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Invited Review

Common and Uncommon Cytochrome P450 Reactions Related to Metabolism and Chemical Toxicity

F. Peter Guengerich*

Department of Biochemistry and Center in Molecular Toxicology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

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Cytochrome P450 (P450) enzymes catalyze a variety of reactions and convert chemicals to potentially reactive products as well as make compounds less toxic. Most of the P450 reactions are oxidations. The majority of these can be rationalized in the context of an FeO^{3+} intermediate and odd electron abstraction/rebound mechanisms; however, other iron−oxygen complexes are possible and alternate chemistries can be considered. Another issue regarding P450-catalyzed reactions is the delineation of rate-limiting steps in the catalytic cycle and the contribution to reaction selectivity. In addition to the rather classical oxidations, P450s also catalyze less generally discussed reactions including reduction, desaturation, ester cleavage, ring expansion, ring formation, aldehyde scission, dehydration, *ipso* attack, one-electron oxidation, coupling reactions, rearrangement of fatty acid and prostaglandin hydroperoxides, and phospholipase activity. Most of these reactions are rationalized in the context of high-valent iron−oxygen intermediates and Fe^{2+} reductions, but others are not and may involve acid−base catalysis. Some of these transformations are involved in the bioactivation and detoxication of xenobiotic chemicals.

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1. Introduction

P450 enzymes are the major catalysts involved in the oxidation of xenobiotic chemicals, a significant focus of scientists in the areas of toxicology, drug metabolism, and pharmacology. The effects of these oxidations can be manifested in poor drug bioavailability and various acute and chronic toxicities, including adverse drug interactions, cancer susceptibility, and birth defects. Information about which human P450 enzymes are involved in the metabolism of new drug candidates is required for new drug approval submissions in most countries, and information about the induction of P450s is a part of large-scale toxicogenomics efforts (*1*, *2*).

The field has certainly advanced in the 27 years since the author entered it, and some explanation should be given about the impetus for this review. I began this review at the suggestion of the feature editor, Paul Hollenberg. Although I have written a number of reviews on several aspects of P450 research over the past two decades, the only one I wrote for *Chemical Research in Toxicology* was with Tsutomu Shimada in 1991 on the subject of metabolism of carcinogens by human P450s (*3*). It occurred to me that an entire generation has passed since Professor Hollenberg and I began our own studies on P450s. I have a reasonably good idea about what most students and postdoctoral trainees at Vanderbilt know about P450 by the time they leave, for better or worse. However, in interactions with trainees at other institutions and with scientists in the chemical and pharmaceutical industries I have found a great diversity of backgrounds.

To me, the metabolism of xenobiotic chemicals has always been an interesting field of research. Some of my more biologically oriented colleagues seem to have the view that all aspects of metabolism were discovered by 1960, give or take a few years. However, the continuing onslaught of new xenobiotics has provided a tremendous source of new projects and also yielded needs for new explanations. Much of this continuing search with xenobiotics has had major "spin-offs" in the generation of important biological signaling agents, e.g., nitric oxide synthase. Many students learn some fundamentals of P450 enzymology in drug metabolism or toxicology courses, if they have taken these. My impression is that most university-based programs in the general area deal with some of the more fundamental types of P450 reactions, e.g., carbon hydroxylation, epoxidation, and a few others. However, in the actual practice of xenobiotic metabolism researchers are confronted by numerous "unusual" reactions that they may not understand or be able to retrieve examples of from the literature. In some recent cases, the author has not been able to resolve some issues encountered in practical pharmaceutical problems, and new paradigms continue to be added to our repertoire.

In the past 5 years, several reviews of the catalytic mechanisms of P450s have been published, both brief (*4*−*10*) and more extensive (*11*, *12*). In particular, the comprehensive review of Ortiz de Montellano (*11*) is recommended; it discusses most aspects in detail. The present review will touch on the fundamentals of general P450 mechanistic enzymology, with some added discussion of a personal interest that has not been reviewed recently, i.e., the nature of rate-limiting steps in reactions catalyzed by mammalian P450s. When one has a fundamental knowledge of how P450s work, most of its reactions can be understood, or at least rationalized. The bulk of the review will be devoted to a series of somewhat unusual P450 reactions, divided into 13 types. Most of these were unknown 25 years ago. Many are included in the extensive treatments of Ortiz de Montellano (*11*, *13*) and Testa (*12*). In this review I have several purposes regarding these reactions: (i) to update the two 1995 treatments just mentioned, (ii) to stimulate P450 practitioners to learn more about some of the apparent diversity of the repertoire of the P450 family, and (iii) to interest the readers in reading and learning more about the chemistry of these interesting enzymes. The scientific views I present here are based on my own research, my own experience, and my interpretations of the work of others. The approach I will use in presenting the article is based on my experience teaching classes at Vanderbilt, my experiences with my own students and postdoctoral fellows, and my experience in consulting with the pharmaceutical and chemical industries.

2. General Information about Cytochrome P450 Enzymes

Mixed-function oxidations were first observed in the late 1940s (*14*−*18*) but the enzymology was not characterized for a number of years. P450 Fe^{2+}·CO spectra were reported in 1958 (*19*); Sato and Omura published on some additional properties of the system in 1962 (*20*) and developed the name P450 (first used with the hyphen, "P-450") for "pigment 450" because of the A_{max} at 450 nm. A classical series of light reversal (of CO inhibition) studies by Estabrook and Cooper (*21*, *22*) established a P450 as the terminal oxidase in an adrenal steroid hydroxylation. Several lines of investigation in the 1960s, mainly involving induction experiments in rats, led to the view that multiple forms of P450 might exist within a single tissue (*23*−*25*).

P450 enzymes were originally discovered through independent research activities involving the metabolism of carcinogens (*15*, *26*), drugs (*18*, *27*, *28*), and steroids

* To whom correspondence should be addressed. Phone: (615) 322-2261. Fax: (615) 322-3141. E-mail: guengerich@toxicology.mc.vanderbilt.edu.

(*21*, *22*, *29*). All of these areas are of interest in issues related to toxicology. Other substrates of note include pesticides, fat-soluble vitamins, and endogenous compounds such as eicosanoids and mammalian alkaloids (*30*−*33*). The general reaction catalyzed by P450s is one of mixed-function oxidation (eq 1)

$$\mathrm{NADPH} + \mathrm{H^+} + \mathrm{O_2} + \mathrm{R} \rightarrow \mathrm{NADP^+} + \mathrm{H_2O} + \mathrm{RO} \quad (1)$$

although we will see several exceptions later.

2.1. Nomenclature and Distribution. Following the basic characterization of the nature of the mammalian P450 system, evidence for the existence of more than one form of microsomal P450 began to accumulate (*23*−*25*). The first successful purification and reconstitution of a P450 system by Lu and Coon (*34*) was followed by extensive efforts in a number of laboratories to purify P450s from experimental animals and, later, from humans (*35*). These efforts led to a considerable collection of P450s and information about their catalytic activities (*36*, *37*). Soon after the cDNA cloning work on P450s began in earnest, the nomenclature system was systematized (*38*). On the basis of sequence identity, P450s are grouped into families (1, 2, 3, ...), subfamilies (A, B, C, ...), and individual P450s (1, 2, 3, ...), e.g., 1A1, 1A2, 1B1, ... For more discussion the reader is referred to the current approach to the nomenclature at the website http://drnelson.utmem.edu/CytochromeP450.html (site accessed April 20, 2001). A compilation of the allelic variants of human P450s is maintained at http://www.imm.ki.se/CYPalleles/ (site accessed April 20, 2001).

P450s are found throughout nature. With the near completion of the sequences in the human genome, the number of human P450s seems to be about 53 and will not change much in the future. For comparison, there are probably at least 20 human glutathione transferases (*39*). With regard to the number of P450 genes in other organisms, even the bacterium *Mycobacterium tuberculosis* has ∼20, the model nematode (rootworm) *Caenorhabditis elegans* has 80, Drosophila has 83, and the plant *Arabidopsis thaliana* has 286 (but the yeast *Saccharomyces cerevisiae* has only 3 and the bacterium *Escherichia coli* has none). Many of the P450s (in mammalian organisms) are devoted to the biosynthesis of specific natural products. In plants, many of these chemicals are used for coloration or defense. Mammals appear to use a smaller set of P450s in that many of the functions are in the catabolism of natural products, aside from the synthesis of important steroids and eicosanoids (e.g., P450s 5, 8, 11, 17, 19, 21, 24, 26, and 27). Of the remainder of the mammalian P450s, a relatively small set accounts for *most* of the metabolism of drugs (i.e., 1A2, 2C9, 2C19, 2D6, and 3A4) and another small set is involved in the metabolism of most of pro-toxicants and -carcinogens that are P450 substrates (i.e., 1A1, 1A2, 1B1, 2A6, 2E1, and 3A4) (*4*, *40*, *41*). Similar subfamily P450s are found in experimental animals, although the catalytic selectivity may be altered (*42*, *43*).

The majority of the P450s to be discussed are concentrated in liver (except 1A1 and 1B1). However, many of these P450s are also found in some extrahepatic tissues and the actions of these within a target tissue may be more important, particularly if a generated reactive product is not stable enough to migrate out of the cell in which it is formed (*44*). The P450s of interest here are all found in the endoplasmic reticulum (isolated as "microsomes"). Avadhani and his associates have identified mechanisms by which proteolytic cleavage in the N-termini of some microsomal P450s can "mark" these for import into mitochondria (*45*−*47*), or phosphorylation of a Ser of rat P450 2B1 can signal the transfer (*48*). In mitochondria these P450s can utilize electrons from the iron−sulfur protein adrenodoxin instead of NADPH−P450 reductase (*49*). The fraction of the pool of some P450s that can move to the mitochondria is considerable in some cases, e.g., $^1/_3$ for P450 1A1 (*45*). Small amounts of some P450s are also able to be translocated to the outside of the plasma membrane (*50*), where they may be important in the development of immune responses. In the 1970s the concept was advanced that P450s in the nucleus would be more likely to generate reactive products capable of DNA alkylation (*51*−*53*). However, despite a flurry of interest in this area, there appears to be little basis, for two reasons. (i) The nuclear P450s are located in the outer nuclear membrane (*54*, *55*), which is contiguous with the endoplasmic reticulum. (ii) Today we know that many reactive chemicals [e.g., aflatoxin *exo*-8,9-epoxide, $t_{1/2}$ 1 s (*56*, *57*)] are able to cross cell membranes and would probably have no trouble entering porous nuclei.

Many P450s are inducible by chemicals; the inducer may also be a substrate but this is not necessarily the case. Some excellent inducers (e.g., 2,3,7,8-tetrachlorodibenzo-*p*-dioxin) are very poor substrates. The most common pattern of P450 induction is a transcriptional one in which a ligand binds to activate a heterodimer, ultimately stimulating RNA synthesis from specific genes. However, in some systems there is posttranscriptional control (*58*) and in other cases a chemical may act by increasing the synthesis of a receptor or transport into the nucleus.

2.2. Structures of P450s. The first three-dimensional structure of a P450, that of bacterial P450 101, was reported by Poulos in 1987 (*59*). At this time, structures of at least eight different P450s are known. Of these, six are now published (*59*−*65*). All but one are of soluble enzymes, and all but two are of bacterial origin. One is from *Fusarium* (mold) (*63*) and one is a modified rabbit P450 (*64*). The reader is referred to the original papers (vide supra) for reference to the actual structures and to discussion of exactly what one can conclude from the structural results (particularly, see refs *65*−*67*).

All of the P450 structures have common elements and have some similarity at a gross level. The proteins are arranged into a similar series of helices and folds, with well-conserved helices denoted A−L. The I and L helices contact the heme. Residues in the B and I helices also contact substrate, and site-directed mutations in these regions have had some dramatic effects (*42*, *68*−*70*). Another region contacting the substrate is the F helix, where mutations can also influence catalytic selectivity (*71*−*73*). Other structural, homology modeling, and site-directed mutagenesis work supports the original proposal by Gotoh (*74*) that the six "substrate recognition sequences" (SRS,[1] three already cited) are generally conserved among most P450s.

[1]Abbreviations: SRS, substrate recognition sequence; b_5, cytochrome b_5; $E_{m,7}$, oxidation−reduction midpoint potential, relative to hydrogen electrode at pH 7.0; $E_{1/2}$, oxidation−reduction potential, relative to saturated calomel electrode; PAH, polycyclic aromatic hydrocarbon; HHT, hydroxyheptatrienoic acid.

The most conserved part of the P450 sequence is in the region containing the Cys that acts as the thiolate ligand to the heme iron (Cys 357 in P450 101). This sequence is used as the identifier of P450s in gene banks. Another highly conserved residue is a Thr corresponding to Thr 252 in bacterial P450 101, which has been postulated to have a variety of functions. The Thr may donate a proton to the $Fe^{2+}O_2^-$ complex to facilitate (heterolytic) cleavage of the O−O bond. Arguments against the general need for such an activity include the high pK_a of Thr, the absence of this Thr in some P450s, and the retention of function of P450 101 in which a Thr *O*-methyl ether was substituted using mutagenesis (*75*). The dominant view today is that the Thr and its neighboring Glu are part of a proton relay system that is useful but not absolutely necessary in all P450s. In P450 108 (P450eryF), the substrate binds and then mobilizes H_2O to serve the same function (*76*).

Crystal structures in themselves do not necessarily provide information about function. Indeed, some of the P450s for which structures are available are not characterized with regard to their electron donor or substrate (*76*). The P450 101 structure has provided the greatest amount of information, in part because of the wealth of knowledge that existed about protein interactions, substrate specificity, and kinetics (*77*). Recently, time-resolved crystallographic approaches have been used to obtain structures of distinct redox forms of P450 101 during the catalytic cycle (*78*). Although the structures of the latter intermediates are not as clear and the molecular identity is not as certain, insight is provided into the structural changes that can occur in the course of P450 reaction. Changes in the structure near the active site occur at every step of the reaction. To some extent then, we can consider each of the redox forms as a slightly different enzyme in the context of binding ligands, etc. In P450 102 there is a long, open substrate channel; conformational changes are also required (*60*, *67*, *79*). However, with several of the other P450s, the substrate is completely enclosed by the protein [e.g., P450s 101, 107, 108, 2C5 (*64*, *67*, *79*)], and obviously a major rearrangement of the protein is necessary. This rearrangement is not well understood, even in P450 101, and has only been approached from molecular dynamics simulations (*80*, *81*). A key question is how fast the protein motion is and exactly where the substrate is bound. A structural change is also required for the product to leave, and similar questions can be raised. Also, the question arises as to whether the product and substrate follow the same paths in entering and leaving (*82*, *83*).

Recently, the structure of an Archebacteria P450, P450 119, has been reported (*65*). Of particular interest are the differences between the structures in the absence and presence of the nitrogenous heme ligand 4-phenylimidazole. These differences, along with the changes in structures mentioned above due to the presence of substrate, raise the possibility that P450s may have some "induced fit" properties and be able to accommodate diverse substrates in this way. However, comparison of more structures with different ligands will be necessary to provide evidence for this view.

The acquistion of a structure of a mammalian P450 was a very useful development. Rabbit P450 2C5 was modified to increase the level of heterologous expression and decrease aggregation (*84*), yielding a protein that could be crystallized in the absence of detergents (*64*). Molecular anomolous dispersion phasing using synchroton radiation was required to solve the structure. The structure is of considerable interest in terms of modeling of other mammalian P450s. In this regard, the structure has been compared to that of P450 102, which has already been employed extensively and shows the greatest similarity (*64*). The spatial arrangement of the major elements diverges from the other structures, with SRS-4 being the most conserved compared to P450 102. SRS-5 diverges significantly, with 3.3 Å root-mean-square deviation, and the shape of the base of the active site is significantly different, affecting the orientation and position of substrates as well as selectivity for different substrates. This is not surprising, in that the substrates of P450 102 and 2C5 are fatty acids and steroids, respectively (*64*, *85*, *86*). The spatial organization of SRS regions 1, 2, 3, and 6 also diverges from P450 102 (4.5−6.0 Å). The high-temperature factors of the P450 2C5 B−C loop might be indicative of dynamic fluctuations related to passage of substrates through this area (*64*).

The original P450 2C5 structure seems to indicate a bound ligand, although no substrate was added. It is possible that this is a reagent utilized in the work (e.g., dithiothreitol). The substrate binding cavity is slightly larger than that for camphor in P450 101 (360 $Å^3$) (*64*), and progesterone was readily docked into this site.

Many P450 modeling efforts have been published, based first on P450 101 and subsequently on P450 102 or aggregate models of the known structures. Undoubtedly, structure modeling will now also be based upon P450 2C5. The usefulness of modeling details of ligand interaction remains to be demonstrated, particularly with P450s outside of the 2C subfamily.

2.3. Roles of P450s in Drug Metabolism. Aside from issues of potential toxicity, the major issue with P450 regarding drug metabolism is bioavailability. P450 catalysis will lower the plasma concentration of a drug and also the concentration of the drug at the target site. Metabolism (by P450s or other enzymes) is not the only issue in bioavailability (also absorption and other factors), but it is one that scientists in the pharmaceutical industry would like to be able to predict better. Poor bioavailability is one of the most common reasons why new chemical entities from drug discovery fail to reach the market, and there is great interest in developing better in vitro predictive methods.

A second but less common role of P450s in drug metabolism is the P450-catalyzed oxidation of drugs to their active forms. This process might involve conversion of a pro-drug to an active form. Approaches are under consideration for the use of gene therapy in cancer chemotherapy as a means of selective delivery of alkylating agents to tumor cells (*87*). A more common scenario involves the conversion of a drug with some inherent activity to a much more active form. Examples include losartan (P450 2C9) (*88*, *89*) and encainide (P450 2D6) (*90*). This type of behavior is often unrecognized until experiments are initiated in vivo. The discovery of such active metabolites provides new leads for the discovery process.

2.4. Roles of P450s in Chemical Toxicity. The following discussion applies to all xenobiotic chemicals, including drugs, pesticides, carcinogens, and any other chemical with potential toxicity (which according to Paracelsus would be all chemicals, whether xenobiotic

or endobiotic). The particular scenario depends on the mechanism of toxicity and, of course, the dose.

If the administered chemical has a direct toxicity of its own (or acts directly on a receptor to produce toxicity), then metabolism of the compound may reduce toxicity, *if* the P450-generated product has less inherent toxicity. Good examples include many drugs, e.g., terfenadine (*91*). With drugs, toxicity is often the result of exaggerated pharmacological response of the drug itself.

Another case, also very common, is the transformation of an administered chemical to another that either (i) binds covalently to macromolecules (usually because of its electrophilic or other reactive nature) or (ii) otherwise interacts with a target to cause toxicity. An example for part i would be conversion of hexane to 2,5-hexanedione, which reacts with protein lysines to generate pyrroles (*92*). An example of ii would be the hydroxylation of trimethylpentane to an alcohol that binds tightly to α_{2u}-microglobulin to produce droplets in male rat kidneys (and ultimately tumors) (*93*). Examples i and ii are also (usually) distinguished by (i) their capability for genotoxic response vs (ii) tendency to act by causing increased cell proliferation, although these two phenomena are not mutually exclusive.

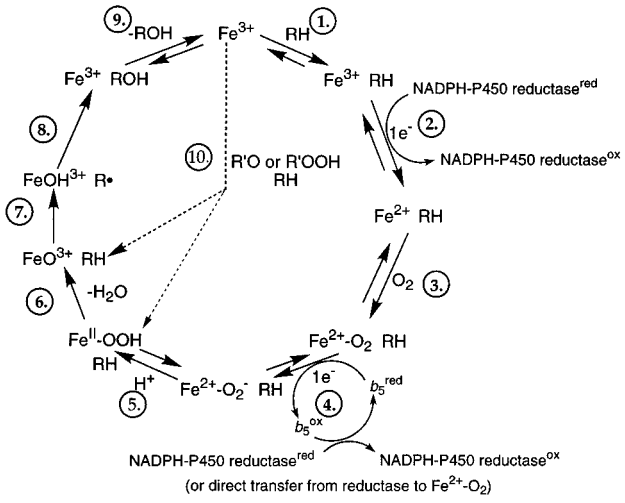
Another mode of P450-caused toxicity involves mechanism-based (or "suicide") inactivation of P450s, which is not an unusual phenomenon (*94*, *95*). This is not necessarily equated with toxicity because, in the case of this phenomenon, only the P450 involved in the activation is destroyed. However, this inactivation may yield a problem in terms of drug−drug interactions, as would even strong competitive inhibition of a P450. For example, inhibition of P450 3A4 by any mechanism can lead to impaired oxidation of terfenadine (to fexofenadine) and its accumulation, resulting in arrhythmias in some individuals (*91*). Another aspect of mechanism-based inactivation is that modified P450s may have toxicities of their own. For instance, *N*-alkyl heme products ("green pigments") can disrupt porphyrin synthesis (*94*). P450s covalently modified with chemicals may behave as antigens and induce auto-immune responses (*96*, *97*). This latter response is correlated with some types of hepatitis, although a causal relationship has not been demonstrated.

The list of potential DNA, lipid, and protein targets for reaction with reactive electrophiles and radicals is extensive. With lipids, there is some understanding of the consequences of lipid peroxidation (*98*). With DNA, understanding at least some of the most relevant gene responses and their mechanisms is becoming possible. In the reaction of electrophiles with proteins, sites of reaction are being defined but establishing biological significance has been difficult (*99*, *100*). An additional complication we have recently dealt with is the instability of protein adducts, as exemplified by the reaction of proteins with acyl halides derived from trichloroethylene (*101*, *102*).

3. General Features of Catalysis

In this section, we will briefly examine some of the base of P450 catalysis, beginning with a general treatment of the reaction cycle, followed by a somewhat classical and unified approach to the oxygenation step, and consideration of alternative chemistry.

Scheme 1. Generalized P450 Catalytic Cycle[a]



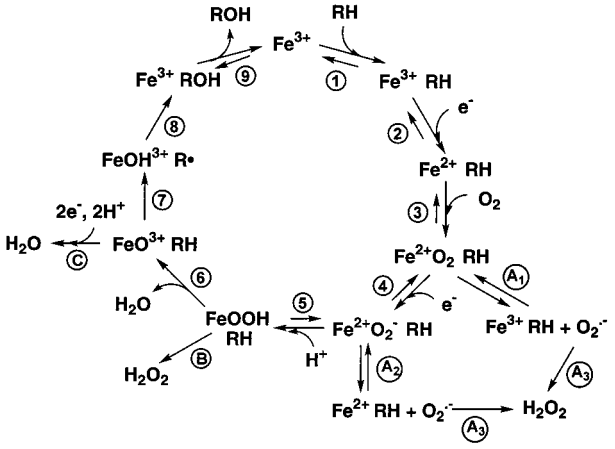
[a] Fe = iron atom in P450 heme, RH = substrate, ROH = product, ox and red indicate the reduced and (1-electron) oxidized states of the reductase involved in the electron transfers (see text).

3.1. General Aspects of the Catalytic Cycle. Most scientists in the field agree that the major events in P450 catalysis are those shown in Scheme 1. These will be presented briefly because they have been treated extensively elsewhere and a discussion of the rate-limiting nature of individual steps follows (section 3.3).

The cycle begins with P450 iron in the ferric state. In step 1, the substrate binds to the enzyme, somewhere near the distal region of the heme. This step may or may not involve a change in the iron spin state (some P450s have low-spin P450 in the substrate-free state; some are high-spin). Step 1 may or may not facilitate step 2, depending on the P450 (*103*); step 1 is shown before 2 because it is faster, even when the kinetics of step 2 are independent of whether substrate is bound. The electrons come from NADPH via the accessory flavoprotein NADPH−P450 reductase, with the flow going from the reductase prosthetic group FAD to FMN to the P450. For further details of electron transfer through the flavins see (*104*−*107*). There appear to be few exceptions to NADPH−P450 reductase as the electron donor. Unnatural donors such as ferredoxins and flavodoxins will work in artificial systems but only relatively slowly (*108*, *109*). The one natural exception among the "microsomal" P450s is the truncated forms of some that are subsequently targeted to mitochondria and appear to use adrenodoxin as an electron donor (*49*).

Ferrous P450 binds O_2 (step 3). This complex is unstable and can generate ferric iron and superoxide anion, $O_2^{\bullet-}$. The $Fe^{2+}\cdot O_2$ complex has been observed in microsomal P450s (*110*−*113*), but beyond this step, the characterization of intermediates is less certain. Inferences have come from some work with bacterial P450s (*77*, *114*) and peroxidases and biomimetic model metalloporphyrins (*115*). A second electron enters the system in step 4. This may come from NADPH−P450 reductase or, in some cases, from cytochrome b_5 (b_5) (*116*, *117*). [b_5 is rather inefficient in supplying the first electron, probably because the first reduction is thermodynamically more difficult than the second (*117*−*120*).] A proton is added in step 5, and the O−O bond is cleaved in step 6, generating H_2O (with the addition of a proton) and an entity shown as FeO^{3+}. This high-valent complex can be

Scheme 2. Generalized P450 Cycle Including Abortive Oxygen Reduction



written as Fe^{V}=O but the precise electronic configuration is unknown. With reference to the precedent of peroxidase compound I, which is stable and has been extensively characterized, the most general view is that the iron is Fe^{4+} and the porphyrin ring is one-electron deficient ($Fe^{4+}O_2$-porph$^{\cdot+}$).

The FeO^{3+} complex can be used to rationalize most of the P450 reactions, although alternate possibilities will be considered later. In step 7 this electron-deficient complex either abstracts a hydrogen atom or an electron from the substrate or else forms a sigma complex with the substrate (vide infra). A subsequent collapse of the intermediate or intermediate pair in step 8 generates the product. (In the case of a hydrogen abstraction mechanism, step 8 is referred to as an "oxygen rebound.") In step 9, the product dissociates from the enzyme.

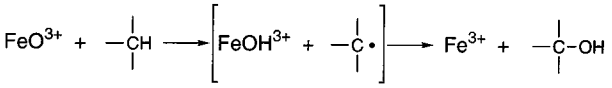
In artificial systems, an "oxygen surrogate" (R′O or R′OOH) may be used to convert the ferric iron to one of the high-valent iron−oxygen complexes (Scheme 1). As we will see later, some of these reactions are relevant to the "unusual" reactions of P450s.

Even with nine steps, the paradigm presented in Scheme 1 is an overall simplification. A slightly expanded version is shown in Scheme 2 with some, but not all, of the transformations that can be involved in the reduction of O_2 without product formation. Another important point to consider is that Schemes 1 and 2 make no provision for important conformational changes. In the case of bacterial P450 101, time-resolved crystallographic studies show changes at each identifiable step of the catalytic cycle (vide supra) (*78*).

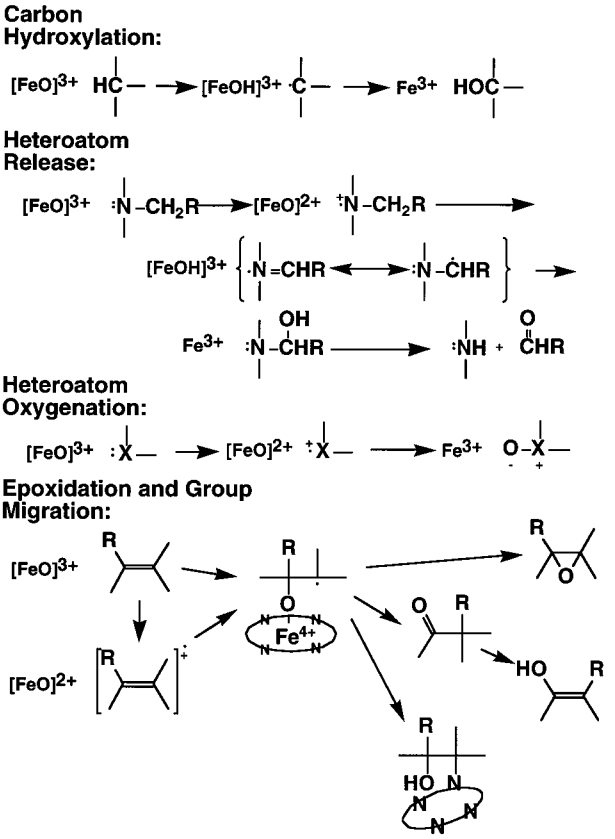
3.2. Rationalization of Major Reactions with a General Mechanism. In the early history of the P450 field, there were various proposals to explain P450 catalysis, including the generation of mobile oxidants such as $O_2^{\bullet-}$ that could react directly with substrates in P450 cavities (*121*). In the period 1978−1980, a number of studies, driven primarily by Groves' work with biomimetic models and rabbit P450s (*122*, *123*), led to the development of the system discussed above, in which FeO^{3+}, the "equivalent" of peroxidase compound I, abstracts a hydrogen atom and then completes the reaction by a radical recombination (oxygen rebound) (Scheme 3).

This scheme was expanded somewhat with the view that the putative FeO^{3+} complex could also abstract nonbonded electrons, which seemed very reasonable in light of the measured high oxidation−reduction potential

Scheme 3. Carbon Hydroxylation (H· Abstraction/ Oxygen Rebound)



Scheme 4. A "Unified" View of Major P450 Reactions (*131*)



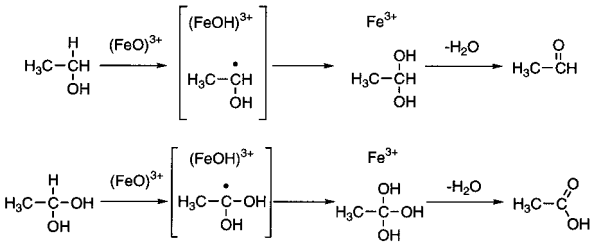
($E_{1/2}$) values of horseradish peroxidase compounds I and II (*124*) and subsequent work by Ortiz de Montellano and our own group on one-electron oxidations (*125*−*130*). With extension of the system to include the formation of σ complexes in oxygenation of π systems, we presented a unified system for rationalization of P450 catalysis (Scheme 4) (*131*−*133*). This system will be presented briefly, with some revision of our first major review (*131*). This systematic approach does not consider all of the alternative mechanisms proposed (section 3.2) or include some of the unusual reactions (section 4), which will be discussed. However, it does provide a rational approach to describing many of the P450 reactions and serves as a basis for discussing more complex views of the chemistry. Only the major P450 reaction groups will be presented at this point.

3.2.1. Carbon Hydroxylation. Carbon hydroxylation (or C-oxidation) is a very common reaction. The simplest case is the formation of an alcohol (e.g., from a steroid, alkane, etc.). The mechanism shown is the classic hydrogen abstraction/oxygen rebound (Scheme 3).

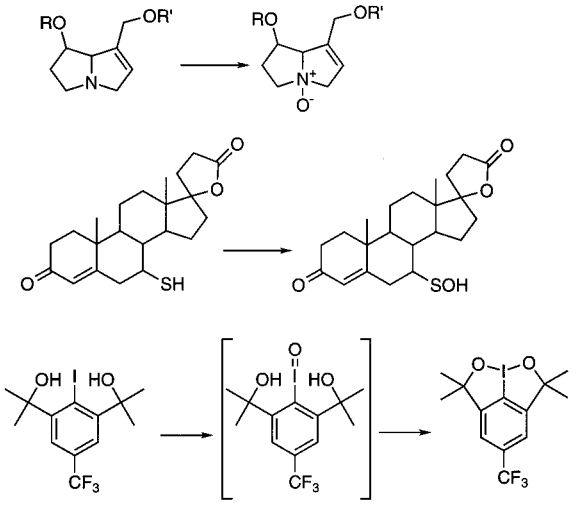
Evidence for the mechanism includes the high intrinsic kinetic deuterium isotope effects (*6*−*10*) and the extensive scrambling of regio- and stereochemistry seen in many reactions (*13*, *122*, *134*, *135*).

The basic mechanism may be readily extended to explain the oxidation of an alcohol to a carbonyl and the

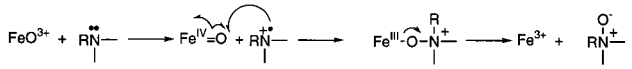
Scheme 5. Possible Mechanisms for Oxidation of Ethanol and Acetaldehyde by P450 2E1 by FeO^{3+} H· Abstraction (*136*)



Scheme 6. Heteroatom Oxygenations by P450s (*143*−*145*)



Scheme 7. A "Homolytic" Mechanism for N-Oxygenation (*131*, *147*)



subsequent oxidation of a hydrated aldehyde to a carboxylic acid (Scheme 5).

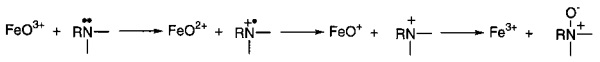
Carbon hydroxylation is generally considered to be a detoxication reaction but there are examples of a role in bioactivation, e.g., *N*-nitrosamine α-oxidation and benzylic oxidation of safrole (*137*, *138*).

3.2.2. Heteroatom Oxygenation. Heteroatoms are those atoms other than carbon. P450s have been shown to add oxygen to N, S, P, and I (Scheme 6) (*139*−*143*). Many of the N and S oxygenations had been assumed to be the consequence of catalysis of the flavin-containing monooxygenase in microsomal preparations (*146*) but are P450 reactions.

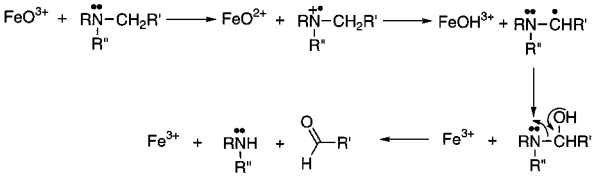
These reactions can be rationalized in the context of the FeO^{3+} chemistry. A direct attack of a nonbonded electron pair on the oxygen atom of FeO^{3+} seems unlikely in the context of the expected electron density, and a stepwise electron transfer is preferred, particularly in the context of the low $E_{1/2}$ values of these compounds. A one-electron transfer/recombination mechanism can be written (Scheme 7). However, some theoretical calculations (*148*) and the lack of a Hammett relationship (*147*) may be used in favor of a process in which a second electron transfer occurs prior to the rebound (Scheme 8).

Many *N*-oxides and *S*-oxides are less toxic than the parent molecules, but N-oxygenation of arylamines and heterocyclic amines is an important bioactivation step (*149*).

Scheme 8. N-Oxygenation (2 × 1-Electron Transfer/Oxygen Rebound) (*147*, *148*)



Scheme 9. N-Dealkylation via 1-Electron Transfer, Proton Abstraction, and Oxygen Rebound



3.2.3. Heteroatom Release (Dealkylation). This process results from the *formal* hydroxylation of a carbon adjacent to a heteroatom to yield an unstable compound that yields cleavage between that carbon and the heteroatom, e.g., a carbinolamine, acetal, thioacetal, *gem*-halohydrin, etc. The mechanism can be written as a "standard" carbon hydroxylation and probably does occur in this manner with fluorides, chlorides, and possibly bromides, as judged by the high kinetic hydrogen isotope effects etc. (*150*, *151*). With amines, sulfides, and some ethers, a stepwise one-electron-transfer pathway is often preferred (Scheme 9).

In this mechanism for P450, the FeO^{2+} complex facilitates the removal of a proton from the aminium radical (*152*) and possibly other radicals. This view is reasonable, because FeO^{2+} should be a reasonably good base (*153*). The ability of the FeO^{2+} entity to readily abstract a proton is rationalized as the basis for the low kinetic hydrogen isotope effects (∼2) (*152*), which contrast with the high intramolecular isotope effects for hydrogen atom abstraction (by P450s) and processes involving only two one-electron transfers (peroxidases) (*154*, *155*).

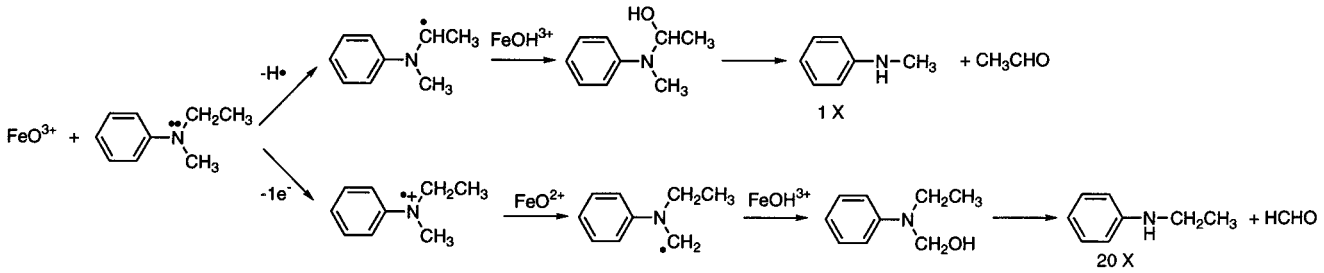
The overall evidence for the role of the one-electron-transfer mechanism has been summarized in detail (*11*, *133*, *156*, *157*) and includes (i) the low kinetic hydrogen isotope effects (vide supra) (*158*), (ii) the evidence for one-electron transfer with other low $E_{1/2}$ substrates (*159*, *160*), (iii) rationalization of the mechanism-based inhibition of P450s by 1-substituted cycloalkylamines (*127*, *128*, *139*), (iv) the trapping of extruded radicals in the oxidation of 4-alkyl-1,4-dihydropyridines and other results with these compounds (*125*, *126*, *130*, *161*), (v) free energy relationships with 4-substituted *N*,*N*-dimethylanilines (*129*, *162*−*164*), and (vi) the preferential N-demethylation of *N*-ethyl,*N*-methylaniline (*147*, *156*), a test based on the relative stabilities of H atom- and one-electron-derived intermediates (*165*) and consistent with the electrochemical studies of Shono (*166*) (Scheme 10). It should be noted that kinetic hydrogen isotope effects increase when an amine is acylated, with the $E_{1/2}$ increased and indicative of a shift to a hydrogen abstraction mechanism (*167*).

The oxidation of 1,4-dihydropyridines (Scheme 11) has some of the features of a vinylogous *N*-dealkylation reaction (*125*, *126*, *130*, *161*).

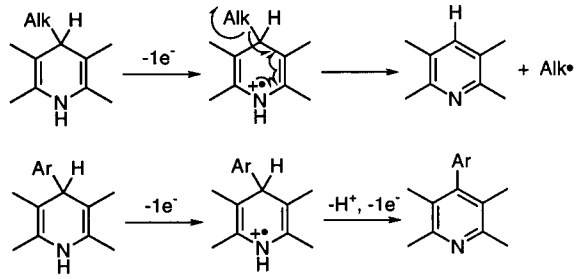
One-electron oxidation is a possible alternative mechanism for oxidations of alcohols or phenols (Scheme 12) and polycyclic aromatic hydrocarbons (PAHs) (vide infra).

Heteroatom release can result in bioactivation or detoxication of chemicals. For instance, the drug terferadine is detoxicated (*168*) but N-demethylation of *N*,*N*-dimethylformamide sets the compound up for desaturation to methyl isocyanate (Section 4.2).

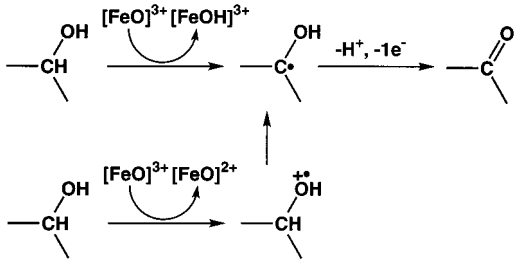
Scheme 10. Oxidation N-Dealkylation of *N*-Ethyl,*N*-methylaniline by P450 2B1 (*147*)



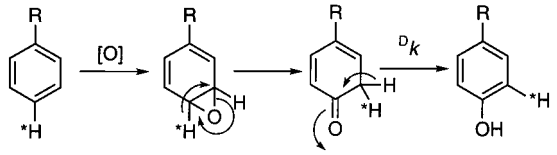
Scheme 11. Oxidation of 1,4-Dihyropyridines by P450s (*125*, *161*)



Scheme 12. Alternate Mechanisms (H· abstraction, 1-electron transfer/deprotonation) for Oxidation of an Alcohol



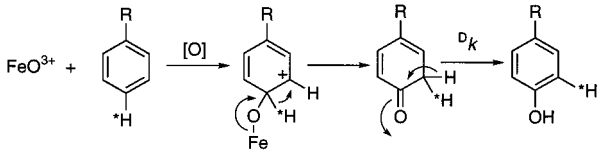
Scheme 13. Arene Epoxidation Followed by a 1,2-Migration to a Phenol



3.2.4. Epoxidation. Epoxidation is of particular interest in toxicology in that the products can be unstable and react with nucleophilic groups in macromolecules to initiate biological effects. Epoxidation is of considerable interest in organic synthesis, and the mechanism of chemical epoxidation has also been of considerable interest to chemists.

Early work in the P450 field generally treated the process of epoxidation as a rather concerted process, for both arenes and olefins. An often-described process is the "NIH Shift," with the name coming from the National Institutes of Health, where it was discovered (*169*). The process, originally described with a 4,3-shift of the hydrogen, was encountered during an attempt to use release of the 4-^{3}H to H_2O in an assay (*170*, *171*). The existence of this shift is often interpreted as evidence of an epoxide (in the formation of a phenol) (*172*) (Scheme 13). However, alternate mechanisms involving electrophilic FeO intermediates are also consistent with the observation of such migration (Scheme 14). Thus, the

Scheme 14. Phenol Formation by a Stepwise Mechanism Involving Attack by an Electrophilic FeO Complex, Followed by 1,2-Migration to a Phenol



demonstration of the "NIH Shift" is in itself not conclusive evidence for the existence of an epoxide; better support comes from the isolation of a dihydrodiol or a hydroxy-GSH conjugate.

Several mechanisms are possible for the epoxidation of an olefin (with a metallo−oxo complex). The same applies to arenes, although this is a thermodynamically more difficult reaction. Mechanisms considered for epoxidation with biomimetic models, P450s, and other metalloprotein oxygenases include concerted processes and intermediates with a carbon radical, a carbocation, or metal−carbon bond (*173*−*186*). The lack of changes in stereochemistry is difficult to interpret in enzymes due to the potential constraints on rotation.

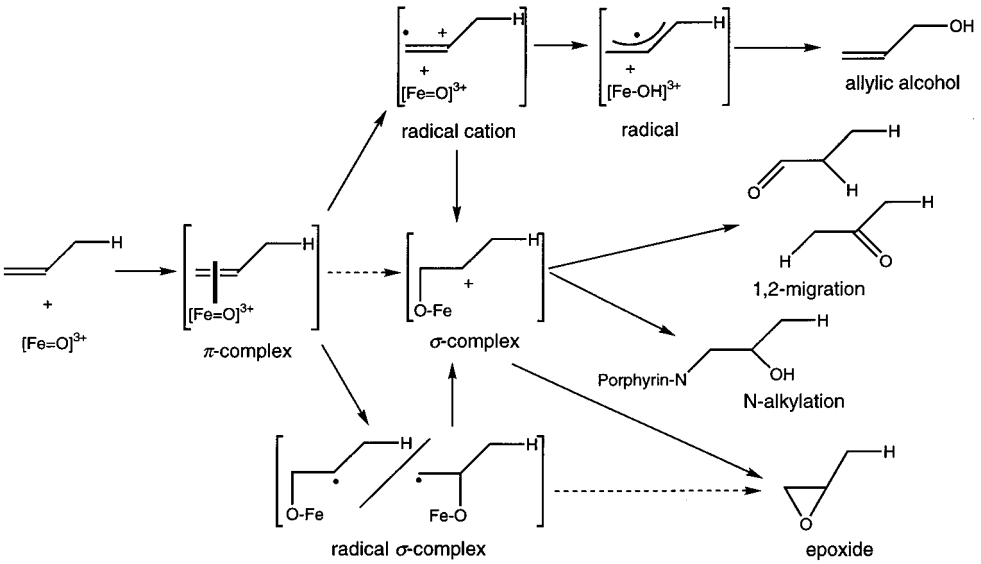
Biomimetic model studies have not given an unambiguous answer as to how epoxidation occurs in those systems (*183*, *184*, *187*). Some of the work argues against a role of initial one-electron transfer in the epoxidation of olefins, at least with the models employed. In our own work, we did not find evidence for one-electron transfer in the epoxidation of low $E_{1/2}$ PAHs (*188*). Much of the thinking regarding enzymatic epoxidation is dominated by the other reactions associated with many epoxidations, i.e., 1,2-shifts and heme modification (vide infra). A generalized paradigm is presented in Scheme 15 (*187*).

One phenomenon seen with only a few olefins and P450s is the incorporation of hydrogen from H_2O into the epoxide (*181*). This result can probably be explained only by the existence of an Fe−C bond (Scheme 16).

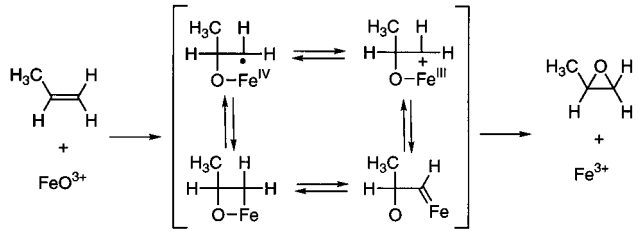
3.2.5. 1,2-Group Migrations and Heme Inactivation in the Oxidation of Olefins and Acetylenes. These reactions have already been alluded to (Scheme 4). With regard to both olefins and arenes, the shifts can be rationalized in terms of rearrangements of epoxides, e.g., "NIH Shift" (vide supra). Work with epoxides of vinyl halides revealed products ascribed to chloride or hydride migration which were not seen in the degradation of the epoxides, even in the presence of P450 or metalloporphyrins (*189*, *190*). These results are best accommodated by the cationic intermediate $Fe^{III}-O-C-C^+$ (Scheme 17), because migration of a radical would be highly unlikely.

The mechanism-based inactivation of terminal olefins has been studied in detail by Ortiz de Montellano and his associates (*94*, *95*). The results are rationalized in a

Scheme 15. P450-Catalyzed Oxidations of Olefins Rationalized with an Initial π-Complex



Scheme 16. Oxidation of Propylene by P450 2B4 and Rationalization of H_2O ^{18}O Exchange with Fe−C Bonding (*181*)



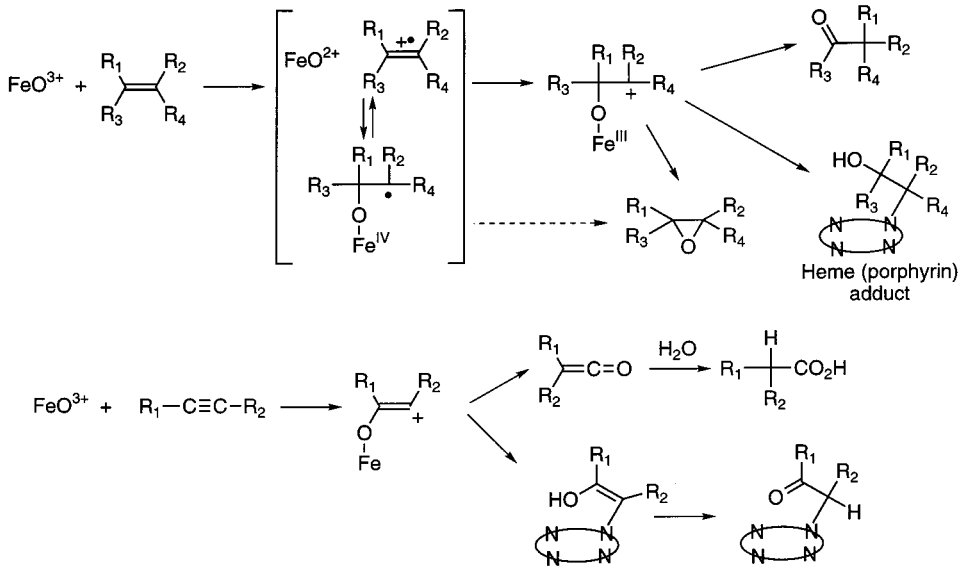
model where the heme pyrrole nitrogen can attack either $Fe^{IV}-O-C-C^{\cdot}$ or $Fe^{III}-O-C-C^{+}$ (Scheme 17). Either is a possibility, although there may be some theoretical preference for the radical. One possibility is that the $Fe^{IV}-O-C-C^{\cdot}$ is only involved in heme alkylation and that this undergoes electron transfer to yield $Fe^{III}-O-C-C^{+}$, which is involved in the shift of an anionic moiety.

Acetylenes show behavior similar to olefins (Scheme 17). Komives and Ortiz de Montellano (*191*) showed a 1,2-hydrogen shift rationalized with an ionic complex of the same type presented for olefin oxidation, Fe−O−CH=C^{+}−. The intermediate can yield either heme modification or migration to generate a ketene. Indirect evidence for an oxirene (epoxide of an acetylene) has been presented recently (*192*). Ketenes react with amines to yield amides, with alcohols to give esters, and with thiols to give thiol esters.

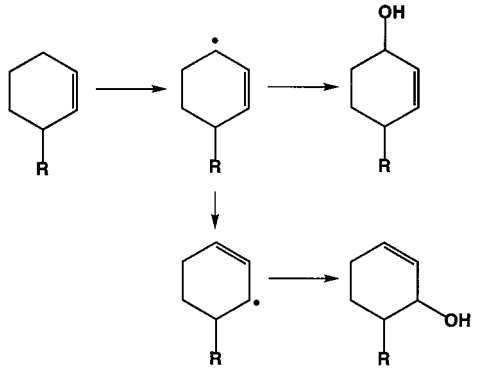
The reaction of an olefin yields a porphyrin adduct of the type N−C−C−OH from an olefin and N−C−CHO from an acetylene, where N represents one of the pyrrolic nitrogens in the porphyrin. The regioselectivity is influenced by the shape of the protein binding site, as elegantly shown by Ortiz de Montellano and his associates in experiments involving other heme modifiers (*94*, *95*).

The point should be emphasized that epoxides are not intermediates in yielding either the 1,2-shift products of olefins or the heme adducts, as shown by experiments with various epoxides (*94*, *95*, *189*, *193*). Epoxides do not give the same 1,2-shifts, even when added to purified P450. The shifts can only be seen with strong Lewis acids, which metalloporphyrins are not (*189*, *193*). Epoxides are

Scheme 17. Oxidation of Olefins and Acetylenes



Scheme 18. Rearrangement during P450 Oxidation Rationalized with Allylic Radicals



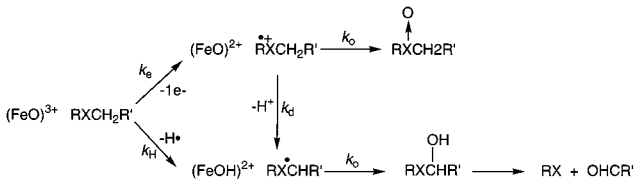
not very efficient at causing heme destruction, and even when *N*-alkyl prophyrins can be formed the stereochemistry is opposite that of the enzyme-catalyzed reactions (*194*). The possibility exists that epoxidation, 1,2-shifts, and heme alkylation are mechanistically unrelated events, although many similarities are observed and the collection of results is best explained by a mechanism with at least some common intermediates (*95*, *131*, *187*, *189*, *193*, *195*).

3.3. Alternate Oxidation Mechanisms. Thus far, we have utilized an entity depicted as FeO^{3+} to explain all P450 oxidations. Such an entity is analogous to peroxidase compound I. However, such a P450 complex has not been characterized (other than possibly by UV−vis spectra obtained by adding oxygen surrogates), presumably due to its suspected instability. Much of the thinking regarding such a complex is the result of work with biomimetic models.

Early work in this field involved mobile reactive oxygen species (e.g., $O_2^{\bullet-}$) (*121*) or an "oxene," which is a carbene-like proposition that was not well-described but was considered to be able to insert into C=C and C−H bonds (*196*−*198*). Some of the logic regarding these proposals was driven by models involving what is generally termed Fenton chemistry (*199*). The concept of a high-valent FeO entity, akin to peroxidases, that could abstract hydrogen atoms and electrons and undergo oxygen rebounds developed in the late 1970s (*123*, *200*). This paradigm provides reasonable explanations for scrambling of regioselectivity and stereoselectivity (Scheme 18) and for high intrinsic kinetic hydrogen isotope effects (*122*). The model also has found support from mechanistic and spectral studies with biomimetic model metalloporphyrins (*201*−*203*). As we have seen earlier, reasonable mechanisms can be drawn for most of the common P450 reactions. For instance, amine dealkylation and oxygenation can be rationalized easily with common intermediates (Scheme 19). Further, evidence for an oxygenating complex is provided by the reactions of P450s in the presence of the oxygen surrogate iodosylbenzene, which can only yield intermediates with a single oxygen (*204*, *205*), i.e., the transfer of oxygen from iodosylbenzene to iodobenzene (*206*).

One nagging problem with the FeO^{3+} mechanism was that it did not provide an explanation for the last step in steroid deformylation, e.g. the aromatase reaction (*207*). Akhtar (*208*, *209*) and Robinson (*210*) proposed an alternate mechanism involving a peroxyferryl intermediate (Scheme 20), and the work has been extended by Vaz and Coon to simple formyl models (Scheme 21) and the

Scheme 19. Bifurcation of an *N*-Alkylamine between N-Dealkylation and N-Oxygenation (*147*)[a]



[a] k_e = rate of electron transfer, k_H = rate of hydrogen atom abstraction, k_d = rate of deprotonation, k_o = rate of oxygen rebound.

P450s involved in xenobiotic oxidations (*211*−*213*). One of the strongest pieces of evidence for involvement of FeOO(H) is that these reactions can be supported by the oxygen surrogates H_2O_2 and alkyl hydroperoxides but not a single-oxygen donor, iodosylbenzene (*214*). Other evidence for the mechanism has accrued from studies with P450 mutants having a putative heme distal Thr changed to Ala. The logic has been that such mutants are impaired in their ability to cleave the O−O bond. Rates of some reactions are enhanced by these mutations, and the interpretation is consistent with a role of an FeOO(H) oxygenating species (*214*, *215*). Oxidation of an aldehyde to a carboxylic acid can also be rationalized with a peroxyferryl intermediate (Scheme 22).

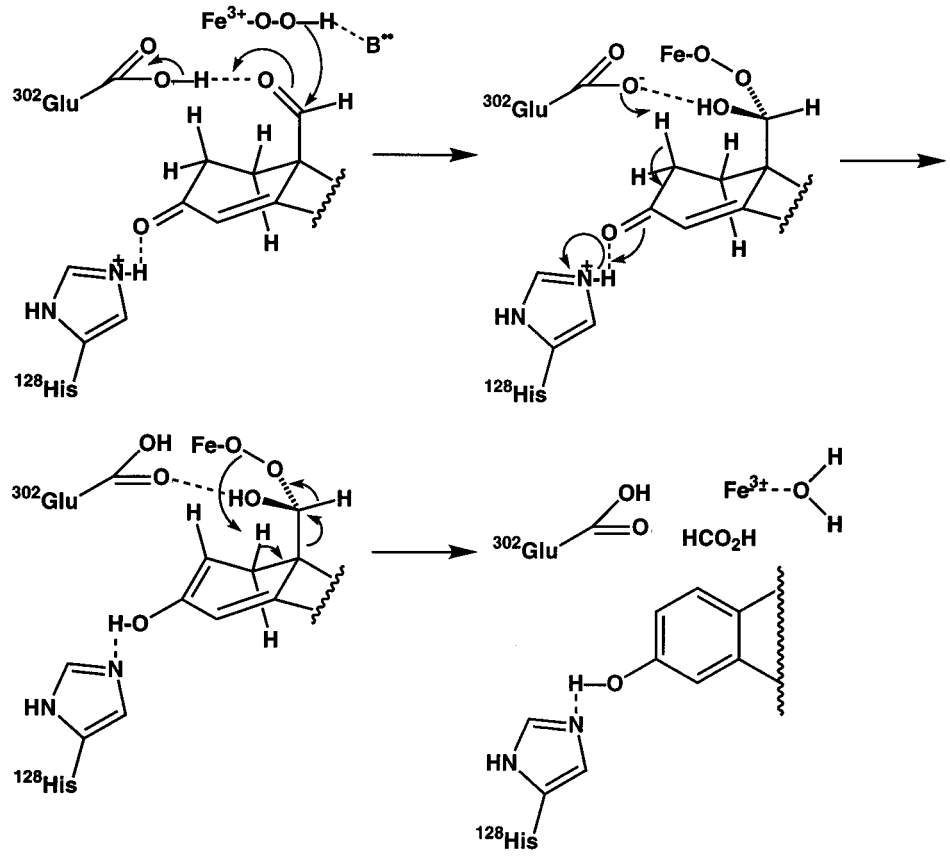
Experiments with strained cycloalkanes have been done with P450s since at least 1978 (*216*), and the rearrangements have been interpreted as evidence of radical intermediates (*11*, *13*, *128*, *160*) (Schemes 23 and 24). Refinements in the approach led to the "clocking" of the putative radicals, in the context of rates measured in solution. The results led to the view that the oxygen rebound is very fast, because some rearrangements are not very extensive (*160*, *217*). Further studies with "faster" clocks led to even less rearrangement, and these results have been interpreted in terms of a concerted mechanism (*218*−*220*). Newcomb has also interpreted results in the context of multiple intermediates, perhaps FeO^{3+} and FeOO(H), which are involved in concerted and cationic-intermediate reactions (*221*). Interpretations of kinetic-hydrogen isotope effects and scrambling of regioselectivity results have also been proposed in the context of this paradigm (*221*, *222*).

Another "alternative" proposal is the agostic model of Collman (Scheme 25) (*223*). This is an alternative concerted mechanism developed to explain some results obtained with some porphyrin models (*223*). This hypothesis has not been examined with P450 enzymes. Collman's group has also recently published evidence that product distribution in reactions catalyzed by metalloporphyrin models is influenced by the oxygen surrogate, arguing against a loss of occupancy by the surrogate in the oxygenating species (*224*), in line with a possible mechanism proposed earlier by Ortiz de Montellano (*13*). This information may also be relevant to P450 systems and the discrepancies that are often seen between reactions supported by NADPH-reductase/O_2 and the surrogates (*225*−*227*).[2]

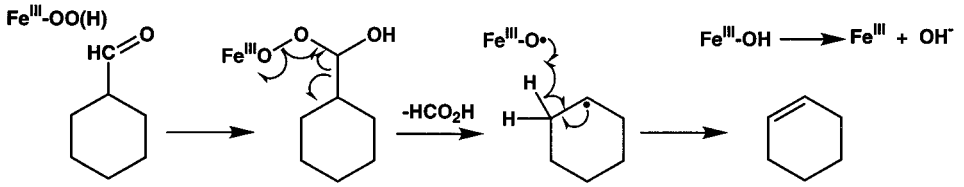
Recently, Shaik and his associates have published an attempt to reconcile all of these results in the context of a more universal model (*228*, *229*). A "two-state reactivity" model is proposed in which the FeO^{3+} complex is

[2] Hanna, I. H., Cai, H., Miller, G. P., Nakamura, K., and Guengerich, F. P., in preparation.

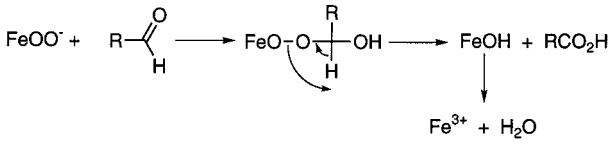
Scheme 20. Third Step in Steroid Aromatization by P450 19 (*208*−*210*)



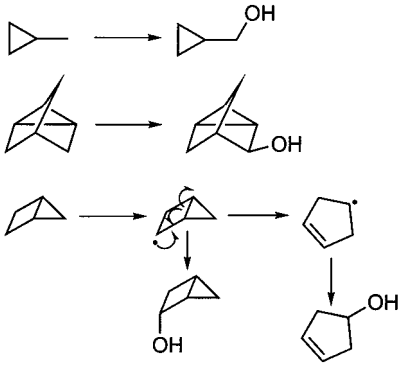
Scheme 21. Oxidative Deformylation of a Model Compound by P450 (*211*)



Scheme 22. A Mechanism for Oxidation of an Aldehyde to a Carboxylic Acid by FeOO−



Scheme 23. Oxidations of Cyclopropyl Derivatives by P450s

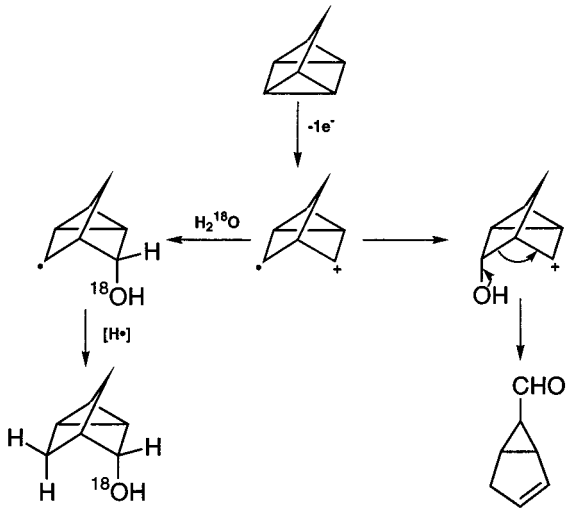


involved in all (or most reactions) but exists in two spin states, high and low. The low-spin complex is proposed to be involved in more concerted reactions, for some unknown reason, without barriers. The high-spin iron complex catalyzes similar chemistry but in stepwise, odd-electron processes. Only these latter reactions are proposed to show the scrambling and isotope effects.

The concept of the existence of multiple oxygenating species has some attraction. For instance, epoxidations might be explained with peroxide-type species, in that synthetic epoxidations can be done with H_2O_2 and alkyl hydroperoxides, and there is precedent for enzymatic flavin C_{4a}-OOH epoxidation (*230*). Heteroatom oxygenations can also be done with peroxides (*231*, *232*).

To date, much of the dogma of P450 catalytic selectivity has involved the view of a rather common chemistry and a reaction selectivity imposed by the juxtaposition of the substrate in the protein, with different atoms proferred to the iron−oxygen complex. We have tended to advance this view in our own past reviews on the subject (*131*−*133*, *233*, *234*), whether right or wrong. This has also been the general logic in all homology modeling and part of the rationale for obtaining crystal structures. However, an alternate view is that we are dealing with a group or a continuum of iron−oxygen species of varying activity (Table 1). Thus, we could have high- and low-spin FeO^{3+} complexes capable of C-oxidation, an FeO^{3+} (or even

Scheme 24. Oxidation of Quadricyclane by P450 (*159*)



Scheme 25. Agostic Mechanism for "Concerted" Carbon Hydroxylation

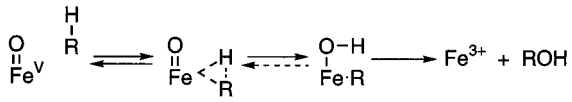


Table 1. Potential Iron−Oxygen Complexes Involved in P450-Catalyzed Oxidations

oxidation/reaction type	some potential oxidations
FeO^{3+}/low−spin (no barrier)	C-oxidation, epoxidation, X-oxidation [a]
FeO^{3+}/high−spin (no barrier)	C-oxidation, epoxidation, X-oxidation
FeO^{3+} (agostic)	C-oxidation
FeO^{3+} ($1e^-$ transfer)	X-oxidation
$FeOO^-$	epoxidation
FeOOH	epoxidation, X-oxygenation

[a] X = heteroatom.

FeO^{2+}) complex capable of one-electron transfer, and $FeOO^-$ and FeOOH species, all with varying capabilities. In this view, catalytic selectivity would be influenced by the nature of the iron−oxygen complex, in the context of interactions with the axial ligand (Cys) and the substrate itself. As Ogliaro et al. (*229*) point out, iron-ligation and the strength of bonds formed after rebound (or its equivalent) greatly influence the theoretical predictions. In the high-spin complex, the R• intermediate is a better electron donor, the resulting C−O bond is stronger, and the Fe−S bond is weaker, according to theoretical calculations (*229*). Thus, the nature of the oxygenating species and also the juxtaposition of the substrate could be influenced by the protein structure. That is to say, the intrinsic iron−oxygen complex and the substrate juxtaposition are not independent variables.

The view that a series of multiple iron−oxygen complexes exist and have different properties has a certain attraction and may explain some apparently conflicting results. However, it should be pointed out that there is no real experimental support for such a view, with the exception of the evidence for FeOO(H) intermediates already cited (*214*). Moreover, any attempts to use modifications of protein structure are not unambiguous in that both substrate juxtaposition and properties of the iron−oxygen complex could be influenced.

3.4. Rate-Limiting Steps in P450 Reactions. Having now reviewed the general features of the chemistry of P450 catalysis and before embarking on a series of specific reactions, consideration of rate-limiting steps within the cycle is in order. Exactly which of the individual steps depicted in Scheme 1 (or 2) is rate limiting has been debated for many years. One of the prevalent views that has developed is that the reductions are rate limiting, either the input of the first or the second electron (Scheme 1). This view seems to have developed from some of the early work by Gillette (*235*) and Ullrich (*236*) with microsomes, in which the concentration of the reductase is low relative to P450, and the rates of reduction of some P450s [but not all (*103*)] are facilitated by the presence of a substrate. However, this explanation is too simplistic, for a rather obvious reason. If reduction is rate limiting in all reactions with each P450, then all reactions should proceed at the same rate. Drug development in the pharmaceutical industry would be much easier because P450 3A4 reactions would all run at the same reaction rates with all potential drugs. However, this is obviously not the case.

In principle, any of the steps in Scheme 1 can limit rates of P450 reactions. We have been very interested in this subject in this laboratory, particularly with human P450s 1A2, 2D6, and 2E1 (*136*, *237*−*239*). If one examines the literature, examples of almost all the observable individual steps in Scheme 1 being rate limiting can be found, if one looks at enough P450s and enough reactions.

Substrate binding (step 1) is probably a rapid, diffusion-controlled reaction. Camphor binding to bacterial P450 101 has a measured k_{on} of 2.5×10^7 M^{-1} s^{-1} (*77*, *240*), and efforts to measure substrate binding to mammalian P450s by observing rates of spin-state changes are consistent, if not as accurate (*241*). These rates are fast, of course, but at low substrate concentrations, the rate of binding substrate may either become rate limiting or, more likely, may be too low to compete with k_{off} (k_{-1}), which will determine the amount of substrate-bound P450. The possibility exists that the initial binding of substrate may not place it directly in an enzyme−substrate complex that can proceed on, and further "delivery" steps might be required [cf., prostaglandin synthase (*242*)]. There is no experimental evidence to support the presence of additional, first-order steps, but their existence is possible. Even in P450s in which considerable protein movement is required to allow the substrate to bind inside the P450 [e.g., P450 101 (*243*−*246*)], rates of movement to a form in which the substrate can elicit iron spin state changes are fast (*77*).

The reduction step involving the introduction of the first electron (step 2) is complex and has been studied extensively. Electron flow from NADPH through the two flavins of the reductase has been described in detail (*104*−*107*). When P450 and reduced NADPH−P450 reductase are mixed together, the interaction to form a productive complex is rather slow, at least in artificial phospholipid residues that support P450 reactions (*103*, *247*). Moreover, if excess P450 is premixed with a limiting amount of reductase, then only an amount of P450 equivalent to the reductase is reduced rapidly and the remainder is reduced slowly (*103*). Such models may have relevance to microsomes, in which biphasic reduction of the P450 is usually observed (*248*, *249*). Thus, the amount

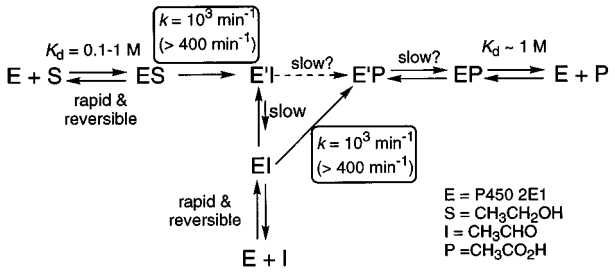
of reductase may be effectively limiting a P450 reaction, even if the actual first-order rate of electron transfer within the reductase−P450 complex is rapid. With regard to the rate of interaction of the P450 and reductase proteins, it is not clear if their second-order rate of encounter is slow or there is a low (first-order) rearrangement following rapid second-order interaction.

In some systems, the presence of substrate can greatly facilitate rates of electron transfer from the reductase to a P450. The effect varies considerably among P450s and their substrates (*103*). The effect may be considerable, e.g., addition of (*S*)-warfarin purified (reconstituted) to P450 2C9 increased the rate from ~4 min^{-1} to 200 min^{-1} (*103*). With P450 101, there is an excellent correlation between binding of the substrate camphor and shifts of the iron to the high-spin form and of the oxidation−reduction midpoint potential ($E_{m,7}$) to a more positive value (*77*, *250*). Although some reviews and primary articles suggest that this relationship applies to all P450s (*12*, *251*), the evidence is limited and the exceptions outnumber the cases where a relationship exists in the literature with microsomal P450s (*103*, *119*). There is no evidence for major changes in $E_{m,7}$ due to substrate binding to microsomal P450s (*119*, *252*), and nearly completely high-spin P450s have very low $E_{m,7}$ values (*119*, *253*). Reductions are driven by the high ratios of $NADPH/NADP^+$ in the combined Nernst equation, and proteins have ways of transmitting ligand binding into more efficient transfers without direct effects on the $E_{m,7}$ of the iron.

The next step in the cycle (step *3*) is O_2 binding. This step is probably fast and diffusion limited. With P450 101, the rate has been measured as 8×10^7 M^{-1} min^{-1} ($K_d = 3 \times 10^{-8}$ M) (*77*). Nevertheless, several reduction reactions of P450s have been described (vide infra) and occur even in the presence of O_2 (at 180−200 μM in solutions, in vitro). It is possible that affinities of some P450 forms are poorer than others, but this possibility has not been explored.

The next step following O_2 binding is the introduction of the second electron into the system (step *4*). This step is often discussed but its direct observation with a mammalian P450 has not been reported. With bacterial P450 101, this step has been observed by mixing the $Fe^{2+}\cdot O_2$ complex with reduced putidaredoxin ($k = 2 \times 10^7$ M^{-1} s^{-1}) (*254*). However, in the case of the mammalian P450s, the formation of a productive complex between the two proteins of interest is slow following mixing (*103*), and this experiment would not work. One of the reasons that step 4 is considered to be rate limiting (in some reactions) is the observed stimulation upon the addition of b_5. This stimulation is usually attributed to the role of ferrous b_5 (which can rapidly accept electrons from NADPH−P450 reductase) in electron transfer. An explanation for the varying effects of b_5 in different P450 reactions has been offered by Pompon in terms of the reversibility of electron transfer between b_5 and the P450 $Fe^{2+}\cdot O_2$ complex (*118*). A corollary to this work is that the $E_{m,7}$ of the redox couple in step 4 is much more positive than that in step 2, because the $E_{m,7}$ of b_5 is −20 mV (*255*,*256*). An $E_{m,7}$ of ~0 mV is predicted from the b_5 transfer and also from comparisons with the $Fe^{2+}\cdot O_2$ and $Fe^{2+}\cdot CO$ complexes (*118*, *119*). However, it should be pointed out that the stimulatory effect of b_5 may not be due only to electron transfer because at least four P450s have been shown to be stimulated by apo (heme-free) b_5 (*252*, *257*, *258*).

Scheme 26. Kinetic Model of P450 2E1-Catalyzed Oxidation of Ethanol to Acetic Acid via Acetaldehyde (*136*)

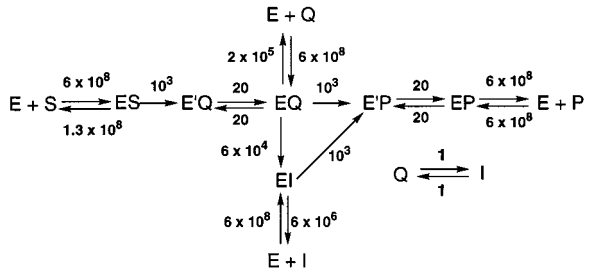


Step 5 involves cleavage of the O−O bond to generate an FeO entity. This step has never been observed directly, and the rate has not been measured. Most of the information about this step has been deduced from the effects of changing Thr and Glu/Asp residues on product formation (*259*−*261*). Substitution of these residues with Ala in the mammalian P450s results in losses of some activities but not others (*214*, *262*, *263*). These latter results have been used in the deduction of a role for iron hydroperoxy complexes in oxygenation ($Fe^{2+}O_2^-$ or $Fe^{2+}OOH$) (*214*, *264*).

The actual oxygenation reaction by a high valent FeO complex is usually described as either (i) sequential hydrogen atom abstraction/oxygen rebound (*122*, *203*), (ii) sequential one-electron abstraction/deprotonation/oxygen rebound (*125*, *131*, *133*, *152*), or (iii) a concerted oxygenation (*223*, *265*). High intramolecular kinetic hydrogen isotope effects have been used as an argument in favor of mechanism i, although mechanism ii may be difficult to distinguish because the isotope effects will depend on kinetic acidity (*152*, *156*, *266*−*269*). Since none of these steps has been observed directly, rate constants are not available. However, the detection of a significant primary competitive hydrogen isotope effect in a noncompetitive (intermolecular) experiment provides evidence that the C−H bond-breaking step under consideration must be at least partially rate limiting. In the past, most efforts in this area had not shown high noncompetitive isotope effects, with some exceptions (*270*, *271*), and the conclusion was drawn that this step is not rate limiting (*272*). Recently, we have found a number of examples of intermolecular kinetic hydrogen isotope effects (in the range of 2−14) (*136*, *237*, *239*).[2] These results argue that this step (C−H bond breaking) is at least partially rate-limiting. Studies with radical clocks (strained cycloalkanes) have been used as evidence for rapid oxygen rebound. This conclusion has been generally accepted in the field. There are caveats, however, in that the apparently very rapid rates may reflect either a change to a concerted mechanism (*219*, *223*) or retardation of rearrangement by the protein (*273*).

Relatively little attention had been given to the possibly rate-limiting nature of any steps following product formation. Hanzlik had interpreted some kinetic isotope effect results on microsomal toluene hydroxylation as indicative of slow product release (*274*). In our own work on the mechanism of the increased P450 2E1, K_m values seen upon deuteration of ethanol (and subsequently acetaldehyde as well), the burst kinetics could only be interpreted in the context of a rate-limiting step (for the steady-state reaction) that must follow product formation (*136*, *239*) (Scheme 26). This rate-limiting step cannot

Scheme 27. Fitting of Individual Rate Constants to the Oxidation of Ethanol to Acetic Acid by P450 2E1 (Scheme 26) (*136*)[a]



[a] First-order rates in units of min^{-1} and second order rates in M^{-1} min^{-1}.

Scheme 28. Kinetic Model Used for Human P450 1A2 Reactions[a]

Kinetic model for P450 1A2 reactions

Mechanism

E + S <==> ES	:	k_1	k_{-1}
ES ---> FS	:	k_2	
FS + O2 <==> FSO2	:	k_3	k_{-3}
FSO2 --> GSO2	:	k_4	
GSO2 --> HSO	:	k_5	
HSO ---> EP	:	k_6	
EP <==> E + P	:	k_7	k_{-7}
GSO2 ---> E + S + H2O2	:	k_8	
HSO ---> E + S + H2O	:	k_9	

Rate constants (min^{-1} or min^{-1} μM^{-1})

k_1 = 6,000, k_{-1} = 120,000
k_2 = 700
k_3 = 6,000, k_{-3} = 6
k_4 = 700
k_5 = 110
k_6 = variable
k_7 = 660, k_{-7} = 6,000
k_8 = 50
k_9 = 50

[a] Simplifed from Scheme 2.[2] E = ferric P450, S = *p*-alkoxyacylanilide substrate, F = ferrous P450, O2 = O_2, GSO2 = $(FeO_2)^+$S complex, HSO = $(FeO)^{3+}$ S complex or an alternate complex capable of oxygenation, P = product (phenol or acetol), H2O2 = H_2O_2, H2O = H_2O. All rates are in min^{-1} (first-order) or min^{-1} μM^{-1} (second-order).

be product release itself, because the P450 2E1 has poor affinity for the product (*136*).

A kinetic scheme for P450 2E1-catalyzed oxidation of ethanol to acetic acid via acetaldehyde has been developed (*136*), although a single rate constant was used to summarize what are steps 2−8 of Scheme 1 (Scheme 27). Recently, we have studied the kinetics of oxidations of *p*-alkoxyacylanilides by human P450 1A2 (*237*, *239*). The steady-state rate constants for the various reactions (O-dealkylation and acetol formation) by different mutants vary considerably (*237*, *275*). A slightly simplified kinetic mechanism was used (Scheme 28). Limits on the system include the known rate of reduction of the ferric enzyme, the kinetic deuterium isotope effects, and the rates of formation of H_2O_2 (measured) and H_2O (calculated by balance between NADPH oxidation and the formation of products and H_2O_2). Substrate and product affinity can be predicted (in the ground state) by spectral titration, although there are caveats about the relationship of the mode of binding to that needed for catalysis.

v vs *S* plots could be fit well by using the mechanism and set of rate constants shown in Scheme 28 (Figure 1). Many different reactions could be fit well by varying only k_6, the rate of substrate oxygenation. Varying this constant yielded excellent fits of protiated and deuterated substrates with wild-type P450 1A2 and mutant E225I, which has enhanced activity (*237*, *239*, *275*).

The sensitivity of the fits to changes in several of the rate constants is shown (Figure 1). Changes in k_4, k_5, or k_6 influenced the fit. Adjustments to k_8 or k_9 yielded estimates of H_2O_2 and H_2O that were not only incompatible with those observed; these changes also changed both the k_{cat} and K_m for the O-dealkylation reaction (Figure 1). The overall set of rate constants is not considered a unique solution and the individual rate estimates must not be considered to be defined values. Nevertheless, these rules do satisfy a number of criteria and should be regarded as approximations. It is important to emphasize that the mechanism is probably more complex than depicted in Schemes 1, 2, and 28. The classic and accepted approach to kinetic modeling is to begin with a "minimal" mechanism and use it until discrepancies must be rationalized, which has been done here.

If the rate constants used in Scheme 28 and Figure 1 are valid, they provide an interesting picture of P450 kinetics. With $k_4 = 350-700$ min^{-1}, $k_5 = 110$ min^{-1}, $k_6 = 33$ min^{-1}, and $k_8 = k_9 = 50$ min^{-1}, we have an enzyme reaction with several transition states of similar heights (Figure 2). The reaction diagram is constructed for the reaction described in Figure 1 and utilizing several assumptions. The energy differences are set up using $\Delta G = -RT \ln K$ for binding equilibria, $\Delta G = -nFE_{(m,7)}$ for oxidation−reduction changes, and $k_{obs} = (kT/h)e^{-\Delta G^{\neq}/RT}$ for transition state barriers (*276*, *277*). (The position of the FeO^{3+}/substrate complex cannot be estimated by this approach; $E_{m,7}$ for the second electron reduction is set at 0 mV, vide supra.) The similarity of the heights of several transition states, if this view is indeed valid, provides a system in which minor changes in the interaction of a P450 with its ligands and accessory factors (e.g., reductase) can alter catalytic specificity.

If this system is applicable to other P450s and substrates, small changes in any of several rate constants can be manifested in altered steady-state rates. It is also notable that, because of the situation, the observed k_{cat} is considerably less than *any* of the microscopic rate constants. The K_m can be altered by changing k_{-1} or k_7 or by changing k_8 or k_9 (*239*). We have not extensively explored which other rate constants can be altered to change K_m (other than k_{-1}, k_7, k_8, and k_9). Another conclusion from this work is that (mammalian) P450s with very high steady-state rates must have higher rate constants for several steps, not only one.

4. Unusual P450 Reactions: General Issues Regarding Reaction Diversity

One of the major issues in studying an enzyme is that of specificity. If an enzyme is completely specific (i.e., absolutely only one substrate and one product) then there is actually not much to say about it. Thus, we are able to impose more studies on enzymes that will accommodate a large group of substrates and inhibitors. The

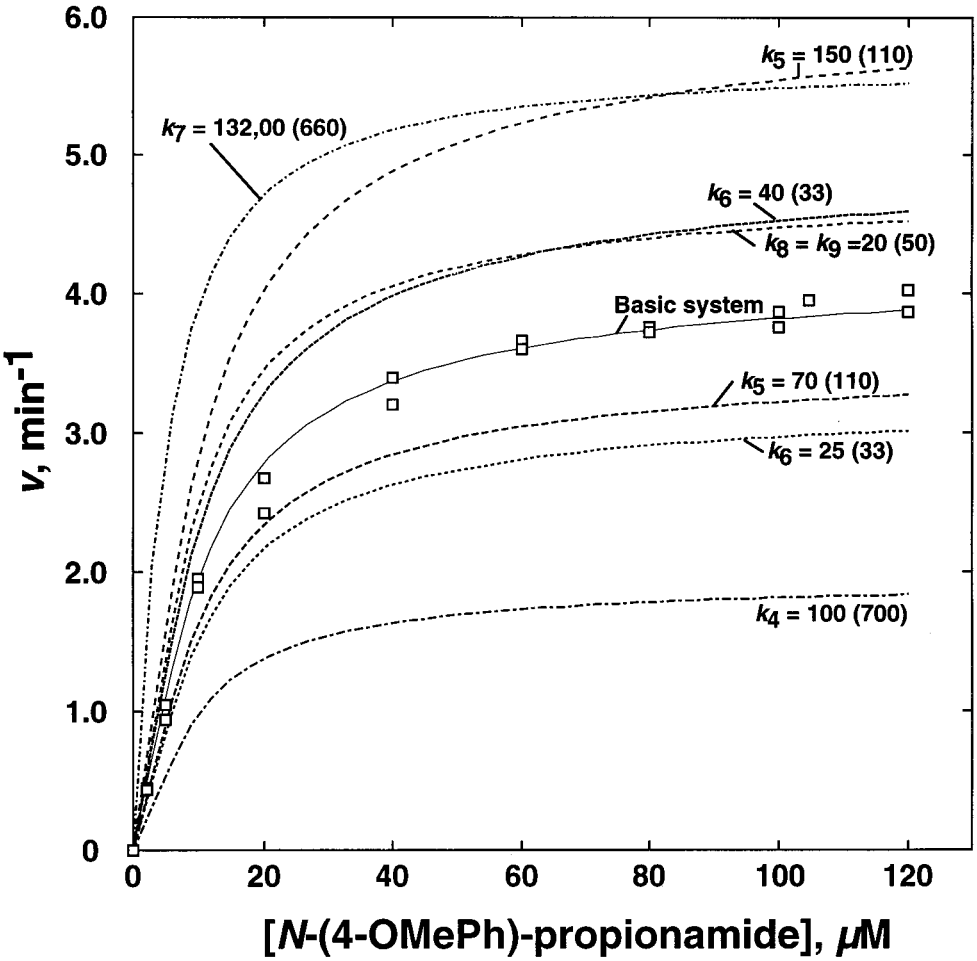


Figure 1. Effects of changes in rate constants on fits to O-demethylation of [*O*-methyl-d_3]*N*-(4-methoxyphenyl)-propionamide by wild-type P450 1A2 (*239*). The basic mechanism and individual rate constants are shown in Scheme 28 (k_6 = 33 min^{-1}) and were used to fit the data points (□) ("basic system"). Traces drawn with modifications of single constants are shown on the figure, with the original rate constant (from "basic system") shown in parentheses.

microsomal P450s have been very accommodating, because of their very nature, and usually accept a great variety of homologues and various diagnostic probes. Because of this promiscuity, we can do many experiments with many of the P450s and subject them to studies involving structure−activity relationships, linear free energy relationships, and searches for rearrangements diagnostic of particular mechanisms.

In one sense, the study of "unusual" reactions provides a window for what might be considered the "mainstream" reactions of the enzyme. For instance, the view that epoxidation is a stepwise and not a concerted process is based in a large part on the demonstration of 1,2-shifts and formation of modified porphyrins (*94*, *95*, *187*, *189*, *194*), events that are not be readily accommodated with concerted mechanisms. However, another side of the picture is that the unusual reaction mechanisms may be simply that, i.e., they may have been selected with the particular probes and not applicable to general reactions catalyzed by the enzyme. These caveats apply to P450 reactions. However, any general mechanisms must somehow accommodate the unusual reactions of an enzyme. In the best of mechanisms, these can be rationalized in terms of particular properties of the probes. Another axiom of mechanistic enzymology is that a positive result has far more impact than a negative one; the possible explanations of how protein structures can attenuate chemistry is myriad.

4.1. Reductions. Although P450s do not participate in biological reductions of endogenous substrates to the extent that flavoproteins do, the diversity of reduction reactions catalyzed by P450s is still considerable. The scope of reduction reactions is somewhat surprising in light of the reactivity of ferrous P450 with O_2 (vide supra). Indeed, anyone who has tried to work with P450s in the Fe^{2+} form might wonder why P450 would *not* be oxidized under minimally aerobic conditions. The general consensus has been that all ferrous P450s bind O_2 tightly and that reoxidation reactions are rapid. However, one consideration is that some of the reactions of interest would be more favorable in veinous sections of tissues, where the O_2 tension is reduced in the gradients. The $E_{m,7}$ for most microsomal P450s appears to be ∼−300 mV, in the absence or presence of substrate (*119*, *252*). Thus, ferrous P450 should be a reasonably good one-electron reducing agent, if a substrate can bind and receive electrons. It is possible that some substrates may bind in such a way as to inhibit O_2 binding; this possibility has not been explored very thoroughly. For instance, "Type II" liganding to iron might be effective in this way. Another consideration is that the $Fe^{2+}O_2$ complex, while it is waiting for an electron, is still a reducing agent. As mentioned earlier, it probably has an $E_{m,7}$ near 0 mV [based on the b_5 work (*118*, *255*, *256*)]. Thus the transfer of an electron to a substrate may be competitive with oxidation even in the presence of O_2.

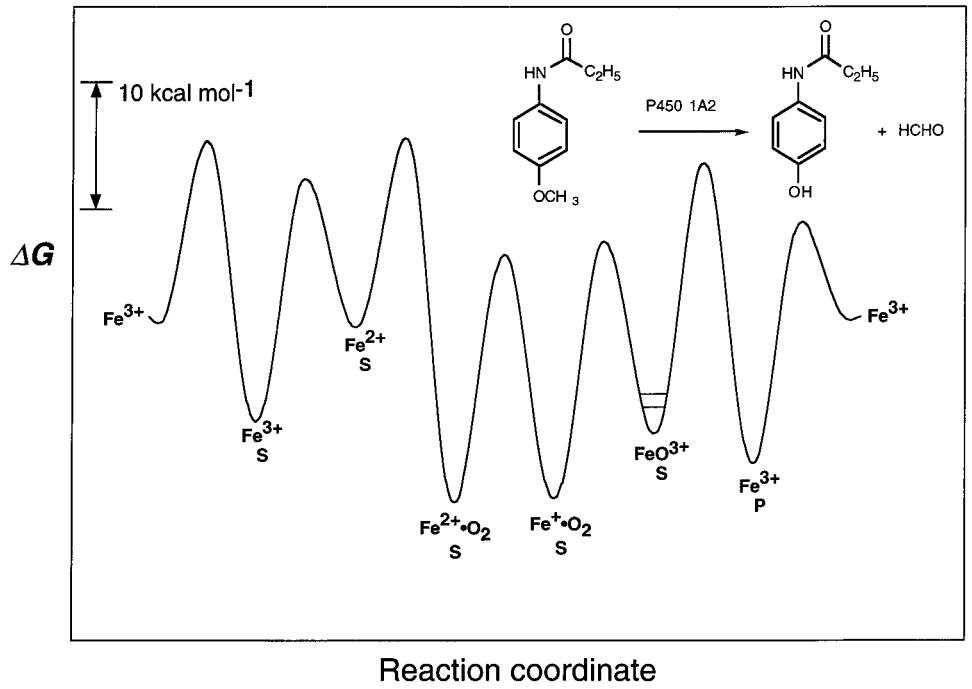
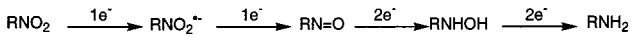
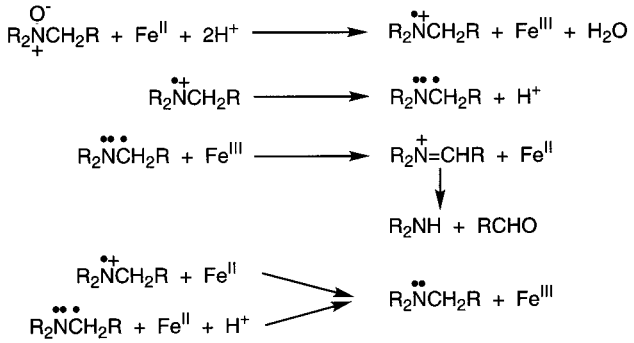


Figure 2. Diagram of oxidation of O-demethylation of *N*-(4-methoxyphenyl)propionamide by wild-type P450 1A2 based on rate constants fit with estimates from fitting to Scheme 28 and known and estimated $E_{m,7}$ values (*239*).

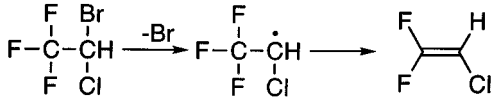
Scheme 29. Reduction of Nitro Compounds by P450s



Scheme 30. Reduction of an *N*-Oxide by Ferrous P450 (*147*)



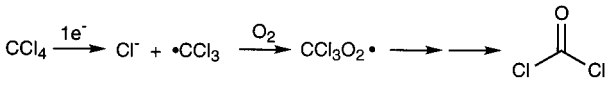
Scheme 31. Reductive Dehalogenation of Halothane



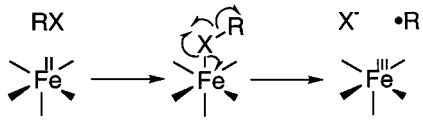
Lists of substrates for reduction by P450s have been compiled by Wislocki et al. (*278*) and more recently by Testa (*12*). In most cases, more mechanistic information about the reactions is not available, and the reader is referred to these reviews and primary references for more details. Some of the reduction reactions are presented in Schemes 29−37 and will be discussed briefly.

Alkyl halides are reduced, with CCl_4 and halothane serving as classic examples (*283*, *284*). Among the products are reduced haloalkanes (halide replaced by hydrogen) and olefins (dehydrohalogenation products), along with products generated from the reaction of generated radicals with oxygen, cellular macromolecules,

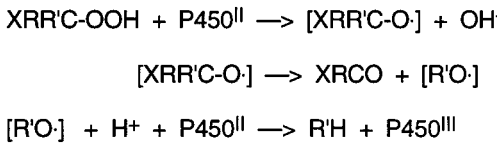
Scheme 32. Reductive Dechlorination of CCl_4 and Reaction of Trichloromethyl Radical with O_2



Scheme 33. A Proposed Mechanism for Reductive Dehalogenation (*279*)



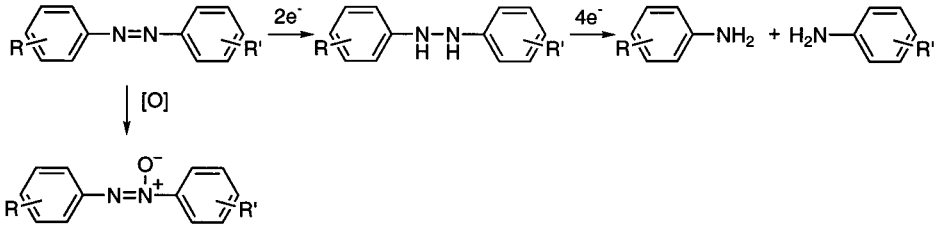
Scheme 34. Reduction of an Alkyl Hydroperoxide to an Aldehyde and CH_4 (*280*)



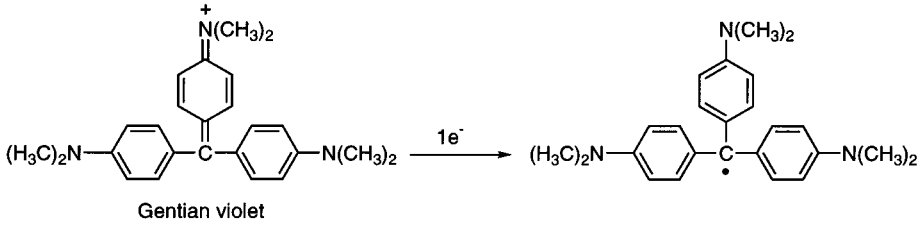
and even other radicals (Scheme 31). Although the reductive defluorination of halothane has been studied extensively, the oxidation is probably more relevant to toxicity (*285*). Some examples of reductive dehalogenation of aryl halides have also been reported. Halide ions are produced in these reductions. A viable mechanism proposed by Castro (*279*) is shown in Scheme 33.

The reaction of alkyl hydroperoxides with ferric P450 has already been mentioned under the general considerations of chemical mechanisms; in this sense the hydroperoxides serve as "oxygen surrogates" (see also section 4.11 for rearrangements of lipoxygenase-catalyzed hydroperoxide products of fatty acids). Hydroperoxides undergo other reactions with ferrous P450, particularly the formation of carbonyls and CH_4 (Scheme 34) (*280*). This chemistry may have some relevance in lipid peroxidation.

Scheme 35. Reduction of an Azo Compound

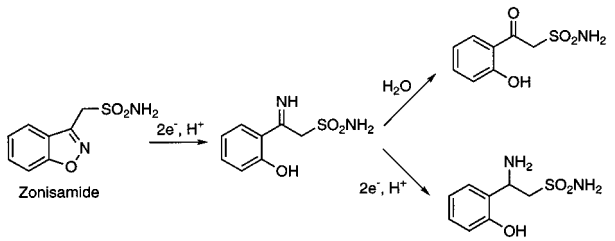


Scheme 36. Reduction of Gentian Violet (*12*, *281*)



Gentian violet

Scheme 37. Reduction of Zonisamide (*12*, *282*)



Nitrogen oxides are reduced by P450s and in many cases, by other enzymes as well. The list of substrates that can be reduced includes *N*-oxides, nitroxides, hydroxylamines, nitro groups, and *C*- and *N*-nitroso compounds (Scheme 29) (*12*, *278*). The deoxygenation/demethylation of *N*-alkylarylamine *N*-oxides by ferrous P450 has been rationalized in a mechanism involving Polonowski chemistry (Scheme 30) (*147*). A rather unusual P450 reaction is the reduction of the drug nitroglycerin (glyceryl trinitrate), which yields nitric oxide (*286*). Another unexpected reduction is that of benzamidoxime, reported by Clement et al. (*287*). The reduction involves the pathway NADH → NADH-b_5 reductase → b_5 → 2D family P450 → benzamidoxime, which seems highly unexpected in light of (i) the unfavorable potential for transfer of electrons from b_5 [$E_{m,7} \approx 0$ mV (*255*, *256*)] to a P450 [$E_{m,7} \approx -300$ mV (*119*, *288*)] and (ii) the low activities of NADH/b_5 systems in supporting P450 reactions that normally proceed via electron transfer from NADPH−P450 reductase (*120*, *289*). One-electron reduction of a nitro compound yields a nitroanion radical which, as in the case of halides (Scheme 32), may react with O_2 to initiate an abortive and destructive redox process.

P450s also reduce nitrogen−nitrogen compounds such as azo compounds, triazines, and hydrazines (Scheme 35) (*12*, *278*). This process may proceed to yield scission of azo dyes into two molecules. [Azo dyes were some of the first carcinogens to be studied in the context of mixed-function oxidation (*290*).]

Quinone-like compounds such as the triarylmethylene dye gentian violet may be reduced by P450 in a one-electron process leading to radicals (Scheme 36) (*12*, *281*). Reduction to semiquinones such as these can be followed by reaction (of the semiquinone) with O_2 to generate reactive oxygen species through redox cycling.

Other P450-catalyzed reductions lead to products whose origins may not be immediately obvious. The 1,2-isoxazole zonisamide is reduced and undergoes ring cleavage (Scheme 37) (*12*, *282*).

P450s reduce not only the typical organic molecules that we normally think of as substrates but also inorganic compounds. P450 reduces SO_2 (or its hydrated form bisulfite, HSO_3^-) to $SO_2^{\bullet-}$ (which is in equilibrium with dithionite, $S_2O_4^{2-}$) (*291*). $SO_2^{\bullet-}$ can then reduce P450 (*292*), and the reaction is reversible (the $E_{m,7}$ for $SO_2^{\bullet-}$ is much less than that of P450, so the process will in principle be subject to equilibrium thermodynamic considerations). P450s (particularly 2E1) reduce Cr^{VI} (to Cr^{V}, Cr^{IV}, Cr^{III}), a process that may contribute to the bioactivation of this carcinogenic metal (*293*). The bacterial $P450_{nor}$ (P450 55) reduces NO· at a very rapid rate (second-order rate constant of 2.5×10^7 M^{-1} s^{-1}) (*294*, *295*). This unusual P450 binds NADH directly and then apparently reduces the Fe−NO complex in a two-electron process (*296*).

4.2. Desaturation. Desaturation, or dehydrogenation, is an important part of fatty acid metabolism and some other aspects of intermediary metabolism, usually involving flavoproteins or non-heme iron proteins. The reaction is electronically equivalent to hydroxylation in that it involves a net two-electron oxidation, with or without H_2O to balance the stoichiometry.

Although desaturation was a well-established process for the so-called fatty acid desaturases, which were known to be mixed-function oxidases, the roles of P450s in desaturation were not clearly recognized in earlier work. In some cases in which olefins were characterized as products, the general feeling seemed to be that these were artifacts of the dehydration of initial alcohol products. For instance, Δ^9-dehydrowarfarin was recognized as a P450 oxidation product of warfarin (*297*, *298*). The Millers found that vinyl carbamate was more carcinogenic than urethan (and formed more DNA adducts) and postulated that dehydrogenation occurred but were unable to detect formation of the olefin (*299*−*301*).

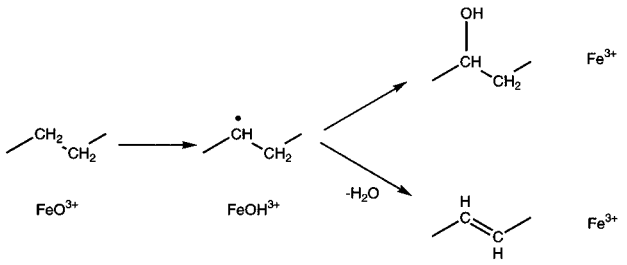
A seminal advance was the characterization by Baillie and Rettie of the desaturation and hydroxylation of the anti-epileptic drug valproic acid (*302*). They clearly demonstrated that both the alcohol and the olefin were stable products and did not interconvert. A purified P450

Table 2. Substrates for P450-Catalyzed Desaturation/Hydroxylation

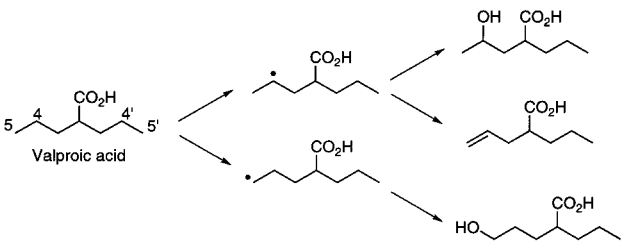
substrate	position of hydroxylation	ref
valproic acid	5	*302*
testosterone	6β	*303*
warfarin	10	*304*
lovastatin	6β	*305*
ethyl carbamate	1′ (also N)	*306*
bufuralol	1′	*a*

[a] See footnote 2.

Scheme 38. Bifurcation in the Oxidation of Alkanes: Hydroxylation and Desaturation



Scheme 39. Oxidation of Valproic Acid (*302*, *308*)



catalyzed both reactions, ruling out contributions from any microsomal fatty acid desaturases. Similar studies have now been done on a variety of other substrates and a list (not necessarily comprehensive) is presented in Table 2.

The mechanism of alkane desaturation is postulated to begin as in the case of C-hydroxylation, with abstraction of a hydrogen atom (Scheme 38). There is a competition between oxygen rebound and abstract of what is formally a second hydrogen atom (or an electron plus a proton). In all known examples of P450-catalyzed desaturation, C-hydroxylation is also observed and is usually the major course of the reaction (Table 2). Support for this mechanism comes from studies with deuterated valproic acid (*307*, *308*). Valproic acid is a symmetrical molecule, and the intramolecular kinetic isotope effect experiments were done with perdeuteration at either the 4- or 5-position on one arm of the molecule (Scheme 39). Apparent kinetic isotope effects [$^{D}(V/K)$] of 4−5 were seen for the two hydroxylated products. An isotope effect of 5.6 was seen with 4,5-^{2}H substrate, consistent with the view that abstraction of a hydrogen atom at C-4 initiates both 4-hydroxylation and desaturation. Removal of the elements of a hydrogen atom from C-5 is not rate limiting. The results do not rule out the possibility that desaturation could be initiated at C-5, with that H abstraction proceeding more rapidly than the second at C-4, although this process would have to be rather independent of C-5 hydroxylation, which shows a large isotope effect. Another interesting aspect of the work is the large intermolecular noncompetitive isotope effect (6.6) seen in comparing rates of desaturation of [4,4,4′,4′-^{2}H$_4$]- with protiated valproic acid (*307*). In our own work on the oxidation of ethyl carbamate (*306*), we found intermolecular noncompetitive isotope effects of 6−8 for the transformation of [^{2}H$_5$-*ethyl*] ethyl carbamate to 2-hydroxyethyl carbamate, vinyl carbamate, and the 1, N^6-ϵ-adenosine formed by trapping the epoxide (Scheme 40). In the deuterium isotope studies with both valproic acid (30*7*, *308*) and ethyl carbamate (*306*), the apparent noncompetitive isotope effects are large but are not segregated into k_{cat} and K_m components, so the parameters may or may not indicate that k_{cat} is limited by the rate of C−H bond breaking [i.e., an isotope effect on K_m could be indicative of an isotopically sensitive step that is not rate limiting in the steady-state reaction (*136*, *276*)].

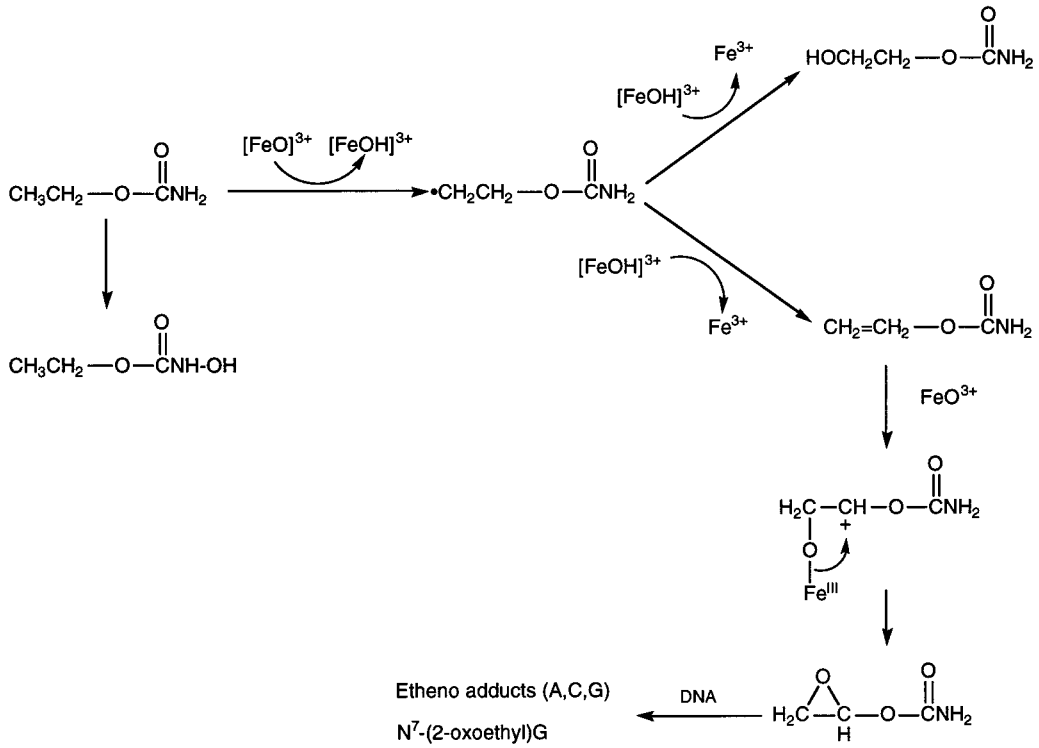
A related reaction is the dehydrogenation of *N*-methylformamide to methyl isocyanate (Scheme 41), catalyzed by P450 2E1 (*309*, *310*). The mechanistic similarity to alkane desaturation is unclear. This reaction is a bioactivation step in that it converts *N*-methylformamide (an N-demethylation product of the common solvent *N*,*N*-dimethylformamide) to a very reactive electrophile, methyl isocyanate, which was involved in a serious industrial accident in Bopal, India, in 1984 in which 3500 people died.

Another situation in which the apparent bifurcation between hydroxylation and desaturation may be applicable is in the oxidation of *N*-nitrosamines (Scheme 42). The oxidative N-alkylation and subsequent generation of an alkylating species has been known for some time (*311*). A reasonable proposal involves initial hydrogen atom abstraction followed by oxygen rebound to generate the α-hydroxynitrosamine, which rearranges to yield an aldehyde plus an alkyldiazohydroxide. A mechanism involving initial one-electron abstraction from the nitrogen is conceivable but probably unlikely in light of the lack of electron density on the atom (*312*) and the relatively high intrinsic kinetic deuterium isotope effects (*313*). The latter species is unstable and yields N_2, OH^-, and what would formally be a carbonium ion (Scheme 42). For many years, the production of nitrite and alkylamines has also been known (*314*, *315*). One postulate for this reaction was the interaction of the nitrosamine with ferrous P450 (*314*, *316*). However, the reaction has been shown to yield nitric oxide, representing an odd-electron removal from the substrate. Further, the same kinetic hydrogen isotope effect [$^{D}(V/K) \approx 5$] is seen for the denitrosation reaction as for the N-demethylation (*313*). [An anomaly in these assays is that the denitrosation pathway shown would also generate HCHO (Scheme 42).] Nitric oxide forms nitrite through oxidation or dismutation (*317*); it can also elicit numerous effects by activating guanylate cyclase or after reacting with $O_2^{\bullet-}$ to yield peroxynitrite.

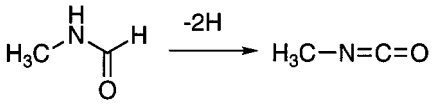
The proposed mechanism (Scheme 42) is actually another case of a collapse of a putative alkyl radical to yield a desaturated product along with the alcohol. However, more direct evidence for this denitrosation mechanism is still required.

The mechanistic information available does not provide a ready answer as to why some C-hydroxylations are accompanied by desaturation and others are not (Scheme 39). At this time, the desaturation reaction is not very predictable, unfortunately, because it can often favor subsequent activation reactions to yield toxic products, as in the case of valproic acid (*302*) and ethyl carbamate

Scheme 40. Oxidations of Ethyl Carbamate Catalyzed by P450 (*306*)



Scheme 41. Dehydrogenation of *N*-Methylformamide (*309*)

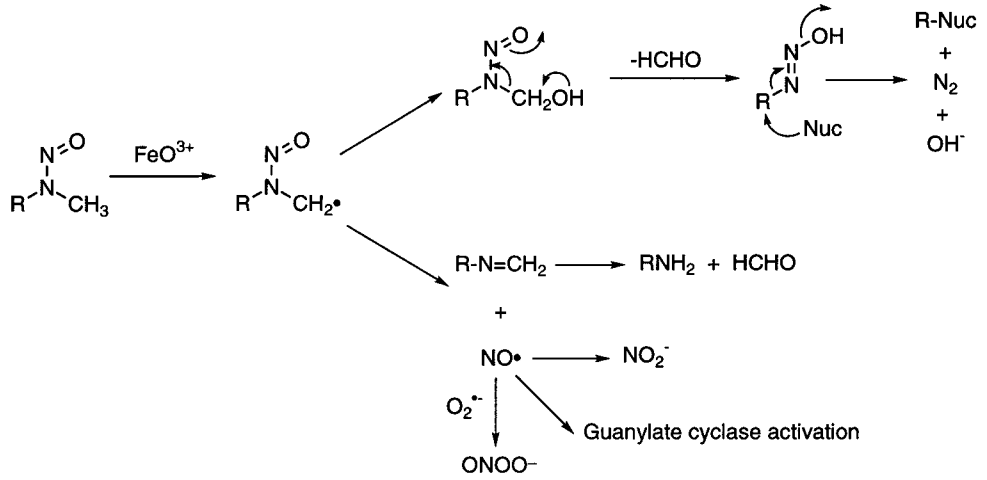


(*306*). One can consider the mechanistic literature of the fatty acid desaturases, which apparently desaturate without releasing any hydroxylated products. These enzymes use di-iron μ-oxo species (*318*). However, di-iron μ-oxo systems also function in monooxygenase enzymes (e.g., methane monooxygenase) and the distinction between oxygen transfer and desaturation in these enzymes is also attributed to uncharacterized effects of groups in the proteins (*318*).

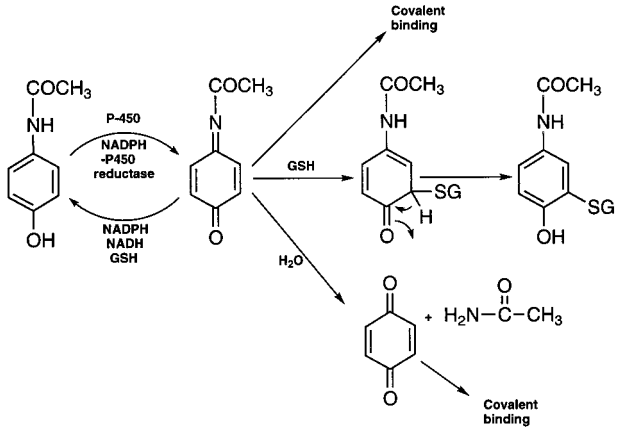
Some other P450 "desaturation" reactions have similarity but are mechanistically different thatn the major systems discussed here. Studies on the oxidation of 1,4-dihydropyridines by Ortiz de Montellano (*125*, *126*) and subsequently our own laboratory (*130*, *161*) identified desaturation as a major event in the metabolism, probably driven by pyridine ring stability. This process is an unusual vinylogous sort of desaturation driven by the ease of one-electron oxidation and more homologous to N-dealkylation (section 3.2.2) rather than C−C desaturation (Scheme 11). Another related reaction is the net two-electron oxidation of acetaminophen to the reactive quinoneimine (Scheme 43) (*319*).

4.3. Oxidative Ester Cleavage. The oxidative cleavage of a carboxylic acid ester by a P450 was observed with rat P450 2C11 in this laboratory (*271*, *320*). The distinction between the oxidative reaction and nonoxidative hydrolysis is demonstrated by the dependence on P450, NADPH−P450 reductase, and NADPH and the observed kinetic hydrogen isotopes (*271*). A carbonyl product is formed instead of an alcohol, although iden-

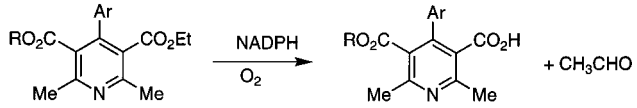
Scheme 42. Oxidation of an *N*-Nitrosamine to an Alkyldiazohydroxide and Nitrite/Alkyamine



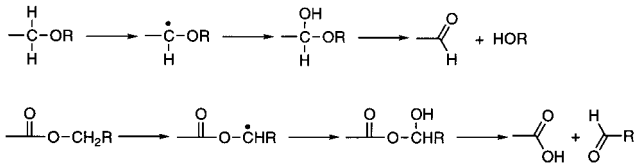
Scheme 43. Oxidation of Acetaminophen to a Quinoneimine (*319*)



Scheme 44. Oxidative Ester Cleavage of Pyridine Derivatives (*271*, *320*, *321*)



Scheme 45. O-Dealkylation of Ethers and Esters (H· Abstraction/Oxygen Rebound)



tification of a carbonyl product in itself might not constitute proof for such a reaction, in that the P450 could have oxidized the alcohol product.

The reaction has been demonstrated with Hanzsch pyridine esters (Scheme 44) (*271*, *320*) and also other acyclic esters (*322*). The high intermolecular kinetic hydrogen isotope effects (~12) (*271*) suggest that the mechanism should be described as a C-hydroxylation, analogous to that often used for O-dealkylation (Scheme 45).

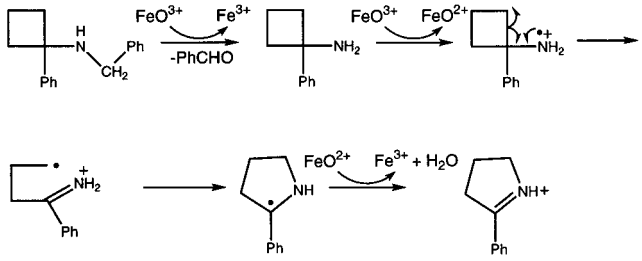
Such oxidative cleavage of esters is not only an in vitro curiosity but also occurs in vivo, as indicated by the deuterium isotope effects on some of the pharmacokinetic parameters measured in rats with a Hanzsch pyridine ester, the oxidized product of nifedipine (*321*).

4.4. Ring Expansions. Ring expansions are fairly unusual P450 reactions. They are rationalized with reaction intermediates or products that rearrange to more stable five- or six-membered rings.

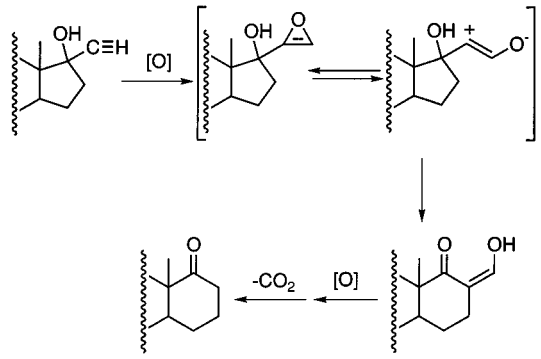
One example comes from work in this laboratory with a cyclobutylamine (*139*). The substrate was designed to undergo ring opening and was based on earlier work by Silverman's group with monoamine oxidase (*323*). The P450 (2B1) oxidation included initial N-debenzylation and then formation of the pyrroline (Scheme 46).

The reaction was rationalized in the context of stepwise electron-transfer mechanisms (Scheme 46). The above reaction is driven by the instability of the four-membered ring and is postulated to involve an enzyme intermediate. Another interesting reaction is the D-homoannulation of 17α-ethynyl steroids (*12*, *324*, *325*), which is rationalized by a mechanism involving a postulated highly unstable

Scheme 46. Proposed Mechanism of P450-catalyzed Cyclobutylamine Ring Expansion (*139*)



Scheme 47. Steroid Ring D Homoannulation (*324*)



reaction product (Scheme 47). Details of the reaction have not been investigated.

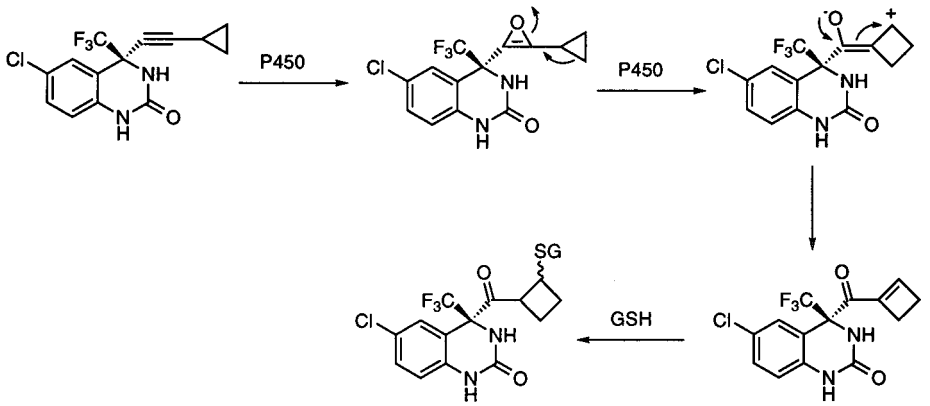
Another reaction already alluded to under the mechanism of acetylene oxidation (*192*) yields a ring expansion from a putative oxirene intermediate (Scheme 48).

4.5. Ring Formation. In some oxidations a new ring system is produced. This is differentiated in this review from the ring expansions discussed in section 4.4 in that a ring did not exist previously. In the examples presented here, products are obtained, but further nonenzymatic reactions occur to generate a new ring.

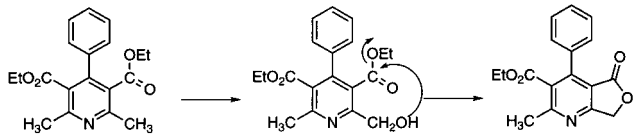
The first example is a relatively simple one we encountered in our own work on the oxidation of dihydropyridines (*271*). Hydroxylation of a methyl group yields an alcohol that can react with an ester at an appropriate position to yield a very stable five-membered lactone ring (Scheme 49). Some of the "unlactonized" hydroxymethyl ester could be isolated when care was taken to avoid exposure to acid and dehydrating conditions.

Another example is found in the activation of pulegone, a toxic terpene in the abortifacient mint oil, pennyroyal oil. This area has been studied extensively by Nelson and his associates (*326*−*331*). (*R*)-(+)-Pulegone is hepatotoxic in mice and several lines of evidence support the view that bioactivation is required. Pulegone is oxidized to menthofuran (Scheme 50). Evidence for the pathway shown is derived from work with ^{18}O and ^{2}H labeling (*326*, *329*, *330*). P450 2E1 is the human P450 most active in this process (*331*). Furans are known to be activated by P450s to reactive products that bind covalently to proteins (*332*, *333*), and menthofuran also showed such behavior (*330*) (Scheme 51). Furans have been postulated to be activated by epoxidation (*331*) or by an electrophilic mechanism that yields a reactive 4-oxoenal (*334*, *335*). Most experiments cannot rule out or definitely implicate an epoxide intermediate, if a dihydrodiol cannot be isolated [note: dihydrofuran epoxides such as aflatoxin B_1 8,9-oxide have actually been easier to handle than

Scheme 48. Proposed Ring Expansion from an Oxirene Intermediate (*192*)



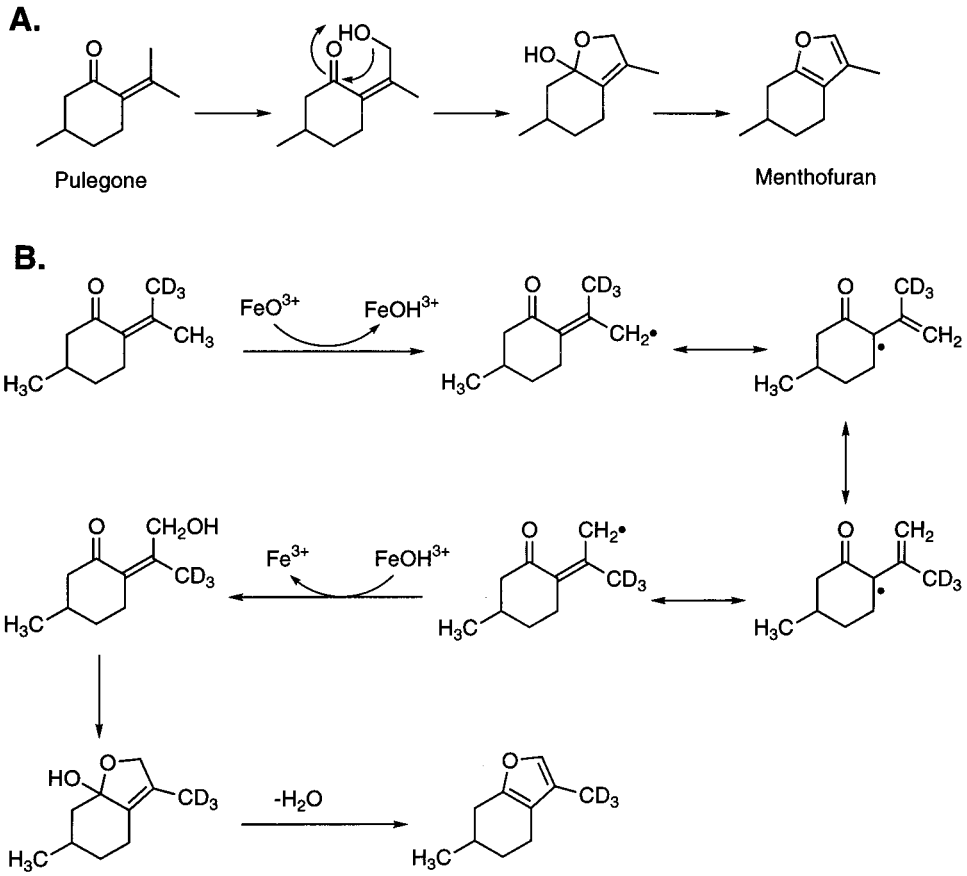
Scheme 49. Cyclization of a P450 Product to a Stable Lactone (*271*)



furan epoxides (*336*)]. Indirect evidence for the formation of furan epoxides by chemical oxidants has been provided by spectral (*337*) and trapping (*338*) experiments.

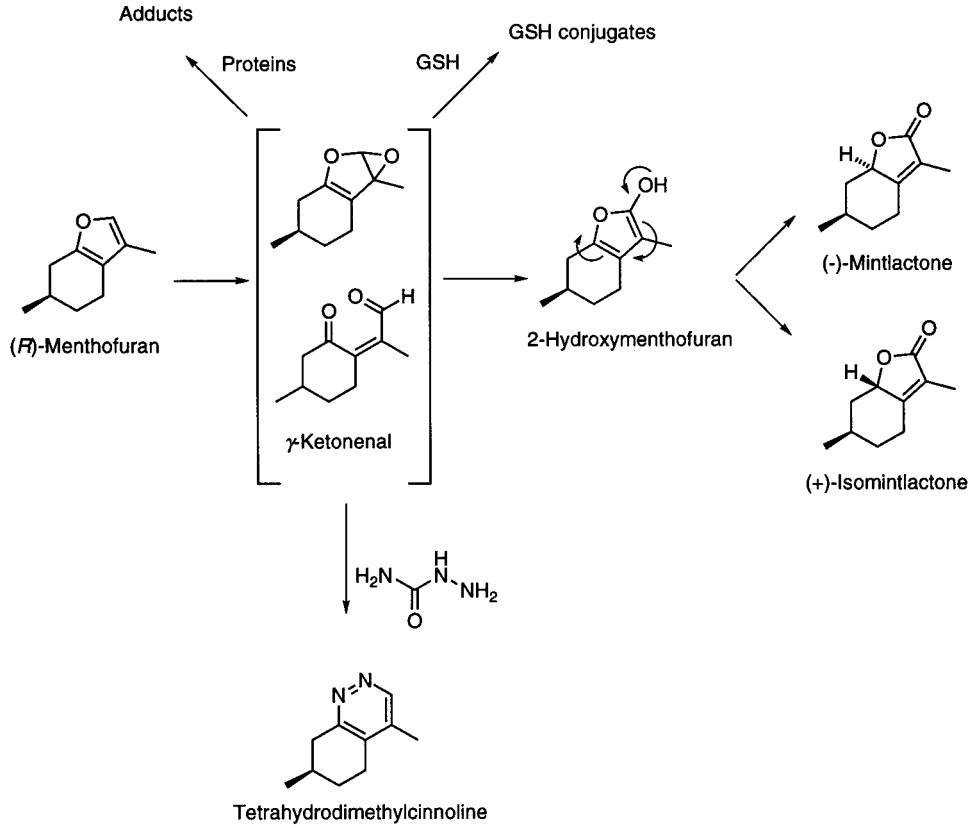
4.6. Aldehyde Scissions. As pointed out in section 3.1, the reduction of hydroperoxides by P450s can generate ketones or aldehydes. Aldehydes (and ketones, to a lesser extent) have long been considered to be potentially deleterious chemicals because of their potential to form Schiff bases with proteins and exocyclic atoms of nucleic acids. However, more recently these have also been shown to undergo oxidative activation to species that cause inactivation of P450s (*339*). Aldehydes also undergo P450 oxidations, not only to acids (*340*), but also some that appear to reflect a different type of chemistry. One impetus to the study of aldehyde oxidation was the long-standing difficulty in rationalizing the final step in the aromatization of androgens to estrogens. Gradual support has developed for a mechanism proposed by Aktar (*208*, *209*) and Robinson (*210*), in which an FeOOH or $FeOO^{-}$ entity catalyzes this step by attack on the carbonyl and subsequent collapse of an intermediate (Schemes 20 and 21). Further development of the field by Coon and his associates (*211*, *213*) led to the characterization of a

Scheme 50. Formation of Menthofuran from Pulegone (*327*)[a]

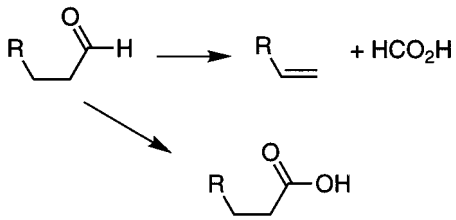


[a] An expanded version fo the postulated mechanism mechanism derived form deuterium experiments is shown in Part B (*327*).

Scheme 51. Oxidation of Menthofuran (*331*)



Scheme 52. Oxidations of Aldehydes by P450 (*280*)



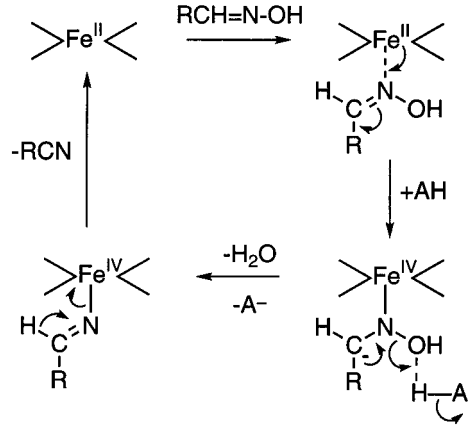
generalized reaction in which aldehydes are converted to olefins and HCO_2H (Scheme 52).

Subsequent considerations have led to the proposal that the FeOOH mechanism may be involved in some other oxidations as well (Section 3.3) (*214*, *221*, *264*). It is possible to rationalize P450-catalyzed oxidation of an aldehyde to a carboxylic acid with either FeO^{3+} or FeOOH, as we have done with the P450 2E1-catalyzed oxidation of acetaldehyde (*136*) (Schemes 5 and 52).

4.7. Dehydration. One example of a formal P450-catalyzed dehydration is the transformation of lipoxygenase-generated fatty acid hydroperoxides to allene oxides. This reaction begins with a reactive substrate and almost certainly involves internal electron transfer between the iron and oxygen atoms during the reaction, and this case will be treated separately under section 4.11, along with prostaglandin H_2 rearrangements.

P450s dehydrate aldoximes to yield nitriles, as shown by Mansuy and his associates (*341*, *342*). This is a key step in the biosynthesis of the cyanogenic glucoside dhurrin in (the plant) *Sorghum bicolor* (*343*). A particular P450 appears to catalyze both the dehydration of an aldoxime to the nitrile and also the subsequent (C-) hydroxylation of the nitrile (at the carbon α to the nitrile). Mammalian microsomal enzymes can also catalyze these

Scheme 53. Dehydration of an Aldoxime to a Nitrile (*341*, *342*)

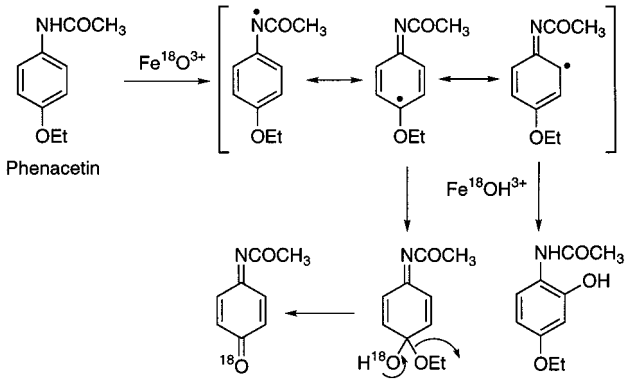


reactions with model aldoximes, e.g., P450 3A4 (*341*). Nitriles can be toxic (*344*); aldoximines are relatively rare chemicals in biological systems, although NADPH-dependent dehydration of a metabolite of the pesticide aldicarb to an aldoxime has been observed with fish microsomes (*345*).

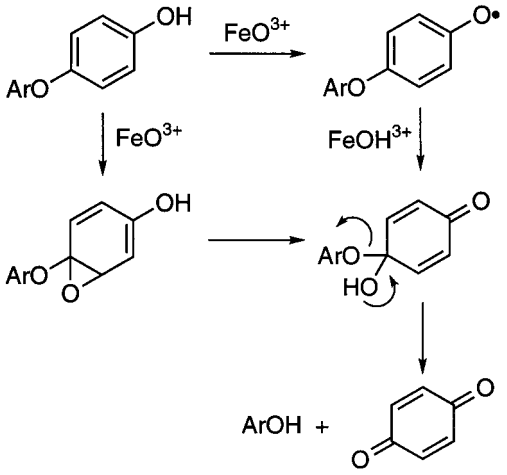
Biomimetic studies have shown that such dehydrations can be achieved with nonprotein iron porphyrin systems, if the iron is in the ferrous form and a proton donor is accessible (*340*). A mechanism is shown in Scheme 53. This reaction involves a charge transfer from the iron into the system; the rate is correlated with $E_{1/2}$ of the iron (*342*). Other examples of this process directly relevant to toxicology are unknown (other than consideration of the cyanogenic glucosides found in plants).

4.8. *Ipso* Attack and Related Reactions of Aromatic Rings. An *ipso* attack is one at the site of the

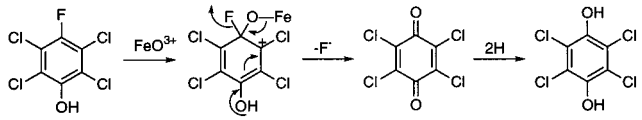
Scheme 54. Proposed Mechanism of Incorporation of $^{18}O_2$ Label into *N*-Acetamidoquinone Formed during Phenacetin Oxidation (*11*)



Scheme 55. Possible Mechanisms of O-Deoxylation (*11*, *12*)



Scheme 56. *Ipso* Attack Mechanism Proposed for Dehalogenation of Phenols, with Subsequent Reduction (*350*, *352*)



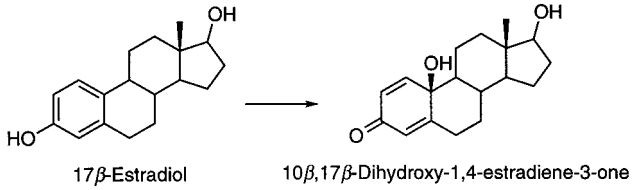
substituent on an aromatic molecule, as opposed to *ortho*, *meta*, or *para* (*346*). *Ipso* mechanisms have been proposed to explain several P450-catalyzed reactions.

One product of the P450-catalyzed oxidation of phenacetin is *N*-acetyl-*p*-benzoquinoneimine, in which partial incorporation of oxygen from $^{18}O_2$ was reported (*347*, *348*). A mechanism to rationalize this is the stepwise one shown in Scheme 54, where the oxygen rebound occurs at the carbon bearing the ethoxy group. This work is consistent with some of our own recent research and the low $E_{1/2}$ of phenacetin (*237*).

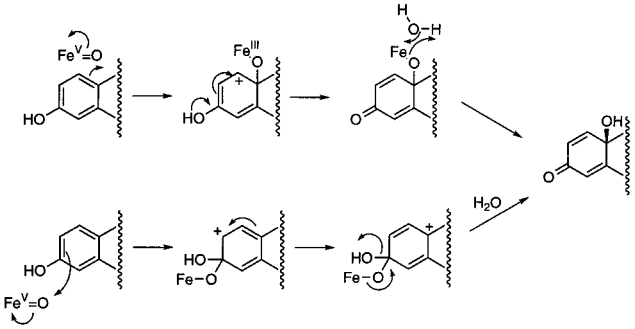
Another type of reaction is N- or O-dearylation. Both reactions are known (*11*, *12*, *349*). Reasonable proposals are presented in Scheme 55. As in many cases of oxidations of aromatic compounds, epoxidations are possible and may not be distinguished from other potential intermediates without additional evidence.

Rietjens and Hirobe and their colleagues have presented an *ipso* mechanism to explain the replacement of aryl halides by hydroxyls (Scheme 56) (*350*−*354*). Fluoride ion is released in the conversion of pentafluorophenol

Scheme 57. Oxidation of 17β-Estradiol at C10 (*357*)



Scheme 58. Possible Mechanisms of C10 Hydroxylation of 17β-Estradiol (Scheme 57)



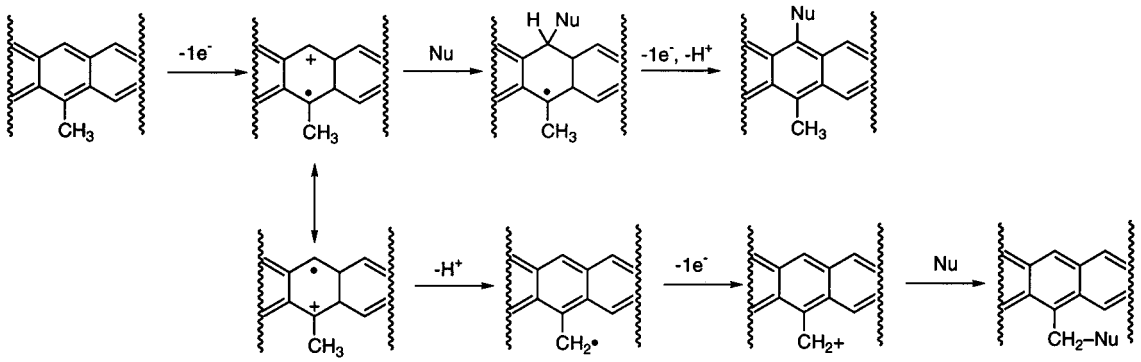
to 2,3,5,6-tetrafluorohydroquinone. Epoxide intermediates cannot be ruled out, but no NIH shifts have been detected (*355*). The loss of fluoride seems reasonable in that fluoride is the best leaving group in aromatic substitution reactions (in contrast to alkyl systems). An alternate pathway to explain the products is oxygenation *on* the halogen (haloso product) followed by loss of halite ($XO^- = X^+$) (*356*), which has not been detected in trapping experiments. Halogen oxygenation can be demonstrated with iodobenzenes (*143*, *206*); with aryl bromides the situation is less clear (*143*). Thus, chlorine and fluorine would be even less likely to be oxygenated because of their electronic properties ($E_{1/2}$ and electronegativity), as pointed out by Rietjens and Vervoort (*350*). One remaining problem with the mechanism (Scheme 56) is that it requires a two-electron reduction to yield the hydroquinone. Nonenzymatic reduction of a quinone or quinoneimine by NADPH or GSH can be efficient (*319*) (Scheme 43).

A possibly related reaction in which an aromatic ring is converted to a quinone-like structure is the oxidation of the estrogen A rings shown in Scheme 57 (*357*). In the absence of ^{18}O labeling data, this reaction can be explained readily by the mechanism shown in Scheme 58A. However, an *ipso* mechanism (Scheme 58B) could be involved to explain incorporation of oxygen from H_2O in the added hydroxyl.

4.9. One-Electron Oxidation. Stepwise electron transfer in the oxidation of substrates by P450s is not a new concept. There is general agreement that this is the dominant mechanism that peroxidases use to catalyze the oxidation of amines, alcohols, and anything else accessible to the $E_{1/2}$ values of 1.06 and 1.08 V estimated for the redox reactions of compounds I and II (*124*). Earlier comparisons of the catalytic mechanism of P450s to peroxidases led to development of the proposal of one-electron transfer by this laboratory, in collaboration with Macdonald (*127*, *131*), and by Ortiz de Montellano (*13*, *125*).

The development of the case for the involvement of one-electron transfer in the N-dealkylation of amines is presented elsewhere (*11*, *133*, *156*, *158*) and will not be

Scheme 59. Proposed 1-Electron Oxidation of PAHs by P450 (*373*)

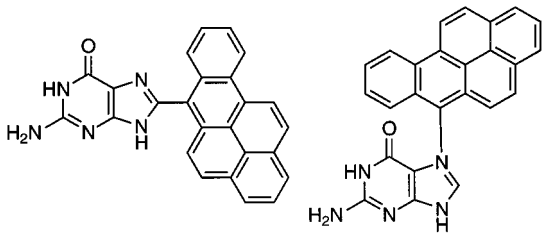


reiterated. The same principles apply to S-dealkylation (*358*, *359*). P450s are capable of both one-electron transfer and hydrogen atom transfer when they oxidize substrates; the mechanism is probably dependent upon the geometry (and the use of FeO^{3+} instead of peroxy forms). In this section, several other issues related to P450-catalyzed one-electron oxidations are considered.

If the premise of one-electron oxidation by P450s is accepted, on the basis of the experimental evidence, then one issue is how strong an oxidant is P450 (i.e., FeO^{3+}; the $FeOO^{-}$/FeOOH forms would probably be weaker oxidants). No exact value can be measured, but there is precedent in measurements of the analogous electronic states of peroxidases and biomimetric models. For horseradish peroxidase, $E_{1/2}$ estimates for both the complex compound I ↔ compound II and compound II ↔ ferric are ∼1.0 V (*124*). The reported potentials for the analogous metalloporphyrin models are in the range of 1.0−1.8 V (*360*−*362*). They are strongly influenced by the solvent and ligands, as well as the metal (Cr, Mn, Fe). We would expect the potentials of P450 FeO^{3+} complexes to fall in this range. In some work on the application of Marcus theory to P450 2B1-catalyzed N-dealkylation reactions with *p*-substituted *N*,*N*-dimethylanilines we were able to fit our results to a model with an $E_{1/2}$ of ∼1.8 V (*164*). This model includes a number of assumptions and, even if these are valid, there is considerable room for error in this value. One point included in the discussion of that work (*164*) is that the effective $E_{1/2}$ is a function of the distance (*r*) between the site of oxidation on the substrate and the FeO^{3+} complex (outer-sphere model) and also effective dielectric constant (*D*). The $E_{1/2}$ increases with decreases in *r* and *D*. Thus $E_{1/2}$ may vary among P450s and among the reactions catalyzed by a particular P450, overcoming arguments such as those of Eberson (*363*) against the ability of P450s to transfer electrons, which is inherently feasible in light of the known abilities of peroxidases to abstract electrons even from PAHs (*364*, *365*). One-electron oxidation can probably be considered a possibility whenever substrates with $E_{1/2}$ of <1.0−1.5 V are considered. The list of substrates for which evidence for one-electron oxidation exists includes amines, sulfides, strained alkanes, anisoles, and polycyclic hydrocarbons. A case can probably be made for some other compounds as well.

Ortiz de Montellano and Stearns examined some strained cycloalkanes, which are known to have low $E_{1/2}$ values (*160*, *366*). Of particular note was quadricyclane, $E_{1/2}$ = 0.9 V (*159*). The P450-catalyzed oxidation was accompanied by a rearrangement that could be only rationally explained by a one-electron oxidation (*159*, *160*) (Scheme 24). These studies with strained cycloalkanes complement the work done with amines, which have similar $E_{1/2}$ values (*127*, *128*).

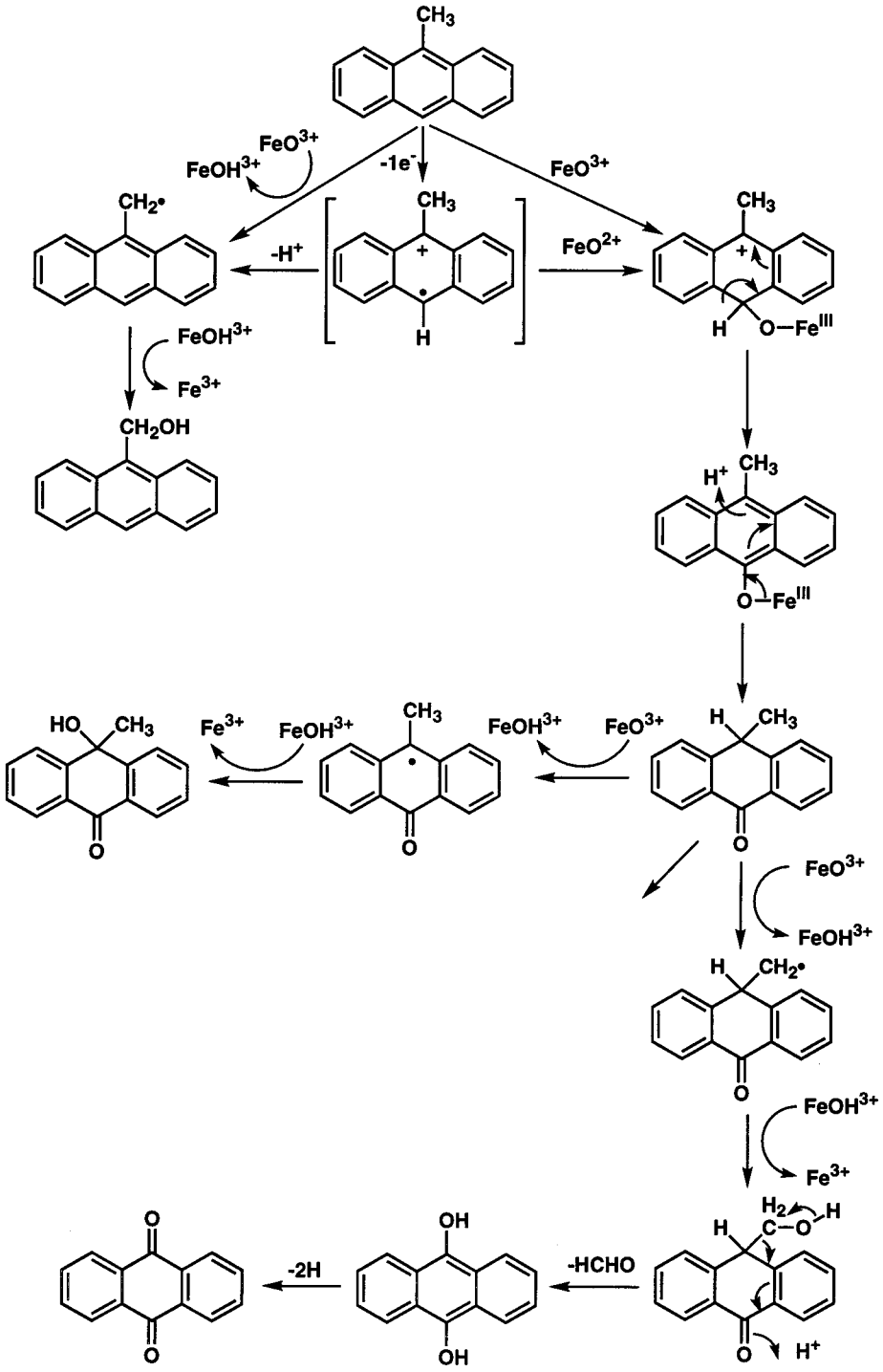
Scheme 60. DNA-PAH Adducts Attributed to P450 1-Electron Transfer Products (*384*)



One group of chemicals long considered as substrates for one-electron oxidation is the PAHs. PAHs are major components of tobacco smoke, soots, tars, and pyrolyzed food and many induce tumors in experimental animals (*367*). The most generally accepted mechanism for activation of PAHs involves the formation of diol-epoxides, particularly in the so-called bay and fjord regions (*368*, *369*). Some alternate possibilities for activation of PAHs include (i) hydroxylation (particularly at alkyl side chains) and attachment of leaving groups such as sulfate (*370*, *371*), (ii) formation of quinones by sequential oxidations (*372*), and (iii) one-electron oxidation to radical cations (Scheme 59). Pathway i can be demonstrated in vitro (*374*, *375*) but the in vivo evidence for its significance is limited (*371*). Pathway ii may follow iii; the quinones are probably not bound well to DNA but may be involved in the formation of reactive, partially reduced oxygen species through futile redox cycling. Mechanism iii, one-electron oxidation, has been considered not only in the context of generating reactive cation radicals but as a part of the generation of quinones and even epoxides.

A case for reactive PAH cation radicals (Scheme 59) is not new and goes back to work in the 1960s by Fried, Ts'O, and others (*376*−*378*). Oxidations of PAHs by model oxidants were described (*379*). The field lost some appeal after reports of DNA adducts, mutagenicity, and tumorigenicity of PAH diol-epoxides appeared (*369*, *380*, *381*). Cavalieri and Rogan have continued to study the issues of PAH cation radicals and their biological relevance (*373*, *382*). The incubation of PAHs with peroxidases (and H_2O_2) in the presence of DNA yields adducts characterized to be the same as those also generated by electrochemical oxidations (*373*, *382*) (Scheme 60). Some of these are found in vivo, although alternate pathways (than formation of cation radicals) to these adducts (e.g., guanyl-N^7-PAH adducts) cannot be unambiguously ruled

Scheme 61. Oxidation of 9-Methylanthracene by P450 2B1 (*188*)

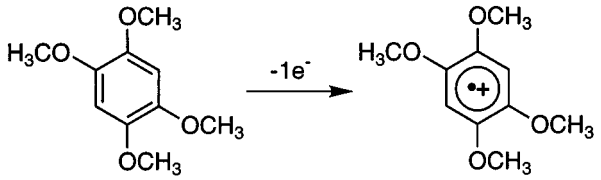


out. Another issue is the instability of PAH radical cations ($t_{1/2}$ in the millisecond range) (*385*). If these were generated by P450s in the endoplasmic reticulum, could they really move to the nucleus? [Perhaps a reference point in this regard is aflatoxin B_1 *exo*-8,9-epoxide, which has a $t_{1/2}$ of 1 s in H_2O yet modifies DNA extensively (*56*, *57*).]

We have explored the basic question of whether P450s can oxidize PAHs (Scheme 61). The simplified models are 9-alkylanthracenes (*386*, *387*). A series of studies were done with P450 2B1, horseradish peroxidase/H_2O_2, and a biomimetic model and the major experiments involved the incorporation of ^{18}O from H_2O into the products (*188*). Even with these simple models, the products were complex. One conclusion is that formation of cation radicals does not seem to be an obligatory step on the pathway to epoxides or phenols. Reasonable schemes could be developed for the observed products, even those involving demethylation of 9-methylanthracene. However, unambiguous distinction between one-electron transfer and "direct" hydroxylation (e.g., via hydrogen atom abstraction) was not possible (*188*). One problem was the potential for H_2O generated within the P450 active site to react with cations before exchanging with the medium.

To resolve the issue of whether P450s could oxidize low $E_{1/2}$ aromatic compounds by one-electron transfer, we turned our attention to "polyanisoles" (polymethoxybenzenes), which had been used extensively in the peroxidase

Scheme 62. 1-Electron Oxidation of 1,2,4,5-Tetramethoxybenzene (*390*)



literature (*388*, *389*). Formation of cation radicals of these compounds is easily demonstrated with peroxidases because (i) the radicals are rather stable at the low pH values used with many peroxidases, (ii) the radicals can be detected by their visible or ESR spectra, and (iii) peroxidases form few other products from these compounds. We demonstrated that several P450s could oxidize 1,2,4,5-tetramethyoxybenzene (Scheme 62, $E_{1/2}$ 0.83 V) to a cation radical (λ_{max} 450 nm) (*390*). The cation radical is hydrolyzed to *o*- and *p*-quinones in both peroxidase- and P450-catalyzed reactions (Scheme 63) (*390*). These experiments establish that P450s are capable of catalyzing one-electron oxidation of low $E_{1/2}$ compounds, and most PAHs have similar potentials (*391*). However, the stability of these radicals at neutral pH and their roles in carcinogenesis remain to be established.

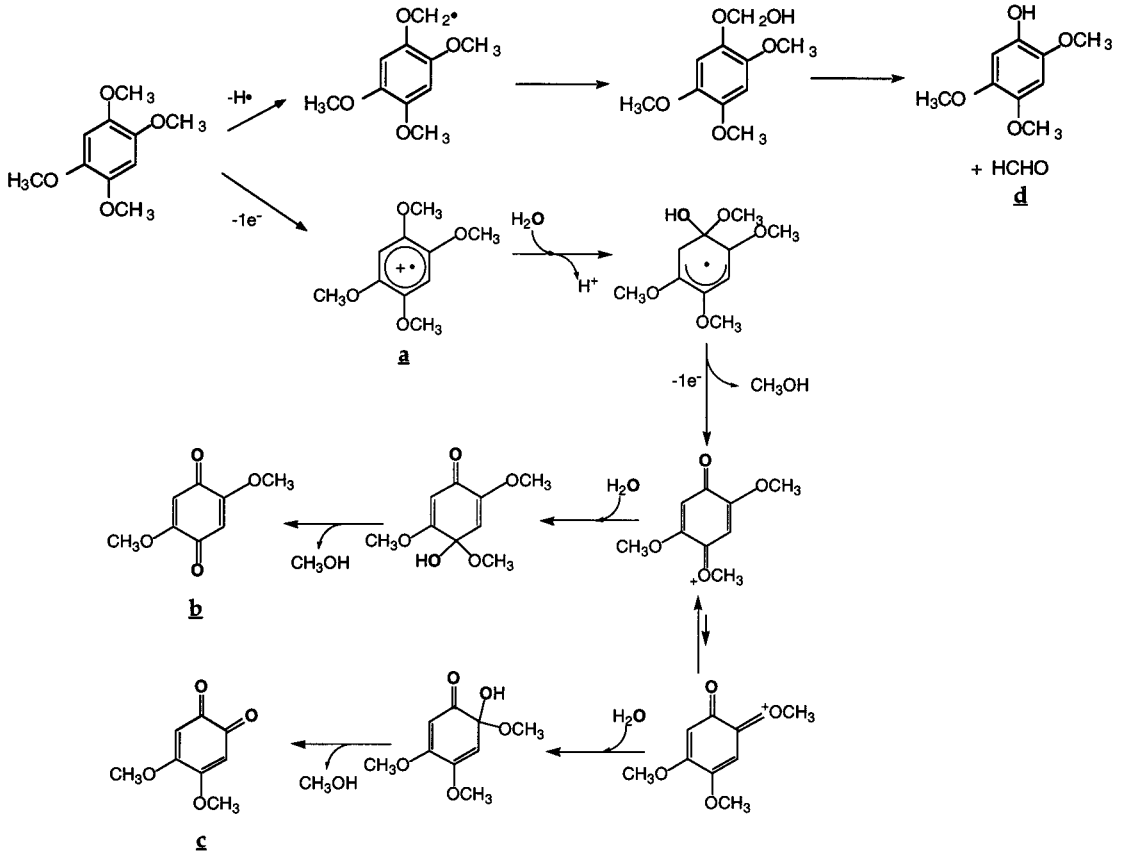
The generality of one-electron oxidation in the oxidation of anisoles and related phenylalkyl ethers remains to be better established. We had tended to consider the $E_{1/2}$ values of such compounds to be outside the range for efficient one-electron oxidation. However, in recent work we found that low $E_{1/2}$ values had been established for phenacetin (*N*-acetylphenetidine) (*392*, *393*) and could repeat these results. The low $E_{1/2}$ is of interest in the consideration of low kinetic hydrogen isotope effects for the O-dealkylation of phenacetin but high isotope effects for acetol hydroxylation (*237*, *239*), even though both reactions are formally C-hydroxylations. These results have been extended to more *p*-alkoxyacylanilides (*239*). Horseradish peroxidase can catalyze O-dealkylations of these substrates (but not acetol formation), although the rates are low.[2] Further studies on the role of one-electron transfer in these reactions are in progress.

4.10. Coupling Reactions. This section describes oxidative couplings, either intra- or intermolecular, catalyzed by mammalian P450 enzymes. These are distinguished from what is termed a "ring formation" in section 3.5, where a stable product rearranges to yield a new ring system (e.g., pulegone). These are fairly rare reactions and only two examples are provided.

One reaction is the intramolecular ring formation involved in the synthesis of morphine (*12*, *394*). Mammals synthesize low concentrations of morphine alkaloids, apparently starting from dopamine. Examination of the pathways (*12*, *33*, *395*) indicates the presence of several oxidation steps, the putative P450s for which have not been identified yet (*396*). P450s (microsomes) catalyze cyclization of (*R*)-reticuline to salutaridine (Scheme 64) (*12*, *397*).

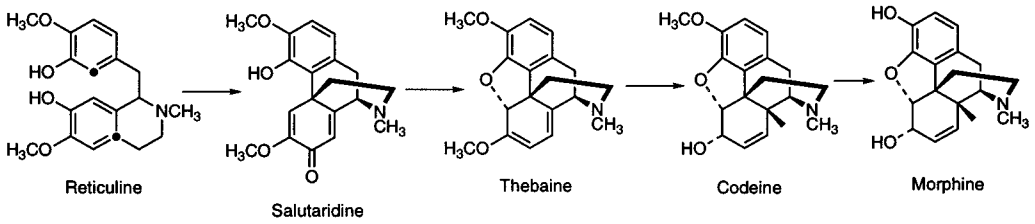
Another possible example is a structure identified as 6*H*-oxazolo[3,2-*a*:4,5-*b*′]diindole (Scheme 65), derived from the oxidation of indole by several P450s (*398*). A mechanism has been proposed for the coupling (Scheme 66). The dye indigo is formed by the nonenzymatic oxidation and coupling of two indoxyls (3-hydroxyindole); in a

Scheme 63. Products of 1,2,4,5-Tetramethoxybenzene Oxidation Catalyzed by Rabbit P450 1A2[a]



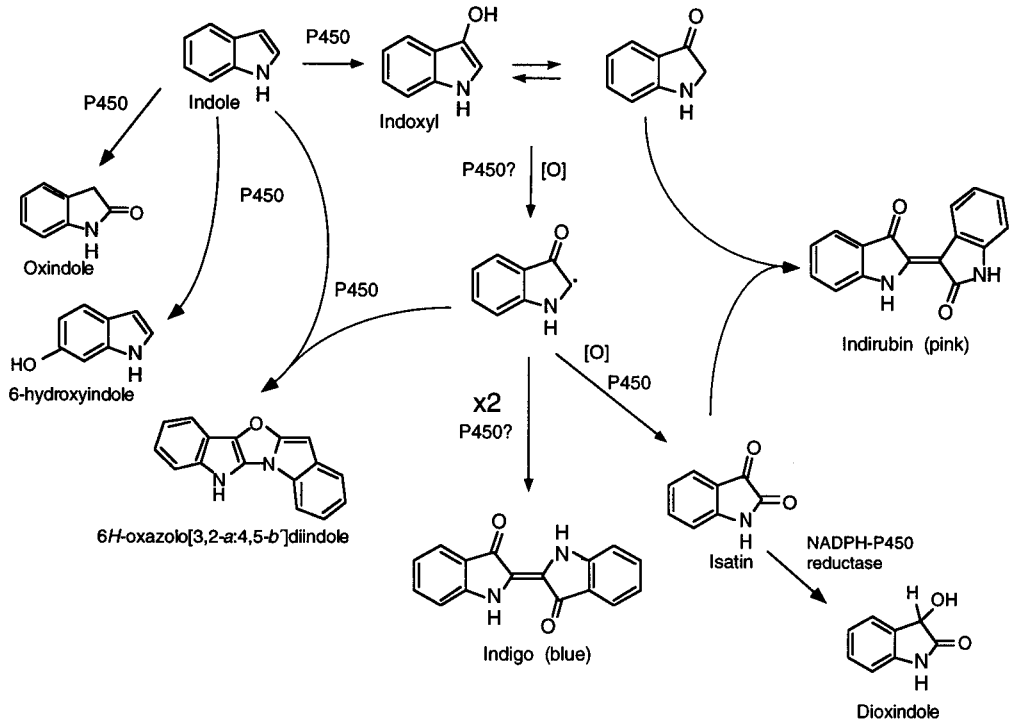
[a] Products labeled with the letters a, b, c, and d were identified (*390*).

Scheme 64. Oxidation Steps in Alkaloid Synthesis (*12, 395, 397*)[a]



[a] The indicated carbon atoms (•) in reticuline are linked to form salutaridine.

Scheme 65. Oxidation of Indole by P450 (*398*)



similar manner indirubin is formed from the coupling of indoxyl and isatin (*398*). It is possible that P450s can facilitate these couplings, although the nonenzymatic efficiency is high. However, indoxyl (and indigo) did not form 6*H*-oxazolo[3,2-*a*:4,5-*b*′]diindole nonenzymatically, so some P450s appear to have binding sites large enough to bind two indole rings and facilitate couplings.

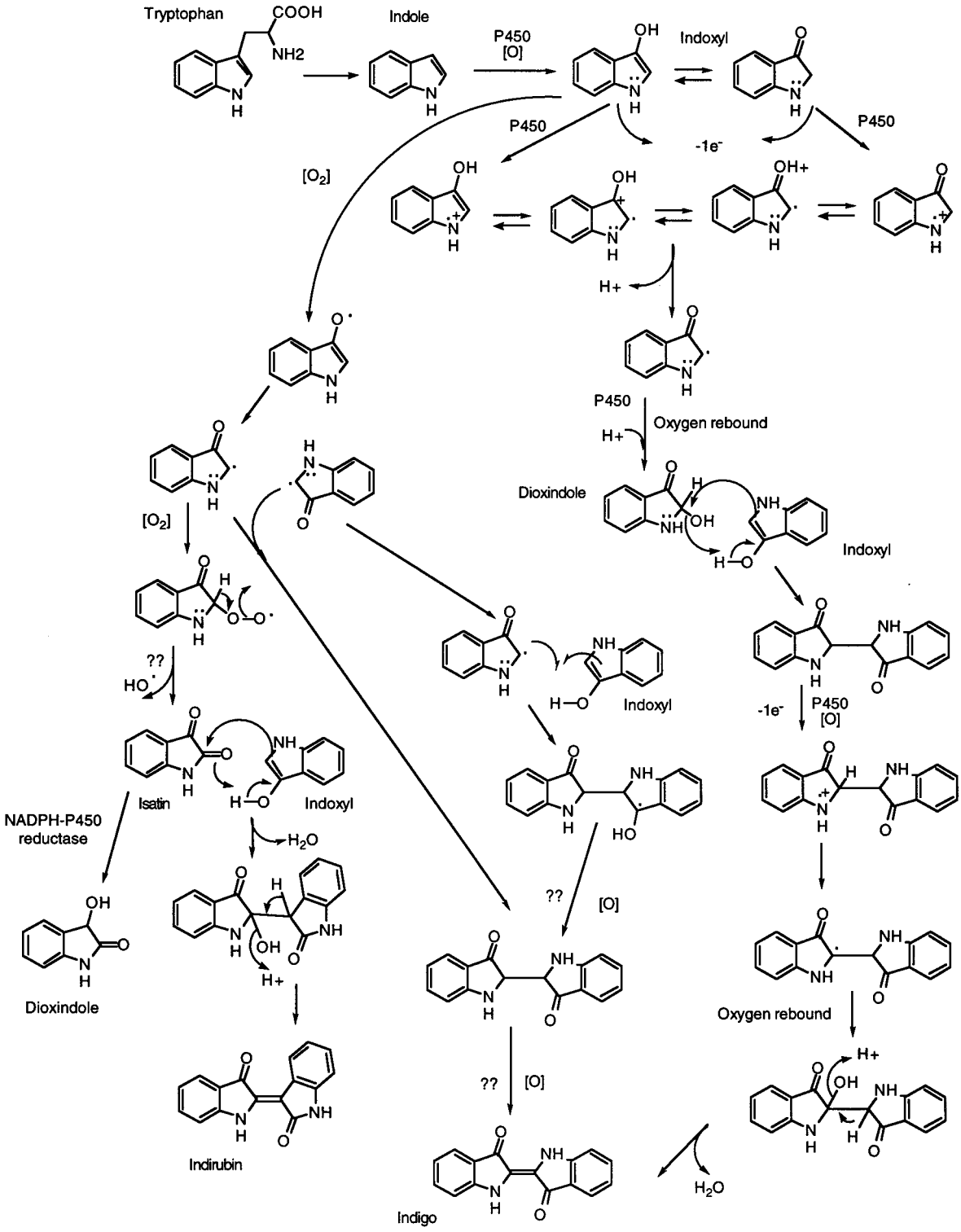
Wakabayashi and his associates have reported the coupling of norharman and aniline in an NADPH-dependent reaction (Scheme 67) (*399*). This reaction, which is tentatively attributed to P450, generates a compound that can be further oxidized to a mutagenic hydroxylamine (*399*). Neither norharman nor aniline is mutagenic itself following incubation with microsomes and NADPH. The coupling process is a formal two-electron oxidation. It is difficult to propose a reaction product of either norharman or aniline that is stable enough to migrate from a P450 and be reactive enough to couple with aniline, or vice versa. An alternative proposal is presented in Scheme 68, in which the two amines are present together in a P450 and the reaction begins with an *ipso* attack on the aniline (see section 4.8). Alternatively, radical mechanisms are possible.

4.11. Rearrangement of Fatty Acid and Prostaglandin Hydroperoxides. These reactions are rearrangements and do not involve the use of electrons added from external sources. Any changes in the iron valence state occur within the course of the rearrangement reactions.

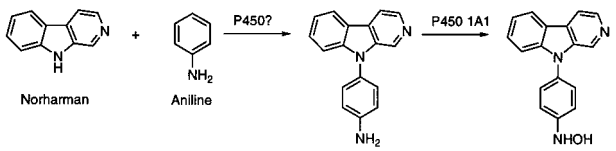
Thromboxane and prostacyclin synthases are P450s 5 and 8, respectively (*400*−*402*) (Scheme 69). These P450s have specialized functions and are localized primarily in platelets (P450 5) and endothelial cells (aorta) (P450 8). Current knowledge does not indicate any other natural substrates for these P450s other than prostaglandin H_2, an unstable hydroperoxide that would probably have to be made in the same cell to be a substrate. However, Ullrich and his associates (*403*) have shown that the enzyme can catalyze monooxygenation reactions on a prostaglandin H_2 analogue in a reaction supported by the oxygen surrogate iodosylbenzene.

Thromboxane synthase (P450 5) converts prostaglandin H_2 to a ∼1:1:1 mixture of thromboxane A_2, hydroxyheptatrienoic acid (HHT), and malondialdehyde (*402*). P450s 1A2 and 3A4 have appreciable catalytic activity in converting prostaglandin H_2 to HHT and malondialdehyde (∼10 min^{-1}), although there is considerable species variation in the P450 1A2 rates (*404*). The chemical mechanism of the rearrangement to form HHT and malondialdehyde by these P450s is presumed to be similar to that postulated for the thromboxane synthase (Scheme 69). These findings may be of relevance in tissues or tumor cells that express both cycloxygenase (which generates prostaglandin H_2) and P450s that

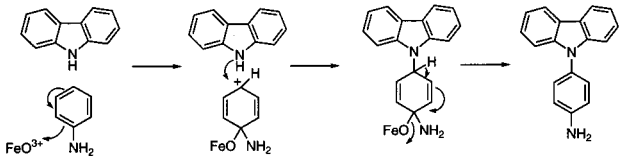
Scheme 66. Possible Mechanisms of P450 Oxidation of Indole to Indigo and Indirubin



Scheme 67. Coupling of Norharman and Aniline to Form 9-(4′-aminophenyl)-9*H*-pyrido[3,4-*b*]indole and N-Hydroxylation to a Mutagen (*399*)



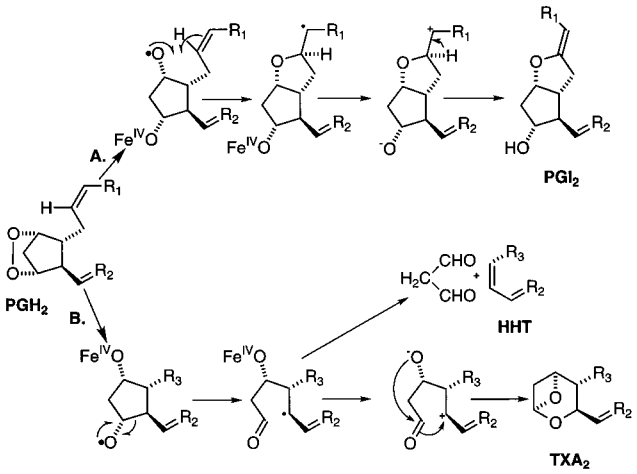
Scheme 68. Possible Mechanism of Coupling of Norharman with Aniline in a P450 Active Site



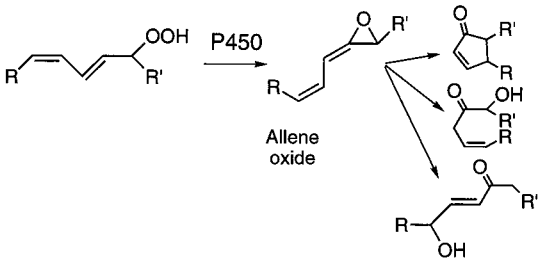
catalyze this formation of HHT and malondialdehyde (*405*).

Another reaction involving the P450-catalyzed rearrangement of oxidized fatty acids is the conversion of hydroperoxyeicosatetraenoic acids to epoxy alcohol prod-

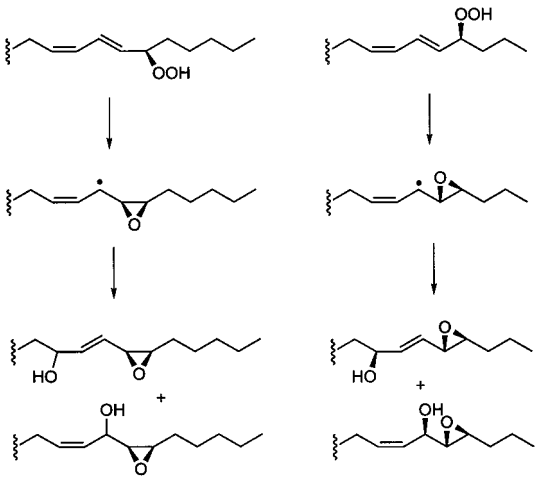
Scheme 69. Postulated Mechanisms for Rearrangement of Prostaglandin H_2 (PGH_2) by P450s (*5*, *8*) to Prostacyclin I_2 (PGI_2) and Thromboxane A_2 (TXA_2) (*402*)



Scheme 70. P450-Catalyzed Rearrangement of a Fatty Acid Hydroperoxide to an Allene Oxide (*406*, *407*)

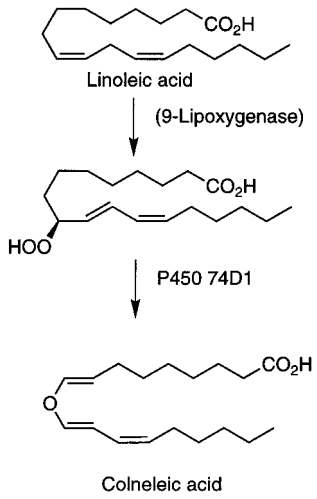


Scheme 71. Mechanisms of Microsomal P450 Rearrangements of Fatty Acid Hydroperoxides (*406*)



ucts, a system explored by Brash and his associates (*406*) (Scheme 70). As in the case of the rearrangement of prostaglandin H_2, this reaction occurs in the absence of external electrons and, again, resembles some of the "oxygen surrogate"-supported P450 reactions, except that the rearrangements are internal. Different microsomal P450s appear to yield products with varying stereo- and regiochemistry (*406*). A general mechanism is presented in Scheme 71. As in the case of NADPH-supported carbon oxidations, desaturation is shown as an alternative. Desaturation yields allene oxides, major products with

Scheme 72. Synthesis of a Divinyl Ether by P450 74D1 (*408*)



plant P450 74A. Allene oxides are highly unstable and undergo rapid rearrangement to other products (leading to jasmonic acid in plants). Allene oxide products have not been detected yet in mammalian systems but the possibility cannot be dismissed (*406*).

Another seemingly unusual variation on this theme is another plant P450 reaction, the formation of divinyl ethers from a fatty acid hydroperoxide, recently demonstrated by Howe (*408*). P450 74D1, a plant enzyme, catalyzes what can be considered a formal dehydration of a 9-hydroperoxydiene (formed from linoleic acid) to colneleic acid (Scheme 72). The trienoic acid formed from linolenic acid yields colnelenic acid (*408*). These products may have biological activities in plants; analogous reactions in mammals are yet unknown.

4.12. Isomerization. A P450-catalyzed allylic double bond topoisomerization has already been alluded to in the work by Nelson related to pulegone oxidation (*327*) and is shown in Scheme 50.

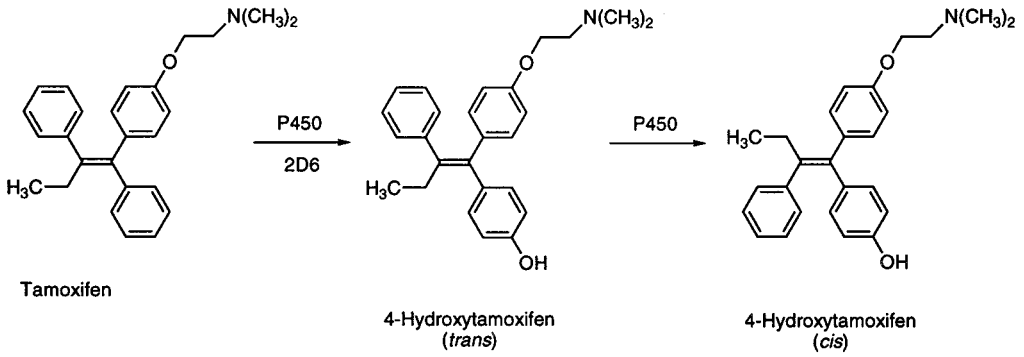
An unusual reaction reported recently is the trans to cis isomerization of 4-hydroxytamoxifen Scheme 73) (*409*).[3] The isomerization occurs with 4-hydroxytamoxifen but not tamoxifen itself, with liver microsomes or with several recombinant P450s, particularly (human) P450 1B1.[3] The reaction is of interest in that an estrogen receptor antagonist is converted to an agonist.

This reaction does not involve a change in the oxidation−reduction state but the requirement for NADPH argues that either Fe^{2+} or an activated iron−oxygen complex should be involved (the requirement of O_2 has not been investigated). A possible mechanism (Scheme 74) involves an *ipso* attack at the 4-hydroxy position and conversion of the olefin to a carbocation, which could invert before collapse to regenerate FeO^{3+}. The FeO^{3+} might dissipate to Fe^{3+} and H_2O as a result of electron input (Scheme 2), or conceivably a change in the juxtaposition of 4-hydroxytamoxifen could occur and be followed by another hydroxylation (e.g., α-hydroxylation to give the known 4,α-dihydroxytamoxifen product).

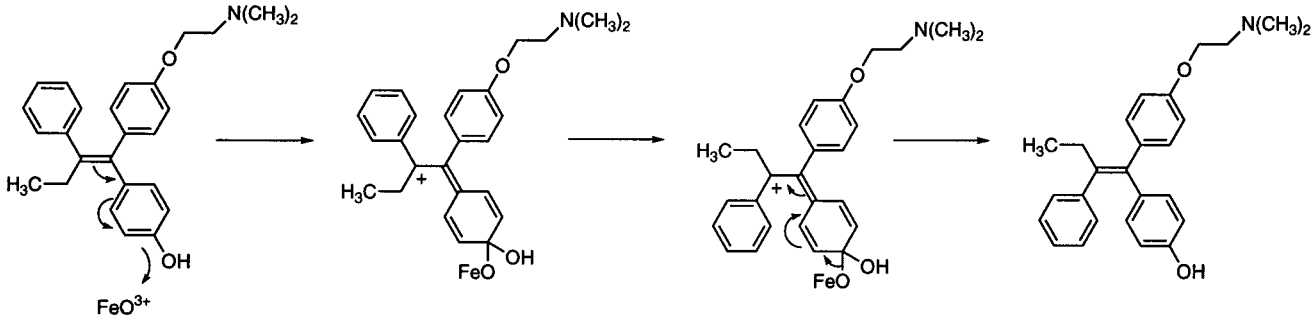
4.13. Phospholipase D Activity. One of the more unusual reactions observed with P450s is the phospholipase D-type hydrolysis seen with phosphatidylcholine (*410*). Phospholipase D has an important role in cell

[3]Crewe, H. K., Notley, L. M., Wunsch, R. M., Lennard, M. S., and Gillam, E. M. J., submitted for publication.

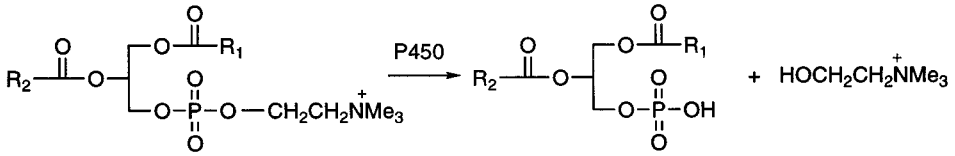
Scheme 73. 4-Hydroxylation of Tamoxifen and Subsequennt trans/cis Isomerization (*409*)[3]



Scheme 74. Possible Mechanism of trans/cis Isomerization of 4-Hydroxytamoxifen



Scheme 75. P450-Catalyzed Phospholipase D Reactions (*410*)



regulation, with the product phosphatidic acid acting as a messenger.

This appears to be a hydrolytic cleavage of the ester linkage, in contrast to the oxidative mechanism described under item 3.3. NADPH is not required, and the reaction products are choline and phosphatidic acid (Scheme 75). Thus, the oxidative ester cleavage mechanism (section 3.3) does not seem operative [this would yield 2-(*N*,*N*,*N*-trimethyl)aminoacetaldehyde, not choline]. The reaction could be demonstrated with several P450s purified from liver or recombinant sources. Antibodies inhibit a significant fraction of the phospholipase D activity of human liver microsomes (*410*). It is a relatively slow reaction (~0.2 min^{-1}) and probably only accounts for part of the phospholipase D activity known to be in liver (*411*). In contrast to other phospholipase D activities, there was no effect of divalent cations on the P450-catalyzed activity.

Exactly how P450s catalyze this reaction is not known. One of the most active of the P450s examined was human P450 1A2, and several mutants had altered activity. One dramatic effect of the mutants was substitution in the N-terminus. Some His residues in the N-terminal region could conceivably be involved in base-catalyzed hydrolysis of the phosphoester (*410*).

5. Conclusions

P450 enzymes catalyze many reactions, and even the major mammalian P450s generally devoted to the metabolism of xenobiotics have been shown to be capable of most of these. One can view P450 reactions as being very diverse (*6*) or very unified (*131*). In this review, an attempt has been made to show that most of the reactions can be understood in the context of some relatively restricted chemical paradigms.

Most of the P450 reactions are oxidations. The vast majority of these can be rationalized by utilizing an FeO^{3+} species, at least on paper. This may be a generally useful way to approach an oxidation that has been demonstrated to occur and for which there is at least a suggestion of P450 involvement. As discussed earlier, rationalization of a reaction in terms of a "classic" FeO^{3+} abstraction/rebound paradigm does not necessarily prove that this is the operative mechanism in the absence of further evidence. The reduction reactions seem to be mechanistically straightforward, involving the transfer of an electron from the iron, although the competition of the substrate with O_2 is still surprising in many cases. More complex and unpredictable are the P450-catalyzed rearrangements. Some of these involve the intermediacy of high-valent FeO complexes and internal electron transfers, either formally one- or two-electron (e.g., section 4.11). Even more difficult to rationalize and certainly to predict are the rearrangements that occur without the FeO complex, e.g. the dehydration of aldoximes to nitriles (section 4.7) and the highly unusual phospholipase D reaction (section 4.13).

In looking at the total collection of P450 reactions that have ever been observed, we can rationalize the vast majority of them in the context of what we do know about

the chemistry of iron−oxygen complexes. However, rationalization in the terms of inorganic biochemistry does not necessarily lead to reliable predictions of regioselectivity in proteins. Although some homology modelers might disagree, predicting the course of new P450 reaction is still not very accurate. Even ligand-free protein structures of the P450s have been a limited guide to prediction of what the ligand-bound structures should be. We have the dilemma (discussed earlier, section 3.3) of deciding whether catalytic specificity is the result of (i) only the influence of protein structure on substrate docking or (ii) an influence of protein structure on substrate docking, the nature of the iron−oxygen intermediate, and the interaction of the substrate with the intermediate. Another issue is rate-limiting steps, which has been discussed (section 3.3) and which are generally not known in P450 reactions. Our own work in this area (section 3.3),[2] suggests that the mammalian P450 enzymes utilize a landscape of barriers of similar free energy in the catalysis of reactions of interest in this field, and small changes may have dramatic effects in terms of influences on product distribution due to tampering with the transition state barriers (Figure 2) (*239*). Another general concern has been raised by some of the reports of stimulation of P450 catalytic activities by added compounds in the context of kinetic cooperativity, especially with P450 3A4 (*412*−*416*) but also with P450s 1A2 (*417*) and 2D6 (*238*) and possibly others. Are we dealing with a system of "induced fit" here? Comparisons of three-dimensional structures of archebacter P450 119 with and without 4-phenylimidazole may be used as evidence in favor of this view (*65*). If this is the case, efforts to use structures and models to predict the course of new reactions will remain problematic.

In conclusion, we will probably remain in an empirical mode of dealing with P450 reactions for some time. Recombinant DNA technology, high-throughput systems, and progress in analytical chemistry and spectoscopy provide experimental approaches to analysis of new chemical entities in regard to P450 reactions. The characterization of the chemistry of the iron−oxygen complexes and P450 kinetics is far from complete and should provide more insight into the details of the reactions that have been discussed. Finally, in regard to the issue of toxicology, new methods are being developed to rapidly provide information about bioactivation in rapid-throughput screening of chemicals, as well as in the prediction of pharmacokinetics and bioavailability.

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