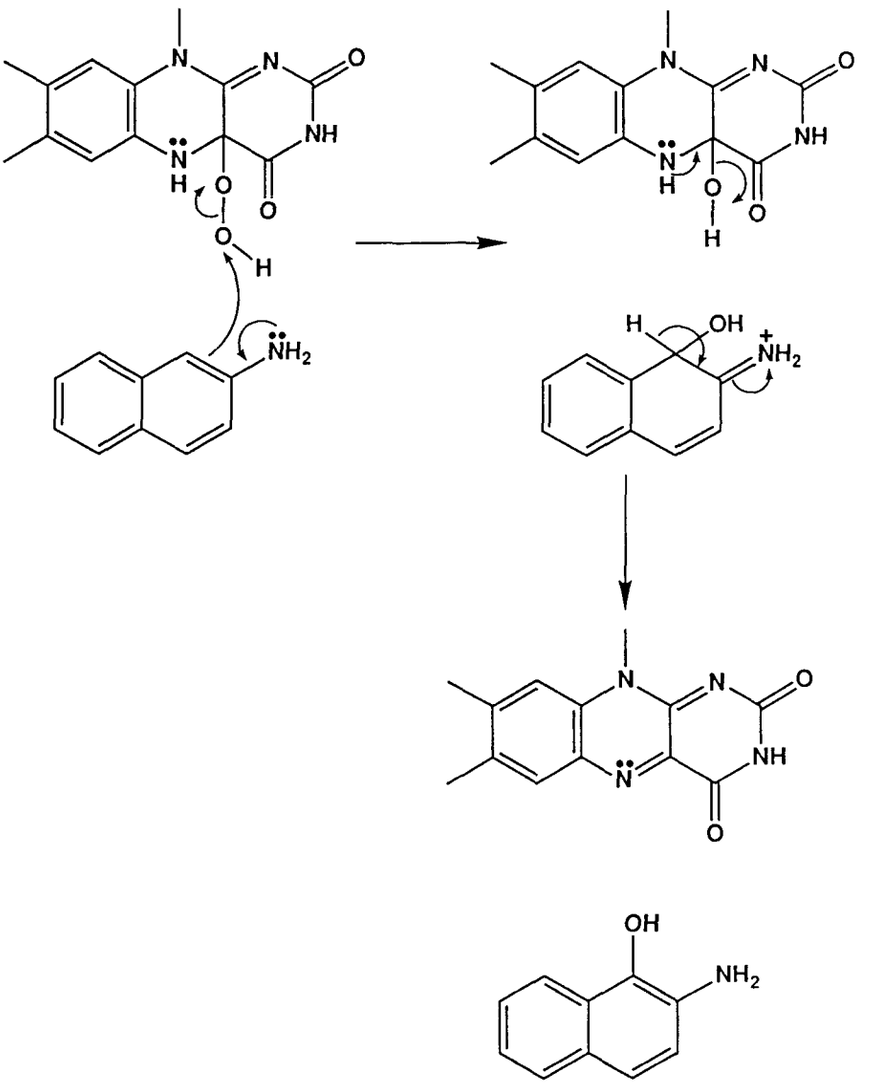

FIGURE 27. Postulated mechanism of 1-hydroxylation of 2-naphthylamine (by FAD-containing monooxygenase).[256,364,365]

3. The A and B activities are the result of posttranslational modification of a protein, i.e., only part of the protein pool is modified and gives rise to the differences in catalytic activity.
4. Monoamine oxidase A and B are related but distinct enzymes that are encoded by different genes.[391]

The evidence is now very strong that monoamine oxidase A and B are indeed distinct gene products. Evidence comes from peptide mapping[392] and studies done with monoclonal antibodies.[384] Bach et al.[393] used oligonucleotide probes derived from sequenced peptide fragments to isolate human liver cDNA clones that encode the A and B proteins. The predicted amino acid sequences are 70% identical and both contain the pentapeptide Ser-Gly-Gly-Cys-Tyr, which had been identified as the site of the covalently bound Cys in both the A and B proteins.[394]

A number of primary amines are substrates for the enzymes. The endogenous biogenic amines, particularly neurotransmitters, appear to be the endogenous substrates, although many xenobiotic amines can also serve as substrates. Although for many years only primary amines were thought to be substrates for the enzyme, more recently monoamine oxidases have been shown to dehydrogenate the tertiary amine MPTP in a stepwise manner to the dihydropyridine and then to the pyridine derivative (Figure 28).[395] This oxidation sequence results in the bioactivation of MPTP, with the pyridinium compound apparently being responsible for the destruction of dopaminergic neurons

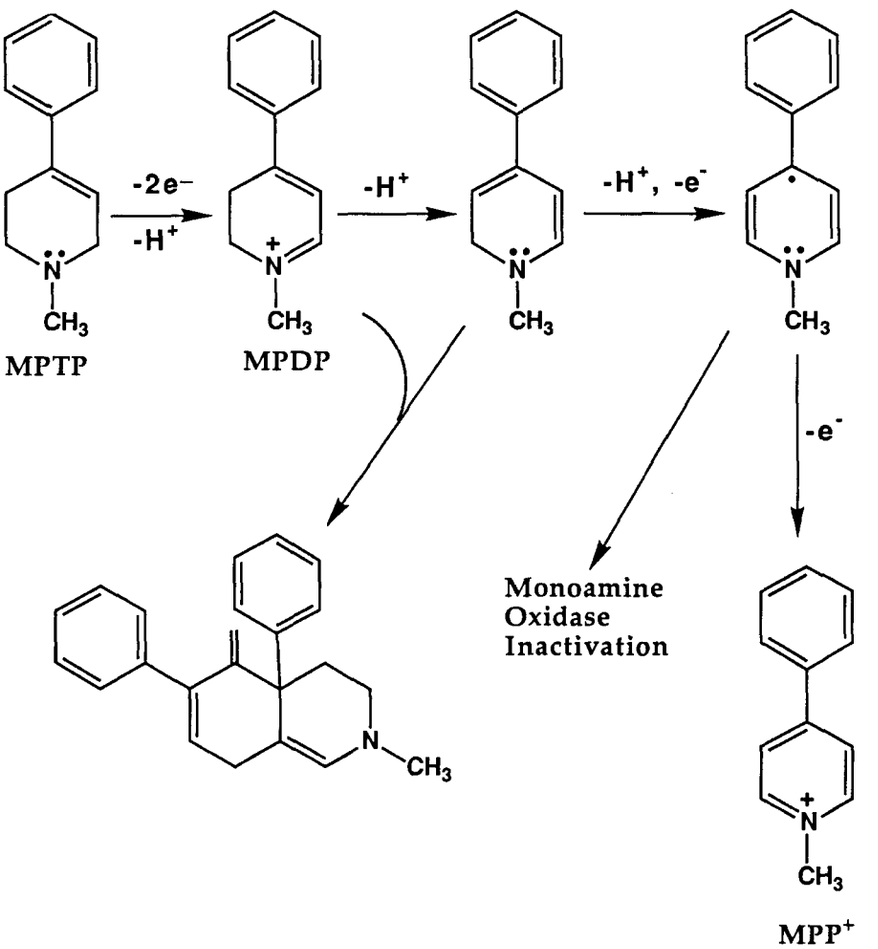

FIGURE 28. Oxidation of MPTP by monoamine oxidase.[370,371]

in the substantia nigra due to inhibition of mitochondrial electron transport. The catalytic specificity of monoamine oxidase A and B has been considered in other reviews.[381,384] For many years, inhibitors of monoamine oxidase have been of interest because of their potential in the treatment of clinical depression. The monoamine oxidase inhibitor deprenyl, used in combination with L-DOPA, has been reported to provide symptomatic relief in Parkinson's disease.[383] Elevated levels of monoamine oxidase B activity have been observed in schizoaffective schizophrenia, unipolar depression, senile dementia, and Huntington's chorea, so it is possible that monoamine oxidase inhibitors may find additional therapeutic uses. However, lowered platelet monoamine oxidase activities have been reported in schizophrenia, bipolar affective disorders, alcoholism, cycloid psychoses, attention deficit disorder in children, epilepsy, insulin-dependent diabetes, Lesch-Nytian syndrome, migraine headache, iron deficiency anemia, riboflavin deficiency, Down's syndrome, pregnancy toxemia, and thyrotoxicosis.[396] It is probably not possible to associate altered levels of monoamine oxidase with the etiology of all of these disorders, and in some cases changes in levels of monoamine oxidase may be a secondary result of a primary lesion associated with the disease. Platelet monoamine oxidase activities have been reported to vary in humans by an order of magnitude, and the significance of this variation in terms of toxicity of xenobiotics is unclear. However, it should be pointed out that a careful comparison of the regulation of monoamine oxidase in different human tissues has not been made, and platelet activities may not reflect activity in sites such as the liver and brain. The literature on monoamine oxidase inhibitors has been reviewed elsewhere.[50,383]

The kinetic aspects of monoamine oxidase B are rather complex. Studies by Singer and co-workers[397,398] indicate that the mechanism may involve either a binary complex ("ping-pong") mechanism or a ternary complex (reduced enzyme, O_2, and substrate or product). With β-phenylethylamine, the reaction of reduced flavin with O_2 is rate limiting; with benzylamine and MPTP, a ternary complex is observed.

Flavoproteins can, at least in principle, oxidize amines by both stepwise and concerted electron transfer processes (see

References 364 and 365). Silverman and co-workers have studied the mechanism of beef liver monoamine oxidase B with cycloalkylamines, which have been utilized clinically as monoamine oxidase inhibitors for a number of years. Inactivation of monoamine oxidase with 2-phenylcyclopropylamine and trapping of the product released from the protein by acid hydrolysis yielded cinnamaldehyde[399] and not 2-phenylcyclopropanone.[400] The mechanism of inactivation is proposed to involve transfer of one electron from the amine to the flavin.[401] The radical generated by scission of the cyclopropyl group is believed to be captured by the flavin radical, and the product of the reaction with *N*-(1-methylcyclopropyl) benzylamine is the imine of benzylamine and 4-flavinyl-2-butanone.[402] The inactivation of monoamine oxidase B (and A)[403] by 1-substituted cyclopropylamine rules out the formation of cyclopropanone/imine compounds in the inactivation process[400] and probably in the general mechanism of oxidation. *N*-Benzyl-substituted cyclopropylamines are effective inhibitors of monoamine oxidase, and the addition of an α-methyl group at the benzylic carbon has a dramatic effect in decreasing the partition ratio between benzaldehyde formation and monoamine oxidase inhibition.[404] Further evidence for stepwise electron transfer in the mechanism of monoamine oxidase was obtained with the substrate 1-phenylcyclobutylamine, which undergoes oxidative ring expansion (to 2-phenyl-1-pyrroline) characteristic of a radical intermediate and also inactivates the enzyme.[213] The nature of the enzyme inactivation is not completely understood, but both a stable flavin product and a protein adduct, which can be released, are formed.[405,406] The flavin product (minor) appears to contain a N^5 alkyl group; for instance, 1-phenylcyclopropylamine gives rise to the (flavinyl-N^5)-propiophenone (see Figure 29).

The fact that the FAD is covalently attached to the apoprotein through the C-8a methylene linkage has precluded unambiguous characterization of the structure. The apoprotein-bound adduct (major) can be released by amines, which apparently cause the adduct to undergo a retro-aldol cleavage from the putative cysteine to which it is attached (Figure 30).[406,407]

Organosilicon and organogermanium compounds have also been shown to serve as mechanism-based inactivators of monoamine oxidase.[408,409]

What are some of the outstanding issues involving monoamine oxidase today? One of the first questions to be considered is how the FAD molecule becomes covalently attached to the apoprotein.[410] This problem pervades all of the proteins containing covalently bound flavin and remains largely unsolved. A likely explanation is activation of the 8a-methyl group (for instance, by hydroxylation and sulfation) and nucleophilic attack by the cysteinyl moiety. Monoamine oxidase appears to exist as a dimer that has only one monomer with flavin bound. Unfortunately, the need for such a posttranslational modification complicates systems to be used for vector-based expression of cDNA clones and, also unfortunately, such systems are needed to understand the catalytic specificity of the individual gene products. Another critical question is how many related gene products are actually expressed in the monoamine oxidase family. While all of the discussion has been about the A and B proteins for some time, the reader should bear in mind that at one point only two cytochrome P-450 enzymes were thought to exist.[99] The work of Bach et al.[393] shows two mRNA bands hybridizing to the monoamine oxidase A probe. Thus, it is possible that more than one gene may exist. Genomic DNA blotting has not been reported; the use of long, nonoverlapping cDNA probes with genomic DNA is one way of estimating the minimum number of gene-like sequences in a multi-gene family.[411] The availability of nucleic acid probes should facilitate searches for other genes as well as studies on factors involved in the regulation of the individual mRNAs and enzymes. In this respect, we still need to know the significance of variation in levels of the enzyme (actually *each* enzyme) among people and what factors control tissue-specific expression. The finding that MPTP is a substrate changed the view that only primary amines are substrates for the enzyme. Are MPTP and the few related compounds only anomalies or are there many more xenobiotics that may prove to be substrates? Several possibilities exist, one being the parasympathomimetic alkaloid slaframine (Figure 31).

Slaframine can be oxidized photochemically in the presence of flavins to a parasympathomimetic form.[412] Although some evidence was obtained that the microsomal flavin-containing monooxygenase can catalyze the oxidation, there are still important considerations about the mechanism and significance of this reaction and a monoamine oxidase reaction should be considered.

Finally, several epidemiological studies suggest that at least some cases of Parkinson's disease may be caused by environmental factors.[413] This view is based upon geographical distribution in the occurrence of the disease. Thus, the possibility exists, by analogy to the severe Parkinson-like syndrome produced by MPTP, that a natural product or synthetic chemical may exist that can be oxidized by monoamine oxidase to produce an active form. However, if such a compound does exist (and finding it will be extremely difficult), there is no reason to conclude *a priori* that it requires activation by monoamine oxidase or, for that matter, any enzyme at all. In summary, much can probably still be learned about the catalytic specificity of monoamine oxidases with respect to the oxidation of xenobiotic chemicals.

VI. DOPAMINE β-HYDROXYLASE

Dopamine β-hydroxylase (EC 1.14.17.1, dopamine β-monooxygenase) is an enzyme located in the catecholamine-secreting vesicles (chromaffin granules) of the adrenal medulla and certain synaptic vesicles. The enzyme catalyzes the hydroxylation of dopamine to the neurotransmitter norepinephrine

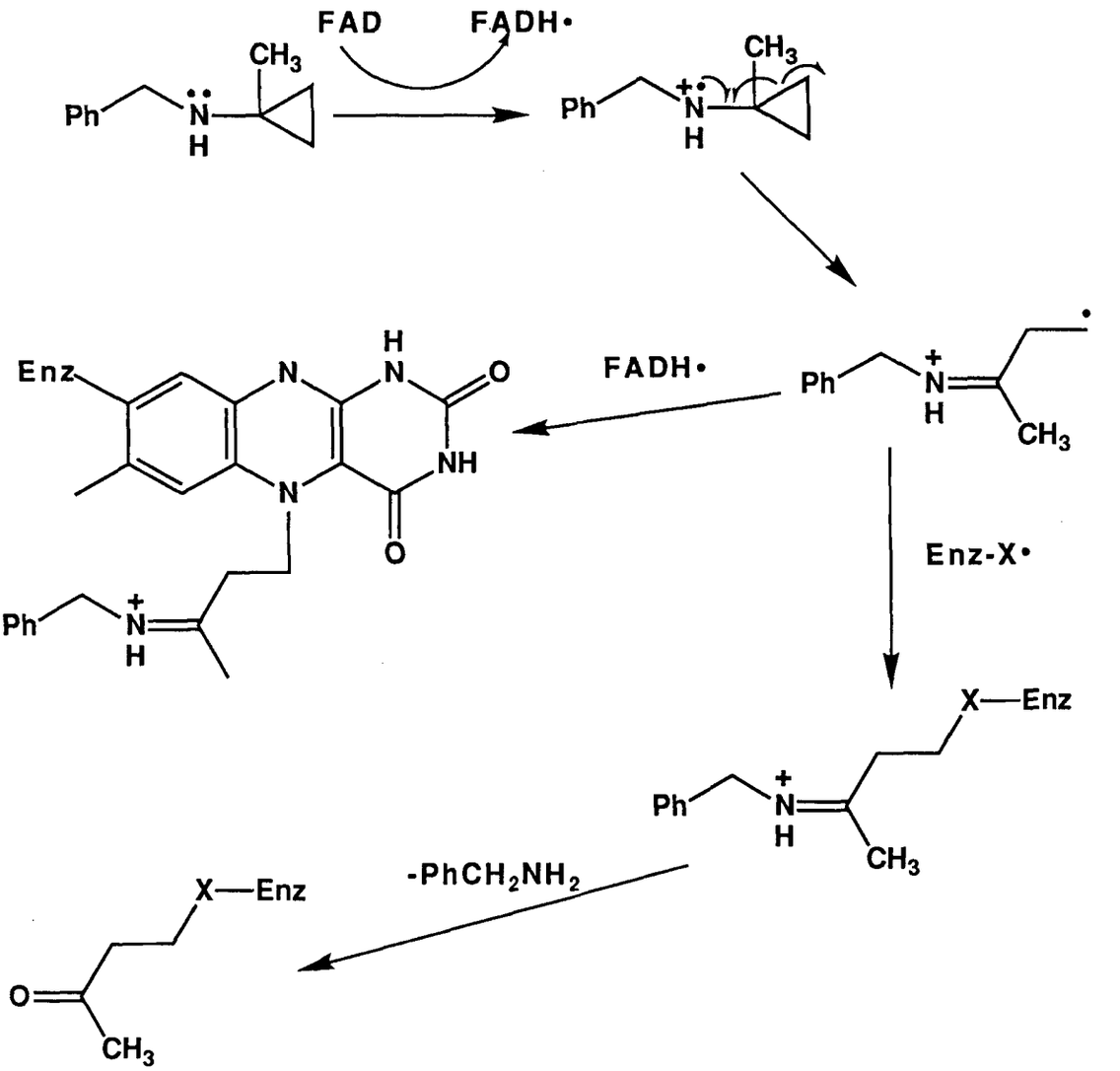

FIGURE 29. Postulated mechanism of inactivation of monoamine oxidase by cyclopropylamines.[402,407]

(Figure 32). The endogenous electron donor appears to be ascorbate, although other possibilities regarding electron transfer proteins cannot be ruled out.

Although the major interest in this enzyme is from a standpoint of neuroscience and a role in the metabolism of xenobiotic chemicals has not been advanced, the enzyme appears to be rather liberal in terms of the potential substrates that it will accept. Recent reviews emphasizing the catalytic mechanism of dopamine β-hydroxylase have been published,[414,415] and the enzyme will be discussed here because of similarities to cytochrome P-450 and some of the other enzymes under consideration.

Recently, the sequence of a full-length human cDNA clone has been published,[416] and structures of previously reported amino acid sequences of tryptic peptides are contained within the predicted coding sequence. A human genomic sequence has also been isolated and sequenced[417]; apparently only a single gene is present and the two sizes of mRNA that are found are the result of alternative use of two polyadenylation signals in the gene. Sequences resembling those invoked as glucocorticoid and cyclic AMP-responsive elements were also found. The existence of soluble and membrane-bound forms of the protein,[415] differing in apparent monomeric M_r, is probably the result of glycosylation. The enzyme can be efficiently purified with the use of concanavalin A affinity chromatography, in the presence of catalase to prevent loss of copper.[418] The fully active enzyme contains two copper atoms per monomer.[418] The ligands to the copper atoms have not yet been defined in the protein. Some studies suggest that each Cu^{II} is bound to four imidazoles (histidines),[415] while other work sug-

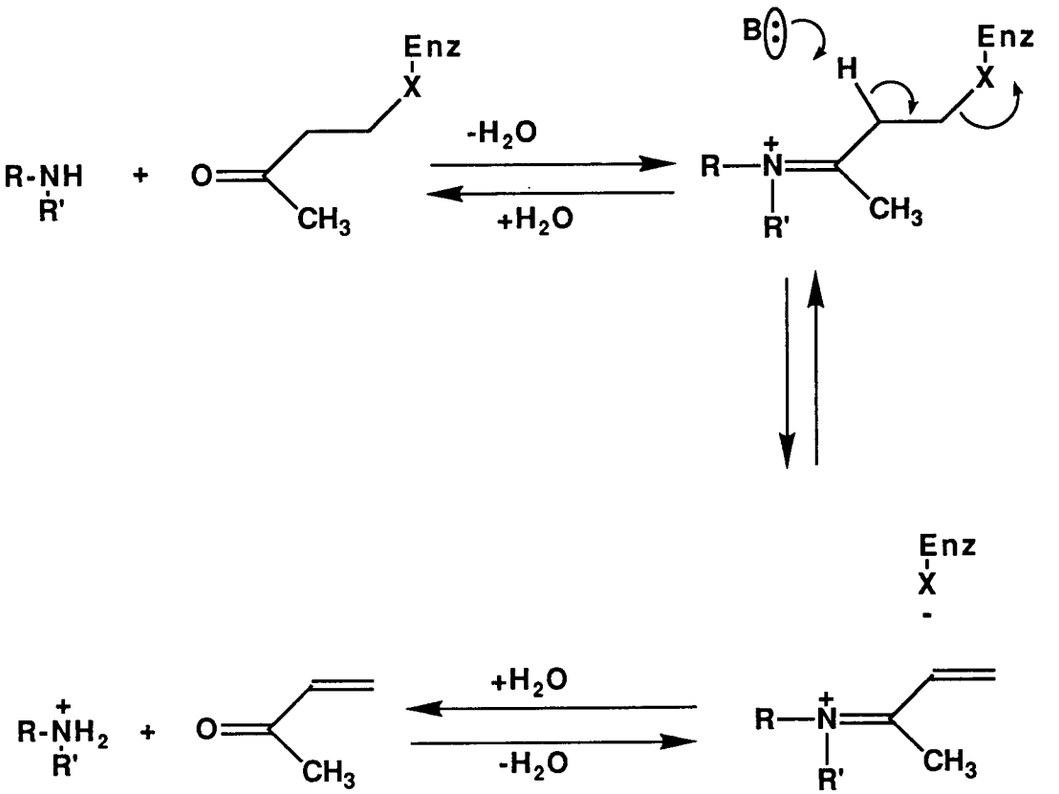

FIGURE 30. Preferred mechanism of reactivation of modified monoamine oxidase by amines (see Figure 29). (From Yamasaki, A. B. and Silverman, R. B., *Biochemistry*, 24, 6543, 1985. With permission.)

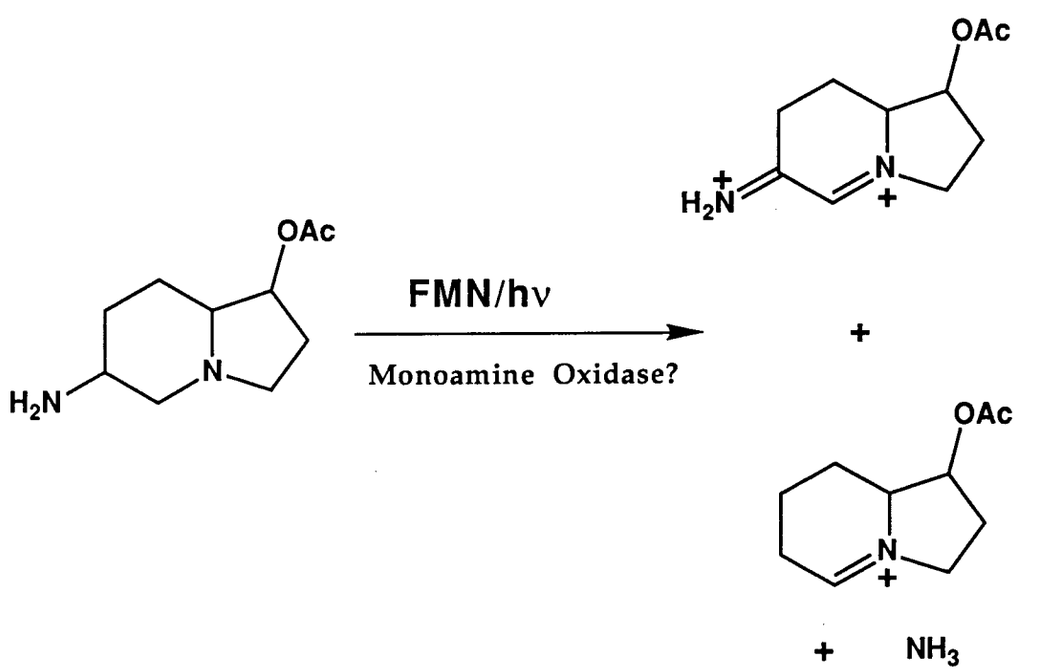

FIGURE 31. Photochemical oxidation and deamination of slaframine in the presence of FMN. (From Guengerich, F. P. and Aust, S. D., *Mol. Pharmacol.*, 13, 185, 1977. With permission.)

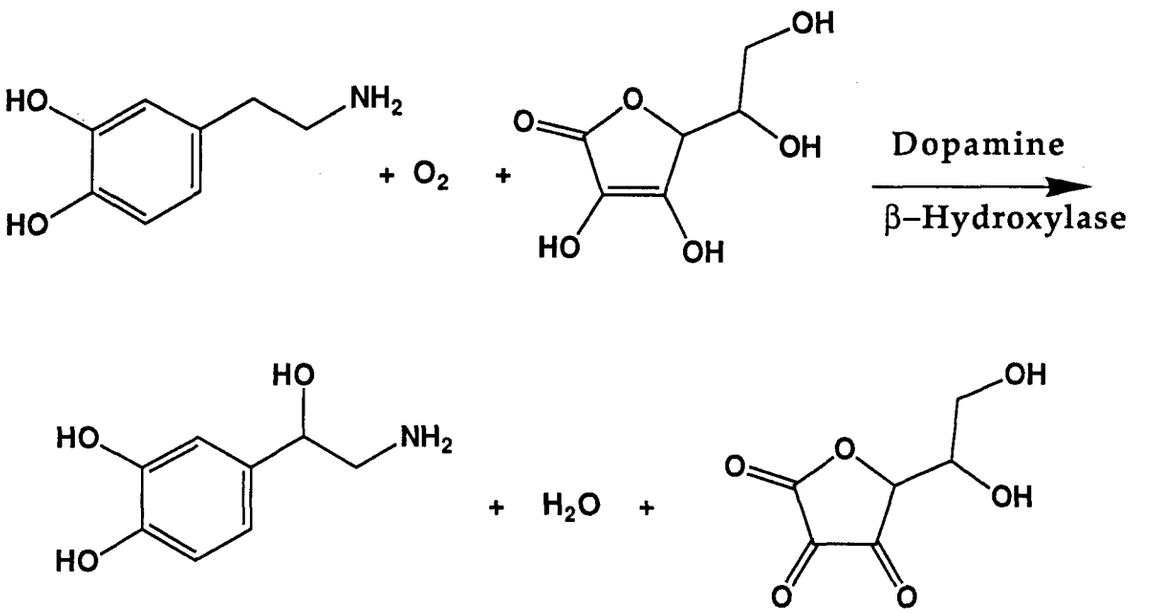

FIGURE 32. Oxidation of dopamine to norepinephrine by dopamine β-hydroxylase.

gests the presence of one or two oxygen ligands, as judged by EPR measurements. Extended X-ray absorption fine structure (EXAFS) studies suggest that the oxidized enzyme has each CU^{II} in a square planar configuration with four imidazoles, while in the reduced enzyme there are only three ligands, two imidazoles, and one sulfur or chloride.[415] Recently, DeWolff et al.[419] have isolated two putative active site peptides labeled with the mechanism-based inhibitor cresol. The adducts appear to have arisen from alkylation by an aberrant 4-hydroxybenzyl radical.

Considerable studies have been done with steady-state kinetic approaches and the results have been interpreted in terms of a random pathway to a ternary complex (reduced enzyme/O_2/dopamine).[414,415] The mechanism of the hydroxylation seems to have many aspects similar to the cytochrome P-450 reaction, although the metals, ligands, and substrates are rather different. First of all, large kinetic deuterium isotope effects are observed; with dopamine, the reverse commitment to catalysis may be ignored (and the application of Northrop's equation[216] yields intrinsic kinetic deuterium isotope effects of about ten).[420] Substituted phenethylamines are also substrates for the enzyme and examination of rates of hydroxylation of these yielded a ρ value of -1.5.[421] These results are similar to those previously reported for cytochrome P-450 carbon hydroxylation (high isotope effects) and *N,N*-dimethylaniline *N*-demethylation (large negative ρ value) and are consistent with the view that a methylene radical is an intermediate, as is the large α-deuterium (secondary) isotope effect (1.19 ± 0.06)[421] (*vide infra*).

In addition to methylene hydroxylation, dopamine β-hydroxylase also catalyzes several other reactions in common with cytochrome P-450, including sulfoxidation,[422] alcohol oxidation,[423] *N*-dealkylation,[424] olefin epoxidation,[424] and mechanism-based inactivation with several types of substrates that also lead to cytochrome P-450 inactivation, including acetylenes,[425-427] olefins,[424,428] and cyclopropyls,[427] all of which are mechanism-based inhibitors of cytochrome P-450.[237,429-432] The results of studies with these and other inhibitors[433-435] have been interpreted as evidence for the involvement of single-electron transfer pathways in dopamine β-hydroxylase, akin to the cytochrome P-450 reactions.[9,150]

How, then, does dopamine β-hydroxylase work? Biomimetic models are known, but they have not been as extensively studied as in the cases of flavins and metalloporphyrins. Obviously, reduction of both copper atoms and binding of O_2 occur first. In one type of mechanism, a Cu^{II}/Cu^{II}-OOH enzyme forms.[415,436] Binuclear model Cu^{I} complexes that reversibly bind oxygen to form μ-peroxo-Cu^{II} complexes have been prepared[437,438] and suggested to be intermediates in the enzyme mechanism.[414] The forms of hypervalent copper shown in each part of Figure 33 are isoelectronic and currently there is no definitive way to distinguish among them.

A species such as $Cu^{IV}=O$ is not considered likely, but as in the case of cytochrome P-450 and the peroxidases, it is not clear that dissemination of the second charge into a ligand or the protein itself may be a possibility.

The above mechanism is adapted from the work of May[436] and Villafranca.[414] Klinman has suggested a somewhat different mechanism.[415] What is agreed upon is the existence of radical intermediates, even though they cannot be observed directly. The $E_{1/2}$ of the form of hypervalent copper that carries out electron abstraction is probably lower than that of cytochrome P-450 and peroxidases, and thus the failure of dopa-

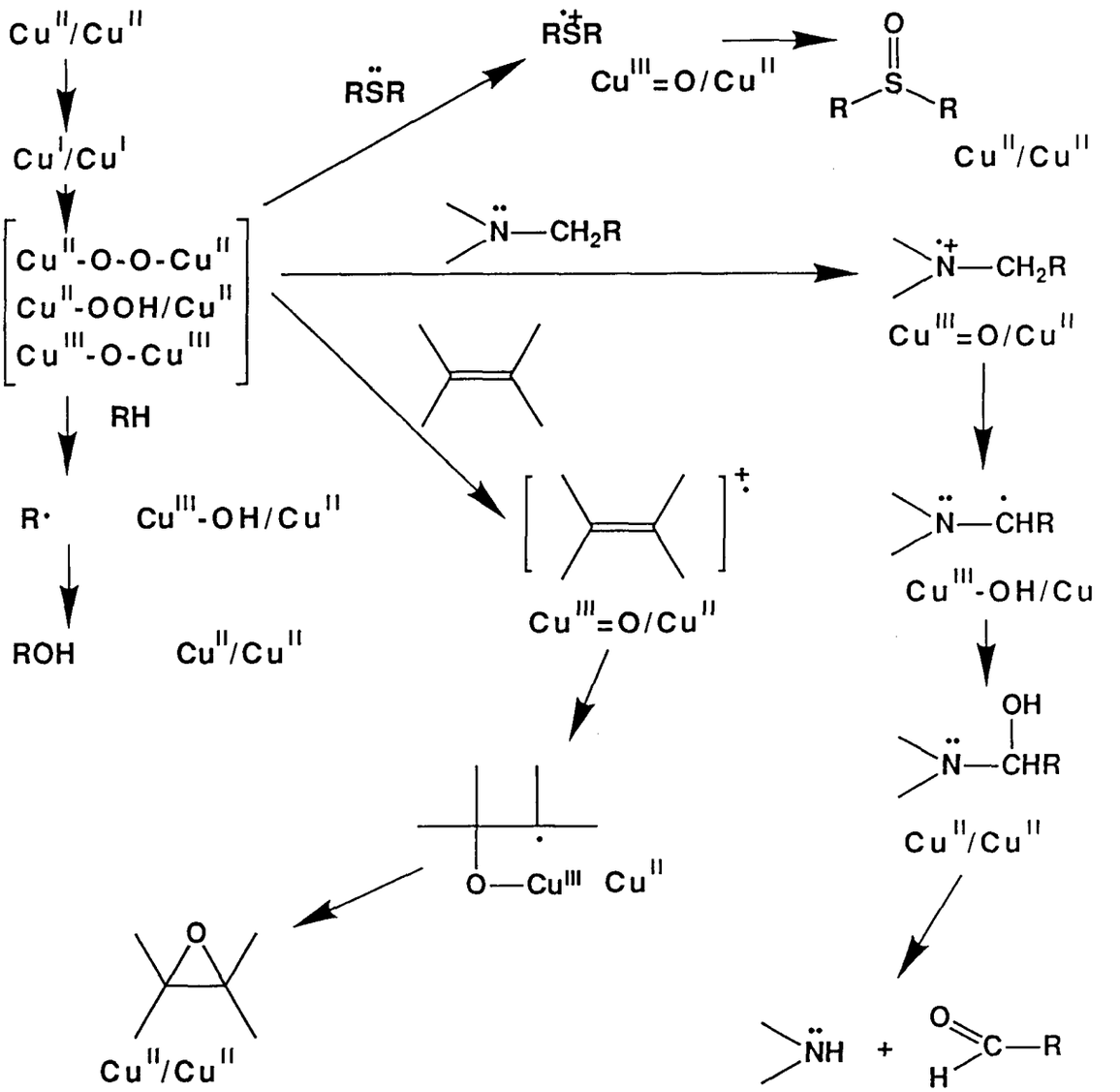

FIGURE 33. Possible mechanisms of various dopamine β-monooxygenase-catalyzed reactions.[414,436]

mine β-hydroxylase to catalyze ether *O*-dealkylations[436] and methylene hydroxylations are difficult than at benzylic sites may be understood. However, if the current view of similar catalytic mechanisms for cytochrome P-450 and dopamine β-hydroxylase is correct, it should be possible to observe some other reactions known to be mediated by cytochrome P-450 enzymes. For instance, strained hydrocarbons with low oxidation potentials are oxidized by cytochrome P-450 via sequential electron transfer processes.[185] Certain amines, particularly those devoid of accessible α-protons, should form *N*-oxides. 1,2-Hydride shifts should also be accessible (e.g., oxidation of styrene to phenylacetaldehyde or methylphenylketone) (*vide supra*). It remains unclear exactly what the forms of hypervalent copper are that participate in the reaction. Further delineation of the chemistry of the Cu^{II}/iodosylbenzene reactions may be of use in the elucidation of mechanisms, and one should bear in mind that the Cu^{II}-bleomycin/iodosylbenzene system catalyzes olefin epoxidation and other reactions.

VII. *Pseudomonas oleovarans* ω-HYDROXYLASE

Bacteria can adapt to growth on single carbon sources by expressing enzymes that catalyze the degradation of the material. In such a manner, a *Pseudomonas putida* strain was isolated that expresses high levels of the enzymes involved in camphor hydroxylation, and the work of Gunsalus and coworkers with the soluble cytochrome $P\text{-}450_{cam}$ (CI) protein has

served as a useful model for work with the mammalian cytochrome P-450 enzymes.[157]

Non-heme iron proteins serve important roles as mixed-function oxidases in mammalian biochemistry (e.g., tyrosine hydroxylase, fatty acid desaturases). An enzyme that has been reasonably well studied is the *P. oleovarans* ω-hydroxylase, and it is discussed here for comparison to some of the other enzymes under consideration.

Coon and co-workers were responsible for elucidating most of the relevant enzymology. Extracts of *P. oleovarans* (grown on hexane as the sole carbon source) were found to catalyze the ω-hydroxylation of the fatty acids octanoate, decanoate, and laurate[439] and octane and other *n*-alkanes.[440] The electron transport chain involved in the reaction includes NADH, the flavoprotein NADH-rubredoxin reductase,[441] the iron-sulfur protein rubredoxin,[442] and the non-heme iron protein termed ω-hydroxylase (EC 1.14.15.3, alkane 1-monooxygenase) (Figure 34).[443,444]

The latter protein, the terminal oxidase, has been purified to homogeneity (apparent monomeric M_r 41,000) and found to contain one atom of iron and one cysteine per monomer. The iron is somewhat labile, and its coordination scheme is still unknown.

Recent studies have shown that the *P. oleovarans* OCT plasmid, which confers the ability to hydroxylate octane and other hydrocarbons and fatty acids to *Pseudomonas* or *E. coli*, carries the *alk* regulon that consists of two gene clusters. The first is the *alkABC* operon, which encodes the enzymes needed for hydroxylation. The same enzyme system can oxidize the alcohol products to aldehydes, and this operon also codes for an aldehyde dehydrogenase. The hydroxylase enzyme system can then attack the carboxylic acid products derived from *n*-alkanes at the ω-position. The *alkR* region encodes the positive regulatory functions required for expression of *alkABC*.[445,446] The *alkABC* region encodes the two rubredoxin species (M_r 14,000 and 18,000) and the 401-residue ω-hydroxylase. The ω-hydroxylase sequence shows nine hydrophobic sequences that may or may not transverse the membrane. Any insight concerning the residues involved in catalysis is not obvious at this time.

The reconstituted enzyme system catalyzes the ω-hydroxylation of fatty acids and *n*-alkanes. Although the rubredoxin component has been found to reduce the alkyl hydroperoxide derivatives,[447] hydroperoxides are probably not intermediates and this reaction is probably not relevant to the mechanism of hydroxylation. May and associates[448] found that terminal olefins are converted to epoxides by the enzyme and that heptyl methyl ether is *O*-demethylated (Figure 35). In addition, octene

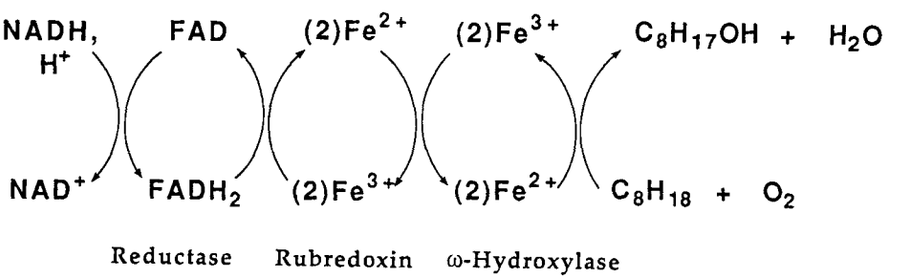

FIGURE 34. Electron transfer pathway in the *P. oleovarans* ω-hydroxylase reaction. (From McKenna, E. J. and Coon, M. J., *J. Biol. Chem.*, 245, 3882, 1970. With permission.)

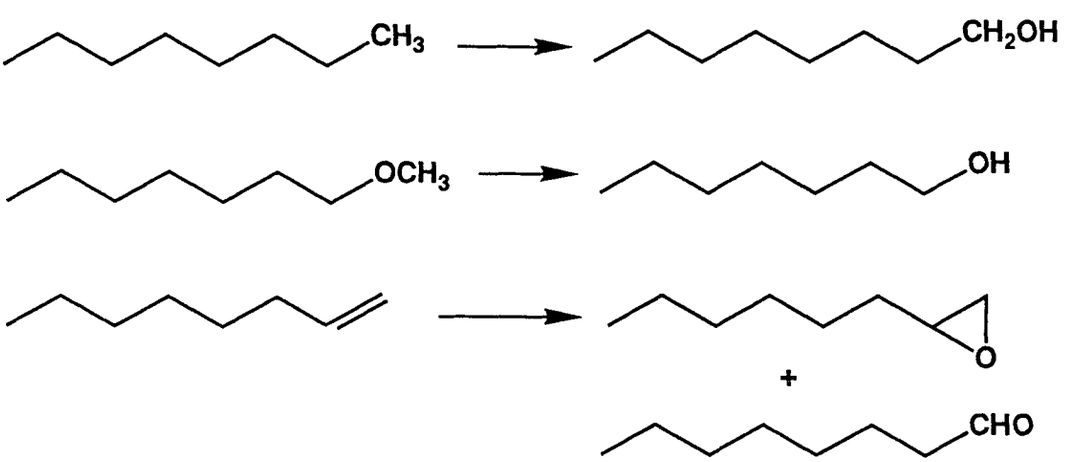

FIGURE 35. Oxidation reactions catalyzed by *P. oleovarans* ω-hydroxylase.

is oxidized to octanal in addition to octene-1,2-oxide, with a 1,2-shift of a vinyl hydrogen. These reactions all resemble those catalyzed by cytochrome P-450 and, since epoxidation proceeds with loss of olefin configuration, stepwise epoxidation must also be invoked. Surprisingly, substrates containing a thioether or thiol at the expected site of attack were not oxygenated by the enzyme.[448]

Unfortunately, the details regarding the nature of the iron-ligand interactions are unknown. Since hypervalent iron chemistry is proposed similar to that for cytochrome P-450 reactions,[448] the distribution of electronic charge into ligands would be of particular interest. Apparently, experiments with oxygen surrogates such as iodosylbenzene have not been done with this enzyme. On the basis of the literature precedents, one might expect iodosylbenzene or peracids to support these reactions. One might also expect reactions such as *N*-dealkylation, aromatic hydroxylation, and perhaps desaturation to occur if appropriate substrates could be found.

VIII. MODEL REACTIONS AND ELEMENTS OF COMMONALITY AMONG THE OXIDATIVE ENZYMES

Considerable effort has gone into the development and study of biomimetic models for several of these oxidative enzymes. The literature can be divided into two parts. The first deals with the use of surrogate oxygen donors for enzymes. The second involves the use of completely chemical models to carry out the same reactions that the enzymes do. A third approach to models, which has not been applied extensively to the enzymes under consideration here and will not be considered, is the attachment of prosthetic groups to proteins that are normally devoid of oxidative activity.[449] This approach has been applied by Kokubo et al.[450] to produce a hemoglobin that hydroxylates aniline after addition of a flavin (details of the catalytic mechanism are unknown).

The surrogate oxygen donor approach has been applied to the cytochrome P-450 enzymes. Normally, the enzyme is activated by the sequential addition of an electron (via the flavoprotein NADPH-cytochrome P-450 reductase), O_2, and another electron, followed by protonation and loss of H_2O. This process can be circumvented by the use of artificial oxidants. Kadlubar et al.[200] first showed that hydroperoxides could support oxidative demethylation in microsomes. Gustafsson and Bergman[451] found that IO_4^- and ClO_2^- could also support hydroxylations. Perhaps the most useful of the artificial oxidants has been iodosylbenzene, first used by Lichtenberger et al.[452] This compound has the advantage of a single oxygen atom and alleviates many of the problems associated with discriminating between heterolytic and homolytic cleavage of peroxides and peracids, a matter that will be mentioned later. In many situations, the rates of cytochrome P-450-catalyzed oxidation are considerably higher than those observed in the presence of the usual system supported by NADPH-cytochrome P-450 reductase,[159,178] although the situation must be considered that the iodobenzene released from the $(FeO)^{3+}$ enzyme can also serve as a substrate.[232] Nevertheless, many features of the enzyme reaction are retained, and there is probably no reason to believe that the intermediate [formal $(FeO)^{3+}$] is not the same as that normally found in the reaction.[150,453] Blake and Coon[454] have added iodosylbenzene derivatives to purified cytochrome P-450 to produce new complexes. The spectra of one of these ("G") was invariant to modification of the iodosylbenzene and may represent a high-valent FeO complex, although the usefulness of the reagents in generating such complexes is limited by the ensuing destruction of the heme prosthetic group.

The other major approach involves the use of model chemicals to mimic reactions that occur with the enzymes. Biomimetic models have been applied extensively in the flavin and heme areas. In the case of the flavin-containing monooxygenase, model N^5-alkylflavin C-4a hydroperoxides can oxygenate amines and thioethers.[351] These models are probably quite appropriate in that considerable evidence has been accrued that UV spectra resembling those of the C-4a hydroperoxide models are seen as intermediates in the reactions catalyzed by flavoprotein oxygenases.[359,455] Other models have been sought that might be transformation products of the C-4a hydroperoxides, such as oxaziridines[456] and carbonyl oxides.[155,364] Evidence has been presented to argue that the (protonated) hydroperoxide itself is the ultimate oxygenating species[351,366,457] and the carbonyl oxides and other uracils are not formed.[458-460]

The matter of how flavoproteins such as monoamine oxidase oxidize amines has been considered at great length and roles for radicals have been invoked through model studies[365] as well as with the purified enzyme (*vide supra*). Other flavoproteins oxidize thiols through mechanisms involving bridged substrate-flavin intermediates, and similar mechanisms can be drawn, at least in principle, for the oxidation of amino acids and amines, if an initial deprotonation to a carbanion can occur.[461] In addition, other observations regarding studies with deazaflavins had been interpreted in light of a mechanism involving transfer of a hydride equivalent, similar to reactions involving pyridine nucleotides.[462] Studies with biomimetic models have been interpreted in terms of single-electron transfer reactions,[365] although with some of the flavoproteins controversy still exists over the enzyme mechanisms.[364,463] In the case of monoamine oxidase, the extensive work of Silverman with the enzyme itself argues that single-electron transfer reactions are probably occurring with the enzyme (*vide supra*).

The work on models for the metalloprotein oxygenases and peroxidases has developed more recently. For a review of early model studies see Reference 155. Precedents for some of the current studies can also be found in the work of Sharpless and others.[464-466] Today, essentially all of the reactions catalyzed by cytochrome P-450 have been reproduced in biomimetic

models, including methylene[467,468] and methyl[469] hydroxylation, epoxidation,[467,470] *N*-[471] and *O*-dealkylation,[472,473] oxidative 1,2-hydride transfer,[474] heteroatom oxygenation (of phosphorus,[230] sulfur,[475] and iodine[535]), and the formation of *N*-alkylporphyrins.[476,477] As might be expected with a large group of models, some have proven to be quite useful in understanding the mechanisms of the enzymes, while others are not so relevant. The early work on cytochrome P-450 models was developed with model metalloporphyrins in which the *meso* positions, sensitive to oxidation, were blocked with phenyl groups. The findings of Lichtenberger et al.[452] and Gustafsson et al.[178] that iodosylbenzene was a good oxygen surrogate served as a basis for model studies. In 1979, Groves et al.[467] reported that a mixture of Fe^{III}TPP (tetraphenylporphin) and iodosylbenzene was effective in catalyzing methylene hydroxylation and epoxidation. Subsequently, Groves reported that the Mn^{III}TPP and Cr^{III}TPP complexes could also catalyze these reactions; it was also possible to isolate somewhat stable high-valent metal intermediates in these reactions [formally $(MO)^{3+}$] and measure spectral characteristics.[478,479]

Subsequently, experiments of this type have been done with almost all of the various cytochrome P-450-catalyzed reactions mentioned earlier. Moreover, the list of metals used in such experiments now includes Fe,[467] Mn,[479] Cr,[478] Co,[480] Os,[481,482] Cu,[483] Ru,[484] Nb,[485] Ni,[486] Pd,[487] Rh,[480] Mo,[488,489] and V.[489,490] Oxygen surrogates now used include iodosylbenzene and its several derivatives (particularly pentafluoroiodosylbenzene[491]), peracids,[492] hydroperoxides,[480] hydrogen peroxide,[493] O_2 plus reducing agents,[494] HSO_5^-,[495] IO_4^-,[496] OCl^-,[497] *N,N*-dimethylaniline *N*-oxides,[498] oxaziridines,[499] and $RSO_2N=IR'$(aryl).[500,501] In the latter case, the RSO_2N group is transferred instead of oxygen (see also Mahy et al.[502]). The high-valent metal oxo complexes have also been formed using electrochemical[503,504] and photochemical[505] means. A number of *meso*-substituted porphins have also been used, including the (tetra) phenyl,[467] pentachlorophenyl,[491] pentafluorophenyl,[472] 2,4,6-trimethylphenyl(mesityl),[506] 2,6-dichlorophenyl,[491] 4-methylpyridinium,[507] and other derivatives,[503,508] such as the chiral 5α,10β,15α,20β-tetrakis{*o*-[(*S*)-2′-carboxymethyl-1,1′-binaphthyl-2-carboxamido]phenyl} iron porphyrin.[506] In these compounds, the presence of electron-withdrawing groups has been shown to have some advantage,[472] but of even more usefulness is the addition of bulky substituents that apparently prevent nonspecific oxidation (destruction) of the porphyrin catalyst by the oxidant. Suslick has prepared metalloporphyrins with bulky groups that force the substrate (guest) into a cavity (of the host). Such a system has been used to shift the preferred site of hydroxylation from a methylene to a less energetically favored methyl group.[469]

It is important to note that a porphyrin structure is not obligatory. Indeed, Hecht and co-workers have found that iron-bleomycin complexes will carry out many of the oxidation reactions mentioned above.[509] In addition, copper-bleomycin complexes also catalyze (iodosylbenzene-supported) epoxidation,[509] and Valentine has shown that copper complexes and even $Cu(NO_3)_2$ catalyze such reactions.[483,510] These observations are very important in that some commonality is suggested between the cytochrome P-450 enzymes and peroxidases (porphyrins) and the monooxygenases that contain non-heme iron and copper prosthetic groups. Such commonality is also considered in terms of experiments with mechanism-based inhibitors and radical probes (*vide supra*).

With respect to the metalloporphyrin models, a number of other interesting observations have been made that may bear on the mechanism of cytochrome P-450 enzymes. Mansuy et al.[480] examined a series of metalloporphyrins in terms of abilities to support hydroxylation reactions. As expected, selectivity was observed when iodosylbenzene was the oxygen surrogate. When cumene hydroperoxide was used as the source of oxygen, no selectivity was seen. The results were interpreted in terms of homolytic scission of the hydroperoxide, with the result that the hydrogen abstracting species was the alkoxide radical instead of the oxometalloporphyrin:

$$M^n + PhIO \xrightarrow{-PhI} (MO)^{n+2}$$

$$\xrightarrow{RH} M^{n+1}-OH + R\cdot \rightarrow M^n + ROH$$

$$M^n + R'OOH \rightarrow M^{n+1}-OH + R'O\cdot$$

$$\xrightarrow{RH} M^{n+1}-OH + R\cdot(+R'OH) \rightarrow M^n + ROH$$

Thus, we see that the final products may be identical, but the mechanism may be quite distinct. Such an explanation is of relevance in the interpretation of many of the results that have been reported over the years in experiments where hydroperoxides were utilized with cytochrome P-450 enzymes. Heme proteins and metalloporphyrins may cleave hydroperoxides either homolytically or heterolytically, depending upon the ligation of the metal and the nature of the porphyrin and protein structure.[274,499,511,512] In this respect, the iodosylbenzenes have proven to be better models (for use as oxygen surrogates) than hydroperoxides.[150] Again, the point should be emphasized that in some cases similar reaction products can be generated by different mechanisms, which may not be relevant to the enzymes under consideration. Fenton chemistry[466] can mimic some of the cytochrome P-450 reaction products.[513] More recently, free iron salts have also been used with H_2O_2 in dry CH_3CN[514,515] (also see earlier studies reviewed in Reference 155). However, the relevance of the proposed ability of Fe^{III} to act as a Lewis acid in direct attack of O_2 must be questioned in light of the poor Lewis acidity of metals complexed in porphyrins.[235,236] *N,N*-Dimethylaniline *N*-oxides have also been used as oxygen surrogates, although the rates of oxygen transfer are very low[214,511] and oxygenation of other

substrates in cytochrome P-450 enzymes has not been observed;[214,516,540] the *N*-demethylation of the oxygen surrogate that does occur may be dominated by Polonowski chemistry in the enzyme.[150,214,517] Another deficiency we have found with all of the metalloporpyrin models examined to date is that none yields *N*-oxygenation of arylamines[514]— all yield either ring hydroxylation (cf. Figure 27) or the production of dimers.

Renaud et al.[518] developed a system involving hydroxylation with *meso*-tetra (2,6-dichlorophenyl) porphins, H_2O_2, and imidazole as a ligand, and others have documented the effect of an imidazole ligand in enhancing rates of oxidation by models in other systems.[517,519-523] Obviously, it would be of great interest to build models in which the thiolate ligand were present to mimic the cytochrome P-450 (and chloroperoxidase) enzymes, but the technical difficulties are great. Free thiols, of course, are very prone to oxidation, and systems with thiols have been largely confined to spectral studies,[524-526] although some reports of catalysis are found.[527] The nature of the models has been probed in several ways. Groves et al.[492] obtained low-temperature Mossbauer spectra on an iron porphyrin intermediate and interpreted this as evidence for a $Fe^{IV}=O$-porphyrin radical configuration, as opposed to $Fe^{V}=O$, which (although useful formally for writing equations) would be unlikely to be formed owing to the high oxidation potential of Fe^{V}.[278] Other formulations can still be considered with the charge distributed into the oxygen and sulfur and cannot be ruled out without further information. It has been possible to generate model compounds of the formal $(FeO)^{3+}$ configuration electrochemically, under conditions where formation of μ-oxo dimers (Fe-O-Fe) is prevented.[503,504] The $E_{1/2}$ values for both of the 1-electron steps are similar to each other and, depending upon the ligands and solvent, fall into the range of +1.0 to 1.7 V (vs. SCE). These values may be compared to $E_{1/2}$ values of about 0.75 V for both steps of the reduction of Compound I of horseradish peroxidase estimated by Hayashi and Yamazaki[280] (using equilibration with chemical indicators). Obviously, an $E_{1/2}$ for the putative $(FeO)^{3+}$ complex of cytochrome P-450 would not be directly measurable at this time (it is not clear that the complex has even been observed). However, we have taken the approach of modeling the relationship between rates of *N*-demethylation of substituted *N,N*-dimethylanilines and their $E_{1/2}$ values for 1-electron oxidation with modified approaches of Marcus theory and predict a value of +1.8 V, which is not inconsistent with the models, considering the influences of the dielectric constant of the protein and the interatomic distances between the reaction centers, which are unavailable in the absence of crystal structures (*vide supra*).[159]

Groves and Watanabe[519] oxidized Fe^{III} tetra(mesityl)porphin with two equivalents of *m*-chloroperbenzoic acid and were able to observe a product (termed intermediate **1**). When cyclooctene was added, intermediate **2** was formed, which EPR measurements suggested did not contain Fe^{III} and had a weak (radical?) signal. The addition of styrene to **2** produced no reaction, indicating the lack of reversibility. Intermediate **2** rearranged to yield ferric tetra(mesityl)porphin and cyclooctene oxide, and this process could be accelerated by the addition of imidazole or methanol. The conversion of **1** to **2** was studied as with a series of ring-substituted styrenes, and a Hammett ρ^+ value of −1.9 was obtained, consistent with an initial electron abstraction process. A formulation consistent with these data is shown in Figure 36.

Further considerations regarding details of mechanisms of epoxidation have been made. Diagnostic substrates (or, more

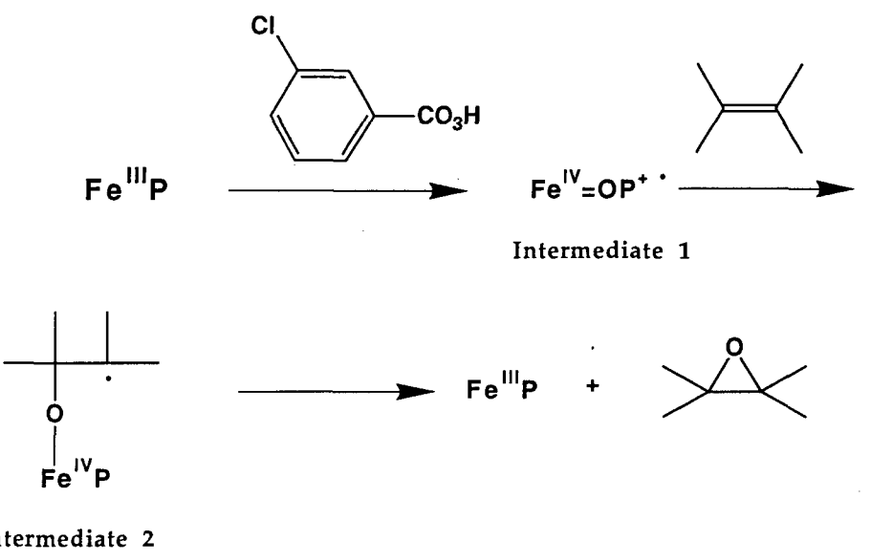

FIGURE 36. Stepwise epoxidation with postulated intermediates in the model of Groves and Watanabe. (From Groves, J. T. and Watanabe, Y., *J. Am. Chem. Soc.*, 108, 507, 1986. With permission.)

accurately in model systems, reactants) have been used to implicate electron transfer processes in the epoxidation of olefins,[528] and Ortiz de Montellano[214] has suggested such radical intermediates on the basis of mechanism-based inactivation studies with cytochrome P-450 enzymes. Another possible mechanism of epoxidation involves cyclic "2+2" intermediates, based on the precedent of model chemical oxidations defined by Sharpless and others.[465,466] Arguments for the existence of such intermediates in model systems have been presented by Collman et al.[529,530] In this respect, Groves[234] has found that one olefinic proton of propylene is exchanged with solvent water in the epoxidation reaction catalyzed by cytochrome P-450, and a "2+2" Fe-carbon bond mechanism would be consistent with the data (Figure 16). It should be pointed out, however, that another explanation for the proton exchange can be considered, that involving formation of the *N*-alkyl porphyrin, which can equilibrate with the Fe carbene-like ligand (Figure 37).[531,532,542]

Thus, the proton exchange results could be rationalized, at least in principle, without invoking "2+2" intermediates. Another consideration is our own ionic intermediate (Fe^{III}-O-C-C+) (Figure 2), which is most useful in explaining the 1,2-shift of hydrogen and chlorine observed in both biomimetic models[506,530] and cytochrome P-450 reactions.[235,236] All of these intermediates are not mutually exclusive, as pointed out by Groves and Watanabe,[519] Collman et al.,[530] and Yamaguchi et al.,[533] and perturbations in the substrates, solvents, and porphyrins may underlie the significance of each in different reactions.

IX. SUMMARY

Studies with biomimetic models can yield considerable insight into mechanisms of enzymatic catalysis. The discussion above indicates how such information has been important in the cases of flavoproteins, hemoproteins, and, to a lesser extent, the copper protein dopamine β-hydroxylase. Some of the moieties that we generally accept as intermediates (i.e., high-valent iron oxygen complex in cytochrome P-450 reactions) would be extremely hard to characterize were it not for biomimetic models and more stable analogs such as peroxidase Compound I complexes. Although biomimetic models can be useful, we do need to keep them in perspective. It is possible to alter ligands and aspects of the environment in a way that may not reflect the active site of the protein. Eventually, the model work needs to be carried back to the proteins.

We have seen that diagnostic substrates can be of considerable use in understanding enzymes and examples of elucidation of mechanisms through the use of rearrangements, mechanism-based inactivation, isotope labeling, kinetic isotope effects, and free energy relationships have been given. The point should be made that a myriad of approaches need to be applied to the study of each enzyme, for there is potential for misleading information if total reliance is placed on a single approach. The point also needs to be made that in the future we need information concerning the structures of the active sites of enzymes in order to fully understand them. Of the enzymes considered here, only a bacterial form of cytochrome P-450 ($P\text{-}450_{cam}$) has been crystallized. The challenge to determine the three-dimensional structures of these enzymes, particularly the intrinsic membrane proteins, is formidable, yet our further understanding of the mechanisms of enzyme catalysis will remain elusive as long as we have to speak of putative specific residues, domains, and distances in anecdotal terms.

The point should be made that there is actually some commonality among many of the catalytic mechanisms of oxidation, even among proteins with different structures and prosthetic groups. Thus, we see that cytochrome P-450 has some elements of a peroxidase and *vice versa*; indeed, the chemistry at the

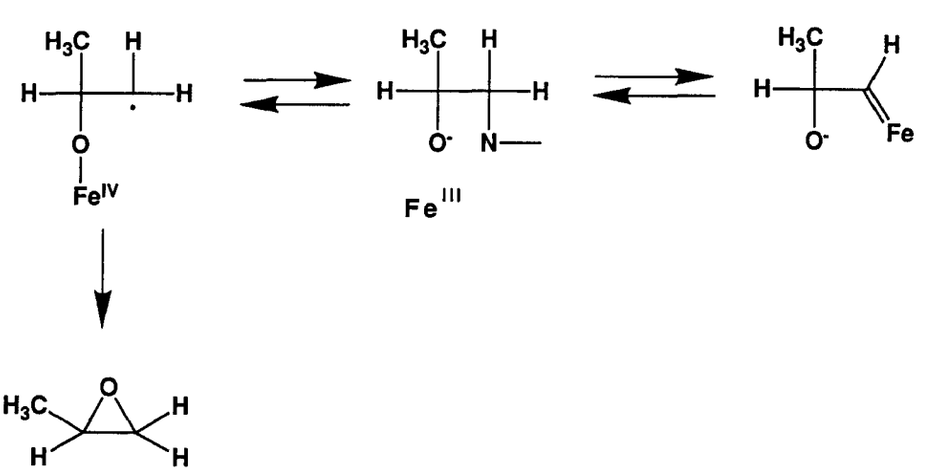

FIGURE 37. An alternate explanation for the exchange of vinylic protons during epoxidation of propylene.[531,542]

prosthetic group is probably very similar and the overall chemistry seems to be induced by the protein structure. The copper protein dopamine β-hydroxylase appears to proceed with chemistry similar to that of the hemoprotein cytochrome P-450 and, although not so thoroughly studied, the non-heme iron protein *P. oleovarans* ω-hydroxylase. Indeed, some of the same experiments regarding single-electron transfer have been done with all of these enzymes, as well as with monoamine oxidase. It is interesting that nature has ways of achieving similar chemical reactions through different systems.

Another point to consider is the diversity of proteins within some of the enzyme families. We have much to learn about details of structure-function relationships in these proteins, for ultimately the three-dimensional structures determine what reactions are catalyzed. Perhaps the reason behind multiplicity of structures in gene families such as cytochrome P-450 and lignin peroxidase is one of dealing with a large variety of foreign chemical structures. Finally, the matter of endogenous and xenobiotic substrates is considered again. We have seen that in some cases there is not a clear case for a particular endogenous natural substrate for a particular enzyme. This can go along with the idea that the enzymes may be present for the purpose of clearing foreign substances that are consumed. In other cases, we have seen that much can be learned about enzymes with clear physiological functions (e.g., monoamine oxidase, dopamine β-hydroxylase, and prostaglandin synthase) from studies with xenobiotics. In at least two cases, we see that enzymes with such clear physiological roles may have important actions in processing dangerous xenobiotics; prostaglandin synthase can oxidize drugs and carcinogens, and monoamine oxidase can activate MPTP. The case may be made that the distinction between enzymes that act on endogenous and xenobiotic substrates is not really very great.

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