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ACKNOWLEDGE

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to my parents, Pil Hyung and Sul Ja Lee

to Julee, with love

ABSTRACT

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ABSTRACT

Nitrogen oxidation catalyzed by cytochrome P-450 is generally believed to proceed through a mechanism involving a radical cation intermediate. A series of 3,5-dicarbethoxy-2,6-dimethyl-4-alkyl-1,4-dihydropyridines has been used as probes to examine the radical mechanism of the cytochrome P-450-catalyzed oxidation of nitrogenous compounds. In addition, the detailed mechanism of the oxidation of the dihydropyridines to the corresponding pyridines has been elucidated. The 4-ethyl analogue (DDEP) catalytically inactivates cytochrome P-450, as measured by destruction of the cytochrome P-450 chromophore, by eliminating the 4-ethyl group as a free radical. The ethyl radical can either alkylate the heme or leave the active site and be spin trapped. The ability of the dihydropyridines to extrude the 4-alkyl groups, destroy the cytochrome P-450 chromophore, and generate spin trapped radicals correlates with the stabilities of the 4-alkyl groups as radicals. Deuterium substitution at the 4-position has no effect on elimination of either the 4-alkyl or the 4-hydrogen moiety. A mechanism requiring loss of the nitrogen proton prior to 4-deprotonation can rationalize these results. Substitution of the nitrogen with alkyl groups, however, results in simple *N*-dealkylation rather than aromatization of the dihydropyridine ring. Substitution of the nitrogen with a phenyl group results in the formation of aromatized products but unfortunately alters the mechanism of the dihydropyridine oxidation from one that has no isotope effect at the 4-position to one that exhibits a large isotope

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effect (k_H/k_D = 6). Mechanisms which are consistent with the results are proposed for the cytochrome P-450-catalyzed oxidation of 4-alkyl-1,4-dihydropyridines.

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1.3 Isozyme

1.4 Structure

1.5 Reaction

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LIST OF ABBREVIATIONS

ALAS	5-Aminolevulinic acid synthase
BSTFA	Bis(trimethylsilyl)trifluoroacetamide
CO	Carbon monoxide
DDC	3,5-Dicarbethoxy-2,6-dimethyl-4-methyl-1,4-dihydropyridine
DDEP	3,5-Dicarbethoxy-2,6-dimethyl-4-ethyl-1,4-dihydropyridine
DDIP	3,5-Dicarbethoxy-2,6-dimethyl-4-isopropyl-1,4-dihydropyridine
DDMQ	2,4-Diethyl-1,2-dihydro-2-methylquinoline
DDPEP	3,5-Dicarbethoxy-2,6-dimethyl-4-(2-phenylethyl)-1,4-dihydropyridine
DETAPAC	Diethylenetriaminepentaacetic acid
DEX	Dexamethasone
DTMQ	1,2-Dihydro-2,2,4-trimethylquinoline
DMSO	Dimethylsulfoxide
EIMS	Electron impact mass spectrometry
EPR	Electron paramagnetic resonance
EDTA	Ethylenediaminetetraacetic acid
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
GC	Gas chromatography
HPLC	High pressure liquid chromatography
LSIMS	Liquid secondary ion mass spectrometry
NAD^+	Nicotinamide adenine dinucleotide (oxidized)

NADH	Nicotinamide adenine dinucleotide (reduced)
NADP+	Nicotinamide adenine dinucleotide phosphate (oxidized)
NADP	Nicotinamide adenine dinucleotide phosphate (reduced)
N-alkylDDEP	*N*-Alkyl-3,5-dicarbethoxy-2,6-dimethyl-4-ethyl-1,4-dihydropyridine
N-ethylDDEP	3,5-Dicarbethoxy-2,6-dimethyl-4-ethyl-*N*-ethyl-1,4-dihydropyridine
N-methylDDEP	3,5-Dicarbethoxy-2,6-dimethyl-4-ethyl-*N*-methyl-1,4-dihydropyridine
NMR	Nuclear magnetic resonance
N-phenylDDEP	3,5-Dicarbethoxy-2,6-dimethyl-4-ethyl-*N*-phenyl-1,4-dihydropyridine
PB	Phenobarbital
PCN	Pregnenolone-16α-carbonitrile
POBN	α-(Pyridyl-1-oxide)-*N*-*tert*-butylnitrone
PPIX	Protoporphyrin IX
SKF525A	Proadifen [(N,N-diethyl)-2'-aminoethyl]-2,2-diphenylpentanoate
THF	Tetrahydrofuran
TAO	Triacetyloleandomycin
TLC	Thin-layer chromatography
UV	Ultraviolet

1.0 INTRODUCTION

1.1 Function and Distribution

Cytochrome P-450 refers to a family of enzymes containing heme as the prosthetic group. The heme iron is ligated to the sulfur of a cysteine residue and forms a carbon monoxide complex with an absorbance maximum at 450 nm (Omura et al., 1965). Cytochromes P-450 occur widely in nature, being found in most animals, plants, and microorganisms. Mammalian cytochrome P-450 can be classified into two general categories; biosynthetic isozymes, which primarily metabolize endogenous or physiological substrates, and xenobiotic isozymes, which primarily metabolize exogenous or xenobiotic substrates. Examples of the biosynthetic cytochromes P-450 are cytochrome P-450$_{SCC}$ (cytochrome P-450 XXIIA1), which catalyzes the side chain cleavage of cholesterol to give pregnenolone (Shikita et al., 1974), and cytochrome P-450 aromatase (cytochrome P-450 XIXA1), which catalyzes the conversion of androgens to estrogens (Fishman, 1982). The xenobiotic cytochromes P-450, which show broad substrate specificity and have molecular weights of 50-55 kD, function to eliminate foreign materials from the body. Water soluble compounds are readily removed from the body but hydrophobic compounds, which accumulate in the membrane and adipose tissue, must be converted to more hydrophilic compounds in order for their elimination from the body to take place. These transformations are conducted by xenobiotic cytochrome P-450 isozymes. The negative aspect of

these metabolic reactions is that relatively non-toxic, hydrophobic compounds may be converted into highly reactive electrophilic species.

1.2 Components and Location

Cytochromes P-450 require not only the heme but also additional electron transport proteins to be active. Cytochrome P-450 systems fall into two general classes, one that is supported by an iron-sulfur protein and an FAD containing flavoprotein dehydrogenase (Sligar and Murray, 1986) and one that is supported only by the flavoprotein cytochrome P-450 reductase (Masters and Okita, 1980). The latter system is found throughout the body, including liver, lung, kidney, adrenal, skin, brain, intestine, and testis (Orrenius and Ernster, 1974, Wickramasinghe, 1975, and Sato, 1978). The cytochromes P-450 are embedded in the membrane matrix of the rough and smooth endoplasmic reticulum. The lipid membrane environment plays an important role since it allows the cytochrome P-450 to be in close proximity to its substrates. Cytochrome P-450 reductase, which has a molecular weight of 79 kD and contains one FAD and one FMN, transfers electrons from NADPH to the cytochrome P-450-substrate complex. FAD accepts the electrons from NADPH and FMN reduces cytochrome P-450 (Vermilion and Coon,1978). Unlike cytochrome P-450, cytochrome P-450 reductase is not deeply embedded in the lipid membrane but is simply anchored to the membrane by a C-terminus hydrophobic tail.

The cytochrome P-450 system supported by an iron-sulfur protein and flavoprotein dehydrogenase is found in the mitochondria and bacteria. The iron-sulfur protein and flavoprotein dehydrogenase mediate electron transport from either NADPH (for the mitochondrial cytochrome P-450) or NADH (for the bacterial cytochrome P-450). Figure 1.2.1 illustrates the electron transfer system for the bacterial cytochrome P-450. The electrons are

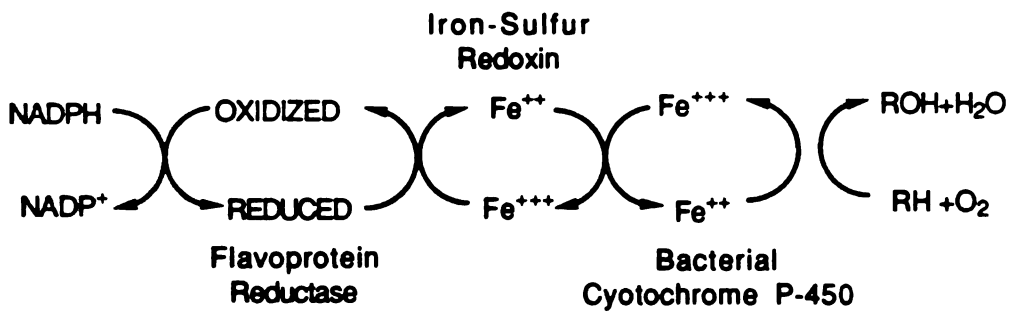

Figure 1.2.1 Electron transport system of bacterial cytochrome P-450.

transferred from NADH to the flavoprotein reductase, which in turn transfers the electrons to the iron-sulfur redoxin. Cytochrome P-450 receives the electrons from the iron-sulfur redoxin. For the mitochondrial cytochrome P-450, the electrons are transported in a similar manner from NADPH through adrenodoxin (the iron-sulfur protein) (Omura et al., 1966 and Suzuki and Kimura, 1965) and NADPH-adrenodoxin reductase (the FAD containing flavoprotein) (Suhara et al., 1972). The only other major difference between the mitochondrial and bacterial systems is that the mitochondrial

system is embedded in the mitochondrial membrane (Omura et al., 1966) whereas the bacterial system is soluble.

1.3 Isozymes and Induction

Rat liver microsomes contain many different isozymes of cytochrome P-450 which are induced by repeated exposure of the animal to certain drugs and environmental agents. Some examples of cytochrome P-450 isozymes and their inducing agents are shown in Table I.3.1 (Dutton, 1988). The ability of the body to metabolize different xenobiotics is due to the multiple forms of cytochrome P-450. These forms exhibit marked differences in substrate-, regio-, and stereoselectivity (Conney, 1986). Approximately a dozen isozymes, each of which is a separate gene product under separate regulatory control (Thomas et al., 1981, Waxman et al., 1985, Bandiera et al., 1986, and Thomas et al., 1987), have been purified from rat liver microsomes (Ryan, et al., 1982, Ryan et al., 1984, Waxman, 1984, Waxman and Walsh, 1982, Guengerich et al. 1982, and Larrey et al., 1984). The amino acid sequence identities of the cytochromes P-450 vary from as little as 3% to as much as 75% (Nebert et al., 1987). The area coding for the heme binding site, near the C-terminus, seems to be the most conserved region among all cytochromes P-450 examined (Nebert and Gonzales, 1987).

The inducing agents can be classified into two major categories: agents that lead to an increase in liver weight as well as increased levels of cytochrome P-450 (Orrenius et al., 1969) and polycyclic aromatic hydrocarbons which increase cytochrome P-450

Table 1.3.1 List of rat liver microsomal cytochrome P-450 isozymes.

Isozyme	Alternate nomenclature [a]	Constitutive [b]	Inducers [c]
P-450a	P-450 IIA1	3-15 % (F > M)	PB, MC 2-3 fold
P-450b	P-450 IIB1	< 2 %	PB ~ 50 fold
P-450c	P-450 IA1	< 3 %	MC ~ 50 fold
P-450d	P-450 IA2	4-10 %	ISO ~ 20 fold
P-450e	P-450 IIB2	1-2 %	PB ~ 30 fold
P-450f	P-450 IIC7	0-10 %	CHL ~ 2 fold
P-450g	----------	0-20 % (M >>> F)	None
P-450h	P-450 IIC11	0-10 % (M >>> F)	None
P-450i	----------	0-20 % (F >>> M)	None
P-450j	P-450 IIE1	5-10 %	Ethanol ~ 5 fold
P-450k	P-450 IIC6	0-5 %	PB ~ 3 fold
P-450p	P-450 IIIA1	0-20 %	Steroids 5-20 fold

[a] Based on the recently recommended standardized nomenclature (Nebert et al., 1987). [b] Percentage of total cytochrome P-450 in liver microsomes from mature rats. F = female, M = male (Thomas et al., 1981, Waxman et al., 1985, Bandiera et al., 1986, and Thomas et al., 1987). [c] PB = phenobarbital, MC = 3-methylcholanthrene, ISO = isosafrole, and CHL = chlordane.

levels but do not increase liver weight (Parke, 1979). Examples of the former and latter are phenobarbital and 3-methylcholanthrene, respectively. The mechanisms of induction have not been fully characterized but the effects of polycyclic aromatic hydrocarbons have been shown to be mediated through the cytosolic Ah receptor (Tukey et al., 1982). The increased levels of cytochrome P-450 upon treatment with polycyclic aromatic hydrocarbons have been shown to arise from increased *de novo* synthesis of the enzyme (Omura,

1979) due to increased transcription of the cytochrome P-450c gene (Kumar and Padmanaban, 1980 and Morville, et al., 1983). The transcriptional activation of the cytochromes P-450b and e genes has similarly been observed with phenobarbital treatment (Bhat and Padmanaban, 1979, Adesnik et al., 1981, and Hardwick et al., 1983), but "phenobarbital receptors" have not been identified. The induction of cytochrome P-450p with triacetyloleandomycin (TAO) has been partly attributed to protein stabilization (Watkins et al., 1986). The mechanism of the inhibition of cytochrome P-450 degradation is not known but the complexation of TAO or metabolites of TAO with cytochrome P-450 to inhibit degradation has been suggested (Watkins et al., 1986).

1.4 Structure and Active Site

Although the different isozymes of cytochrome P-450 have clear differences in substrate specificities and turnover rates, the general active site characteristics are similar. The iron is ligated by the pyrrole nitrogens of the heme and a protein thiolate (Cramer et al., 1978). The thiolate ligand is thought to donate electrons to the heme to aid in cleavage of the molecular oxygen. The sixth ligand of the iron has not been identified but is believed to be a H_2O molecule. For cytochrome P-450cam, the substrate binding site is filled with H_2O molecules, one of which appears to be the sixth ligand. The H_2O molecules are displaced upon binding of the substrate (Poulos et al., 1986).

The substrate specificities of the different isozymes are determined by the protein residues on the sixth ligand side of the heme. The structure of mammalian cytochrome P-450 isozymes has been elusive due to lack of an x-ray structure, but bacterial cytochrome P-450cam has recently been crystallized and its structure analyzed to 1.9 Å (Poulos et al., 1985). This crystal structure has confirmed many of the earlier conclusions regarding cytochromes P-450. The active site has been shown to be hydrophobic (White et al., 1980, Jefcoate et al., 1969, and Canady et al., 1974) and chiral in nature. Some examples of stereoselective action of cytochrome P-450 are: 1) selectivity for the metabolism of one enantiomer of warfarin (Kaminsky et al., 1980) and amphetamine (Cho and Wright, 1978), 2) epoxidation on one side of polyaromatic hydrocarbons (Jerina et al., 1982), 3) hydroxylation on one side of steroids (Waxman et al., 1983 and Wood et al., 1983), 4) removal of a specific prochiral hydrogen (Tullman et al., 1984), and 5) generation of chiral *S*-oxides (Waxman et al., 1982).

Without crystal structures of the mammalian cytochromes P-450, only indirect evidence exists for the topologies of their active sites. For cytochrome P-450b, Ortiz de Montellano and colleagues exploited heme alkylation by olefins and acetylenes to determine the general active site topology (Figure 1.4.1a) (Kunze et al., 1983 and Ortiz de Montellano and Kunze, 1981). This method also led to the determination that the orientation of the 5th ligand in relation to the heme is identical to that of hemoglobin (Ortiz de Montellano, et al., 1983). The substrate binding domain of cytochrome P-450b is not

symmetrical over the heme but rather skewed to cover the pyrrole nitrogen of the B ring. The masking of the B ring is also seen in the crystal structure of cytochrome P-450cam (Figure 1.4.1b) (Poulos et al., 1985).

a) b)

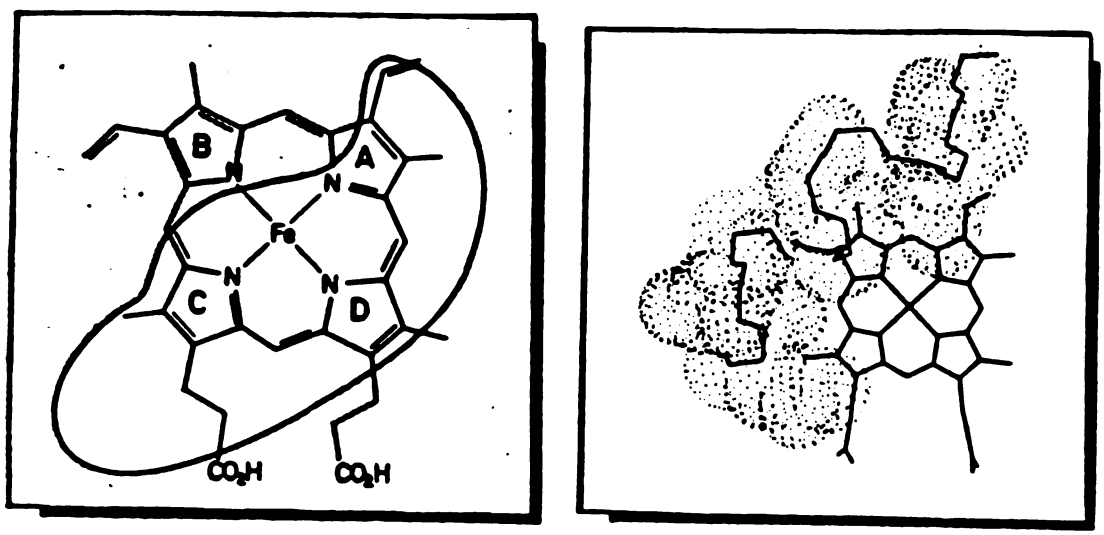

Figure 1.4.1 a) Active site model of cytochrome P-450b and b) crystal structure of cytochrome P-450cam. (Ortiz de Montellano, 1987).

For cytochrome P-450c, the active site shape has been estimated by superimposing the double bonds of benzo[*a*]pyrene (Armstrong et al., 1981 and Boyd et al., 1980), benzo[*a*]anthrene (Thakker et al., 1979 and van Bladeren et al., 1982), chrysene (Nordqvist et al., 1981), and phenanthrene (Jerina et al., 1982). The

regio- and stereospecificity of these hydrocarbons suggests the active site model in Figure 1.4.2 (Yagi and Jerina, 1982). Both of the Figures 1.4.1a and 1.4.2 provide very crude approximations of the active sites and should be viewed as such.

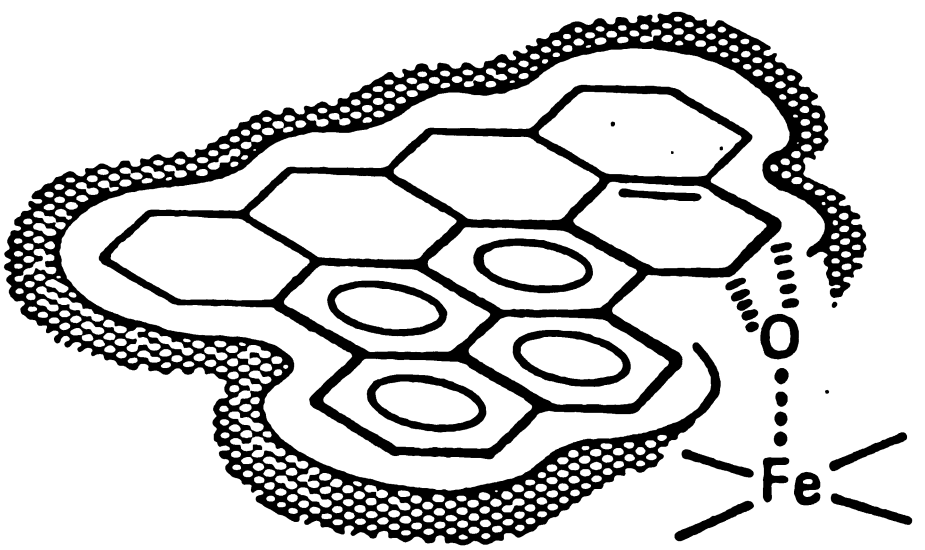

Figure 1.4.2 Proposed active site of cytochrome P-450c (Yagi and Jerina, 1982).

1.5 Reactions and Stoichiometry

The oxidative reactions catalyzed by cytochrome P-450 can be classified into three general categories: 1) carbon hydroxylation (insertion of an oxygen between a carbon and hydrogen bond), 2) olefin epoxidation (insertion of an oxygen atom into an olefin), and

3) heteroatom oxidation (oxidation of the lone pair of electrons on the heteroatom). The general stoichiometry of the reaction is:

$$RH + O_2 + NADPH + H^+ \text{--------} > ROH + H_2O + NADP^+$$

where RH is the substrate. Besides the oxidation reactions cytochrome P-450 has been shown to catalyze reduction reactions under hypoxic conditions (McLane et al., 1983). The reduction reactions compete with activation of the molecular oxygen.

1.6 Catalytic Cycle

Cytochrome P-450, being an enzyme (a catalyst), only facilitates the rate of a reaction and does not alter the energy levels of the reactants and products. For all practical purposes, most of the reactions catalyzed by cytochrome P-450 would not occur without the enzyme. Cytochrome P-450 undergoes several important steps in converting relatively unreactive molecular oxygen to a very reactive electrophilic oxygen species. This process is referred to as the catalytic cycle of cytochrome P-450. A schematic representation of the catalytic cycle is shown in Figure 1.6.1: 1) substrate binding, 2) transfer of the first electron, 3) binding of molecular oxygen, 4) transfer of the second electron, 5) activation of the oxygen, and 6) insertion of the oxygen into the substrate. The intermediates beyond the second electron transfer are hypothetical since they have not been detected and characterized.

1.6.1 Substrate Binding

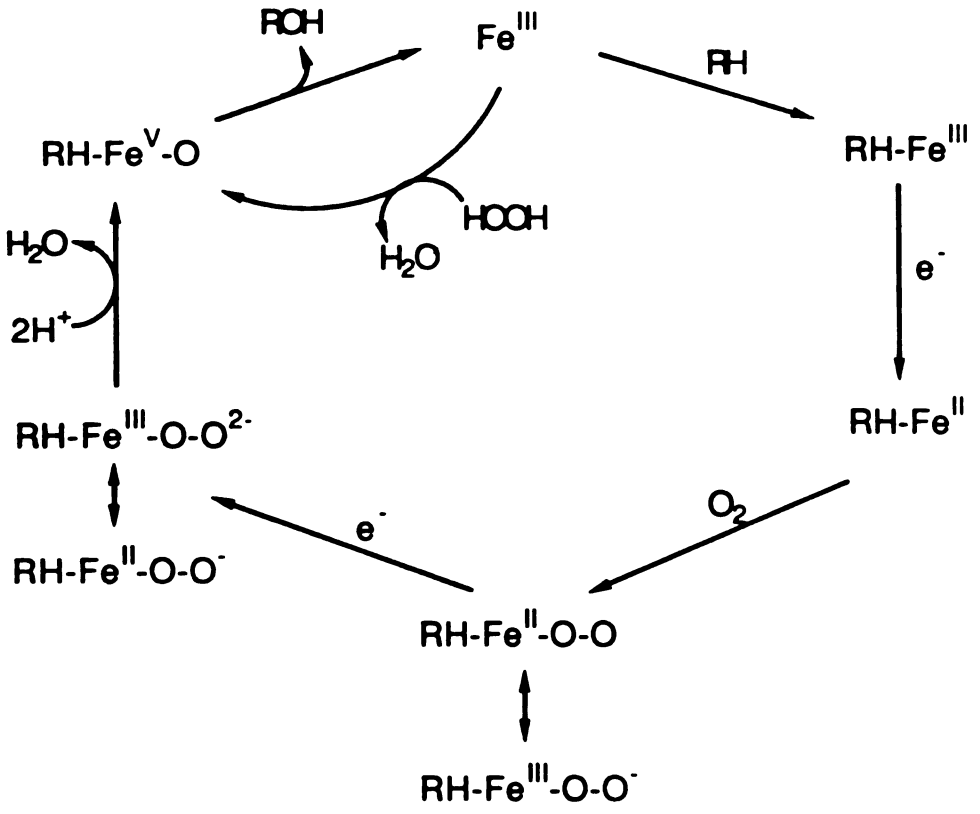

Figure 1.6.1 Cytochrome P-450 catalytic cycle.

The iron of cytochrome P-450 in the native resting state exists as the low spin (S = 1/2) ferric (Fe^{III}) form with H_2O as the sixth ligand. In the low spin state the iron is hexacoordinated and is planar with the porphyrin. Upon binding of the substrate, which is the first step, the H_2O sixth ligand is displaced by the substrate. This causes the iron to be pentacoordinated and change its spin state to high spin (S = 5/2). The change in the iron state has been monitored directly by EPR (Whysner et al., 1969) and absorbance spectroscopy (Schenkman et al., 1967). The binding of the substrate can best be observed by difference spectroscopy, in which the

reference cuvette contains cytochrome P-450 and the sample cuvette contains cytochrome P-450 and substrate. A type I binding spectrum is obtained with a weak ligand substrate and is exemplified by a maximum at 393 nm and minimum at 418 nm. A type II binding spectrum, which exhibits the opposite maximum and minimum, is obtained when the substrate contains a ligating group, such as an amine, which is strong enough to keep the iron in the hexacoordinate state.

1.6.2 Transfer of the First Electron

The Fe^{III} cytochrome P-450-substrate complex is reduced to Fe^{II} cytochrome P-450-substrate complex by cytochrome P-450 reductase (Guengerich et al., 1975), a flavin containing protein utilizing electrons from NADPH. The reduced cytochrome P-450 complex has been shown to bind CO to exhibit a characteristic absorbance at 450 nm (Omura and Sato, 1964).

1.6.3 Binding of Molecular Oxygen

Following the formation of the Fe^{II} cytochrome P-450-substrate complex, molecular oxygen binds rapidly. The Fe^{II} dioxygen species exhibits a characteristic spectrum with absolute maxima at 420 and 558 nm and difference maxima at 440 and 590 nm (Estabrook et al., 1971, Peterson et al., 1972, and Larroque and Van Lier, 1980). It also exhibits properties similar to the iron-oxygen complex of oxyhemoglobin (Sharrock et al., 1976). The binding of oxygen has been reported to occur in two steps. The rate constants

of the first and second steps were reported to be > 60,000 and 210 min^{-1}, respectively (Guengerich et al., 1976).

1.6.4 Transfer of the Second Electron

The Fe^{II} dioxygen species has three fates: 1) dissociation to form superoxide anion with concomitant regeneration of the native cytochrome P-450 (Debey and Balny, 1973, Sligar et al., 1974, Auclair et al., 1978, and Guengerich et al., 1976) (Figure 1.6.4.1), 2)

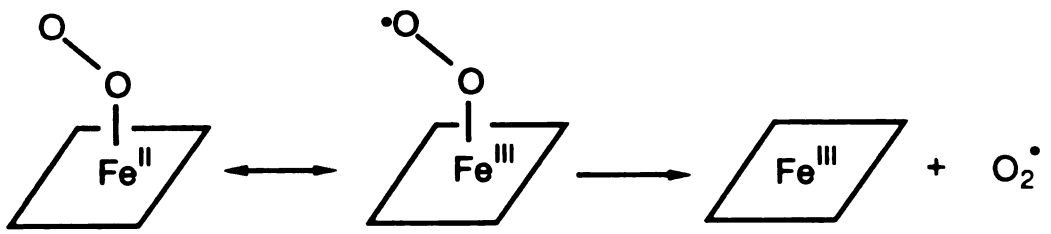

Figure 1.6.4.1 Generation of superoxide anion from the cytochrome P-450-O_2 complex.

transfer of an electron to ferric cytochrome b_5 to regenerate native cytochrome P-450 (Guengerich et al., 1976, Begard et al., 1977, Bonfils et al., 1981, and Pompon and Coon, 1984), and 3) acceptance of a second electron from cytochrome P-450 reductase to form the peroxide-substrate-cytochrome P-450 complex (i. e., normal catalytic function). The Fe^{II} dioxygen species can alternatively accept the second electron from reduced cytochrome b_5 instead of cytochrome P-450 reductase (Hildebrandt and Estabrook, 1971). The peroxide equivalent has been shown to dissociate to generate

hydrogen peroxide and native cytochrome P-450 (Gillette et al., 1957 and Hildebrandt and Roots, 1975) (Figure 1.6.4.2). In addition, Coon (Gorsky et al., 1984) and Sligar (Atkins and Sligar, 1987) have shown that cytochromes P-450 possess oxidase activity where atmospheric dioxygen is reduced to two H_2O molecules.

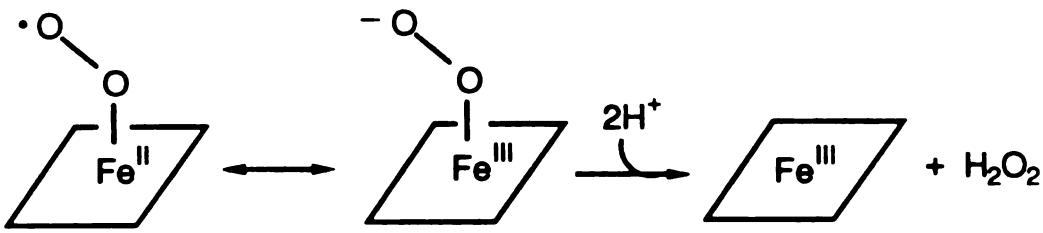

Figure 1.6.4.2 Generation of hydrogen peroxide from the cytochrome P-450-O_2 complex.

1.6.5 Activation of the Oxygen

The transfer of the second electron initiates what is believed to be cleavage of the oxygen-oxygen bond to generate the activated oxygen species and a molecule of H_2O. The nature of the active oxygen and the details of the oxygen activation step are not well understood. The activated oxygen complex is believed to be an iron-oxo species with six of the electrons from the oxygen. The possible mechanisms of oxygen-oxygen cleavage are: 1) cleavage facilitated by donation of electrons by the thiolate ligand (Mansuy et al., 1984), 2) acylation of the the distal oxygen to make it a better leaving group (Hamilton, 1974, Khenkin and Shteinman, 1984, and Sligar et al., 1980), and 3) formation of a three membered cyclic complex

with the dioxygen and iron (McCandlish et al., 1980 and Ataollah and Goff, 1982). The latter two mechanisms are unlikely since cytochrome P-450 lacks the functionalities necessary to acylate the distal oxygen (Poulos, 1986) and structurally related model cycloperoxides failed to oxidize substrates (Welborn et al., 1981), respectively. The possible electron distributions of the active oxygen species are shown in Figure 1.6.5.1 (Ortiz de Montellano, 1986). Porphyrin model systems have produced analogues of the active oxygen species (Tabushi and Yazaki, 1981, Groves and Nemo, 1983, Bajdor and Nakamoto, 1984, and Balch et al., 1984) which are believed to be similar to that of cytochrome P-450 (McMurray and Groves, 1986).

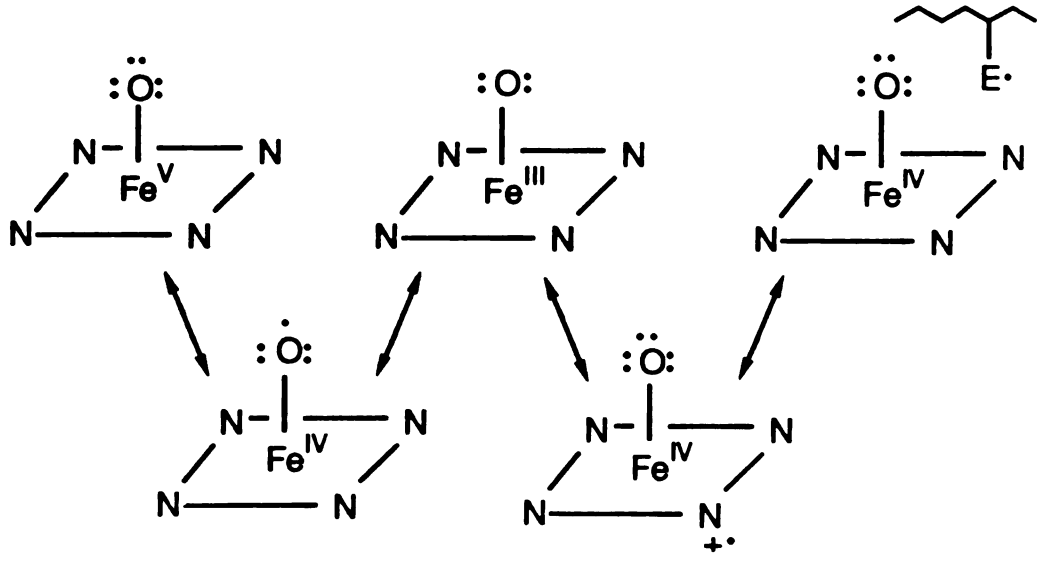

Figure 1.6.5.1 Possible distributions of the electrons of the activated oxygen species of cytochrome P-450.

Cytochrome P-450 reactions, notably *N*-dealkylation (Nordbloom et al., 1976), *O*-dealkylation (Rahimtula and O'Brien, 1975 and Burke and Meyer, 1975), alkyl carbon hydroxylation (Hrycay et al., 1975, Ellin and Orrenius, 1975, and Gustafsson et al., 1976), and aromatic carbon hydroxylation (Burke and Meyer, 1975 and Rahimtula and O'Brien, 1974), can be supported by hydroperoxides instead of cytochrome P-450 reductase, O_2, and NADPH. Cytochrome P-450 reactions are also supported by artificial oxygen donors such as $NaIO_4$, $NaClO_2$, and iodosobenzene (Hrycay et al.,1975, Gustafsson et al., 1976, Gustafsson et al., 1979, Gustafsson and Bergman, 1976, and Lichtenberger et al., 1976). However, the hydroperoxide and iodosobenzene supported reactions are not identical to the NADPH supported reactions (Ellin and Orrenius, 1975, Fasco et al., 1979, Gustafsson et al., 1979, and Berg et al., 1979). Both the results from hydroperoxide and iodosobenzene supported reactions should be taken with some reservations when they are being compared to the NADPH supported reactions.

1.7 Substrate Oxidation

The explicit mechanisms of substrate oxidation by the cytochrome P-450 iron-oxo complex remain unclear to this day, although great progress has been made in the past decade. The principal question debated upon is whether the oxygen transfers are concerted or stepwise, and, if stepwise, whether the reactions proceed via free radical or paired electron steps. Recently, evidence has accrued in favor of the nonconcerted mechanism.

1.7.1 Carbon Hydroxylation

The mechanism of the cytochrome P-450-catalyzed hydroxylation of inactivated carbon-hydrogen bonds has received considerable attention as a result of its high efficiency and specificity. The possible mechanisms for carbon hydroxylation by an iron-oxo species are: 1) direct insertion of the oxygen between the carbon-hydrogen bond (concerted), 2) via heterolytic cleavage of the carbon-hydrogen bond (nonconcerted), and 3) via homolytic cleavage of the carbon-hydrogen bond (nonconcerted). Until the late 1970's, the mechanism of hydroxylation was considered to be a concerted process as a result of the unusual retention of stereochemistry (Bergstrom et al., 1958, Corey et al., 1958, McMahon et al., 1969, and Hamberg and Bjorkhem, 1971) and the relatively low values observed for the primary kinetic isotope effects (Bjorkhem and Hamberg, 1972 and Thompson and Holtzman, 1974). The small isotope effects support the concerted process because the bent transition implicated in the concerted process theoretically yields a small isotope effect (O'Ferrall, 1970). An example is provided by the insertion of a carbene into a carbon-hydrogen bond (Simons and Robinovitch, 1962). Small kinetic isotope effects were calculated from the absolute rates of carbon hydroxylation catalyzed by cytochrome P-450 with deuterated and nondeuterated substrates. However, these isotope effects do not reflect the intrinsic value because there are multiple rate limiting steps in the enzymatic reaction other than the hydroxylation step. Actual values of the intrinsic isotope effects have been measured by comparing the

insertion of oxygen into C-H and C-D bonds on equivalent sites of the same molecule, an approach which avoids the uncertainly of the multiple rate limiting steps. Large kinetic isotope effects were thus obtained for the hydroxylations of [1,1-^{2}H]-1,3-diphenylpropane (k_H/k_D = 11) (Hjelmeland et al., 1978) (Figure 1.7.1.1) and tetradeuterated norbornane (k_H/k_D = 11.5) (Groves et al., 1978) (Figure 1.7.1.2). The large isotope effects observed in these carbon hydroxylations indicate that the hydroxylation is probably not concerted and that C-H bond is being cleaved in the transition state.

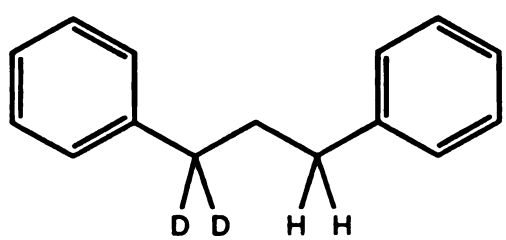

Figure 1.7.1.1 Structure of [1,1-^{2}H]-1,3-diphenylpropane.

Loss of stereochemistry is a measure of nonconcertedness of a reaction (Ortiz de Montellano, 1986). Loss of stereochemical integrity has been demonstrated in several hydroxylation reactions. Groves and colleagues (Groves et al., 1978) have shown that hydroxylation of *exo, exo, exo, exo*-2,3,5,6-tetradeuterated norbornane by rabbit liver microsomes yields *exo*-alcohol with three and four deuteriums (75 and 25 %, respectively) and *endo*-alcohol with three and four deuteriums (9 and 91 %, respectively) (Figure

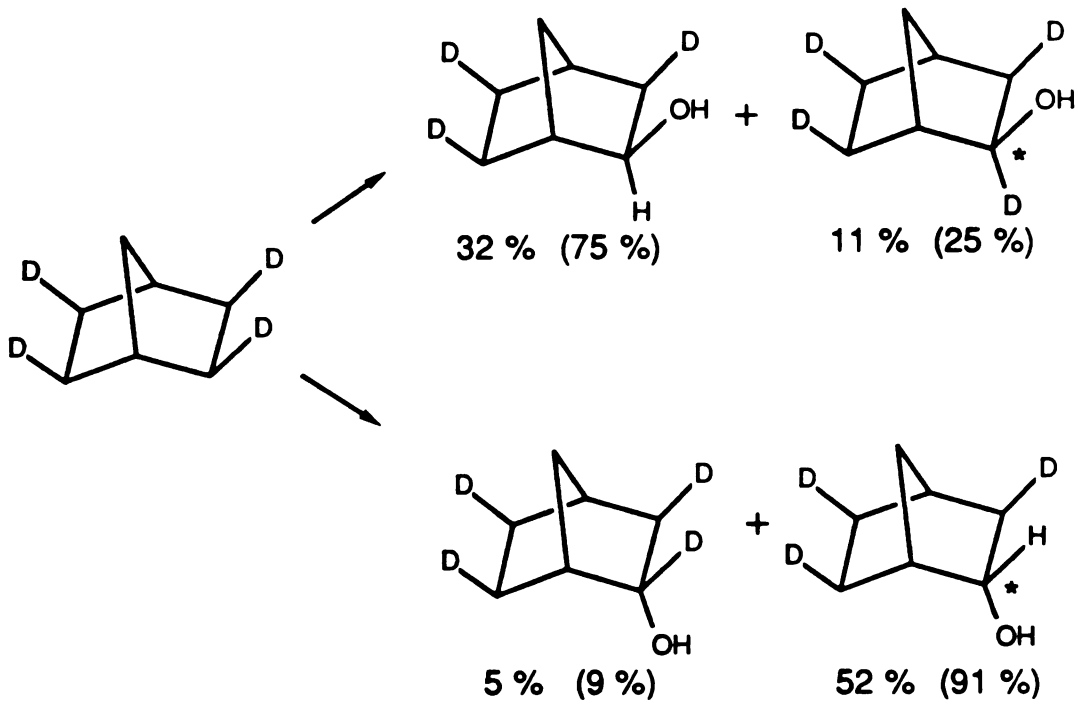

Figure 1.7.1.2 Hydroxylation of *exo, exo, exo, exo*-tetradeuterionorbornane by cytochrome P-450. The percent yields of each alcohol are calculated from the total yield of the alcohol. The percent yields in the parenthesis are the relative yields of *endo* or *exo* alcohol products. * Represents the products formed with loss of stereochemistry.

1.7.1.2). The tetradeuterio-*exo*-5-hydroxy- and trideuterio-*endo*-5-hydroxynorbornanes are products resulting from loss of stereochemistry at the site of hydroxylation. The loss of stereochemistry has also been seen with the bacterial system, cytochrome P-450cam (Gelb et al., 1982). Sligar and colleagues have demonstrated that both the *endo*- and *exo*-5-deuteriocamphors generate the deuterated and nondeuterated *exo*-5-hydroxycamphors (Figure 1.7.1.3). The *endo*-5-hydroxycamphor is not observed

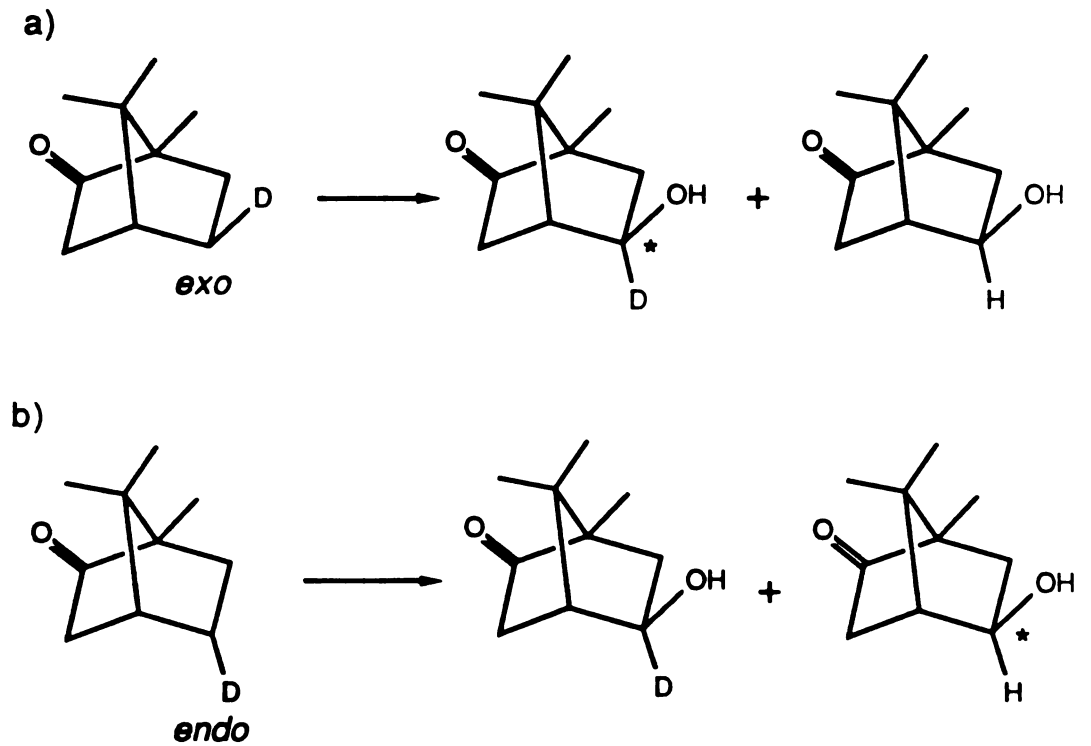

Figure 1.7.1.3 Hydroxylation of 5-*exo*- and 5-*endo*deuteriocamphor by cytochrome P-450cam. * Represents products formed with loss of stereochemistry.

although cytochrome P-450cam is capable of removing the *endo* hydrogen. The oxygen is delivered only from the *exo* side. The presence of *exo*-5-hydroxy-*endo*-5-deuterio- and *exo*-5-hydroxycamphors can only be explained by loss of stereochemistry during hydroxylation of the deuteriocamphor. Recently, ethylbenzene, which was first reported to retain stereochemistry (McMahon et al., 1969), has been reported to undergo stereochemical scrambling (White et al., 1985). The loss of stereochemistry for carbon hydroxylation excludes concerted mechanisms for cytochrome P-450-catalyzed hydroxylations and suggests mechanisms in which

an intermediate mediates the scrambling of stereochemistry. Figure 1.7.1.4 illustrates the now generally accepted mechanism for carbon hydroxylation. The iron-oxo species of cytochrome P-450 is

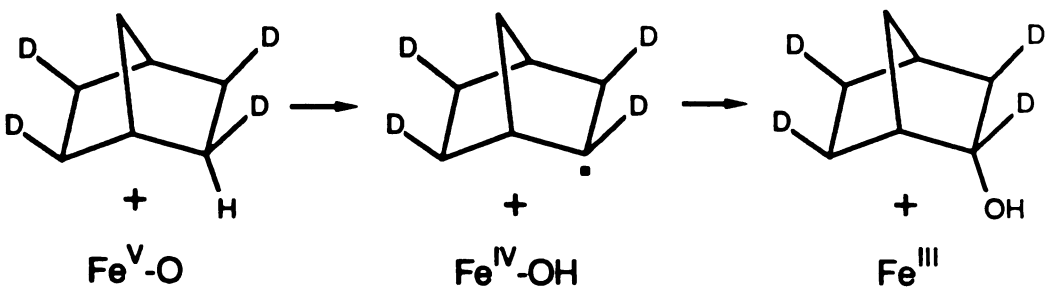

Figure 1.7.1.4 Proposed mechanism for carbon hydroxylation. The loss of stereochemistry occurs from the carbon radical intermediate.

believed to abstract a hydrogen atom to generate an iron-hydroxy radical (Fe^{IV}-OH) species and a carbon radical intermediate from which stereochemical scrambling can occur. The hydroxylated product is obtained by radical recombination of the carbon radical and hydroxy radical. The observed retention of stereochemistry can generally be attributed to the rapidity of the recombination step (Ortiz de Montellano and Stearns, 1987) and physical factors associated with substrate binding in the active site (Ortiz de Montellano, 1986).

Besides the loss of stereochemistry, the loss of regiochemistry also rules out concerted hydroxylation mechanisms because of the necessity of an intermediate. Groves and

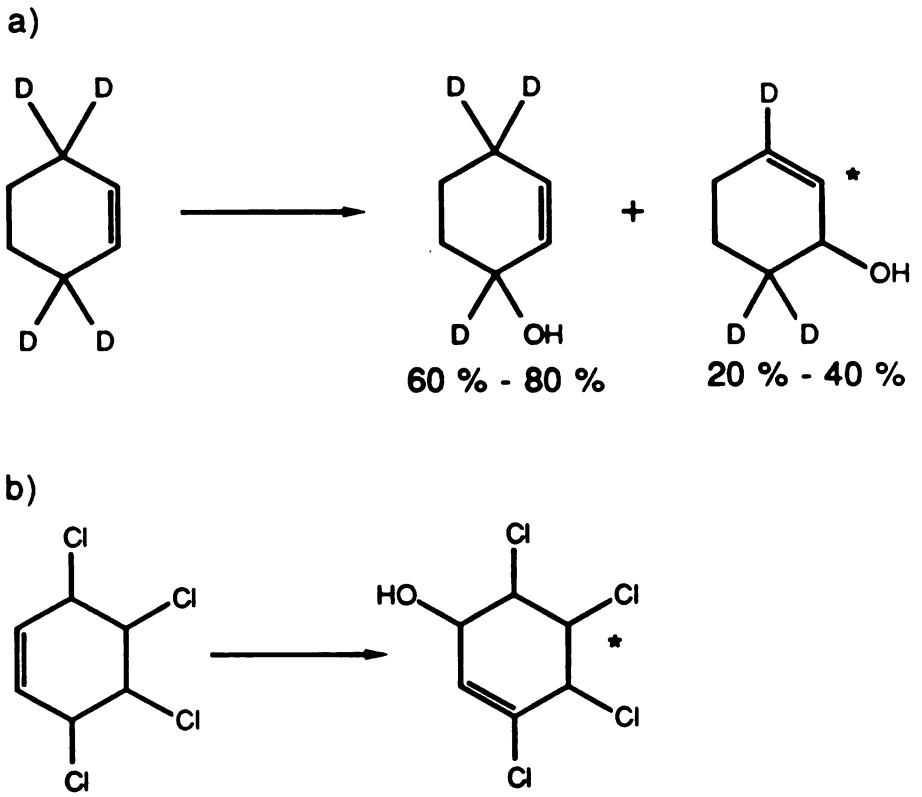

Figure 1.7.1.5 Loss of regiochemistry during hydroxylation of an allylic position of a) 3,3,6,6-tetradeuteriocyclohexane and b) 3,4,5,6-tetrachlorocyclohexane. * Represents the products formed with loss of regiochemistry.

Subramanian (1984) have reported that hydroxylation of the allylic position of cyclohexene results in loss of regiochemistry (Figure 1.7.1.5). Cytochrome P-450LM$_2$ (cytochrome P-450 IIB1) oxidizes 3,3,6,6-tetradeuteriocyclohexene to 3-hydroxy-3,6,6-trideuteriocyclohexene (60-80 %) and the allylic rearranged product, 3-hydroxy-1,4,4-trideuteriocyclohexene (20-40%). Scrambling of regiochemistry was also observed earlier in the hydroxylation of 3,4,5,6-tetrachlorocyclohexene by cytochrome P-450 (Tanaka et al., 1979). The regiochemical scrambling calls for an intermediate that

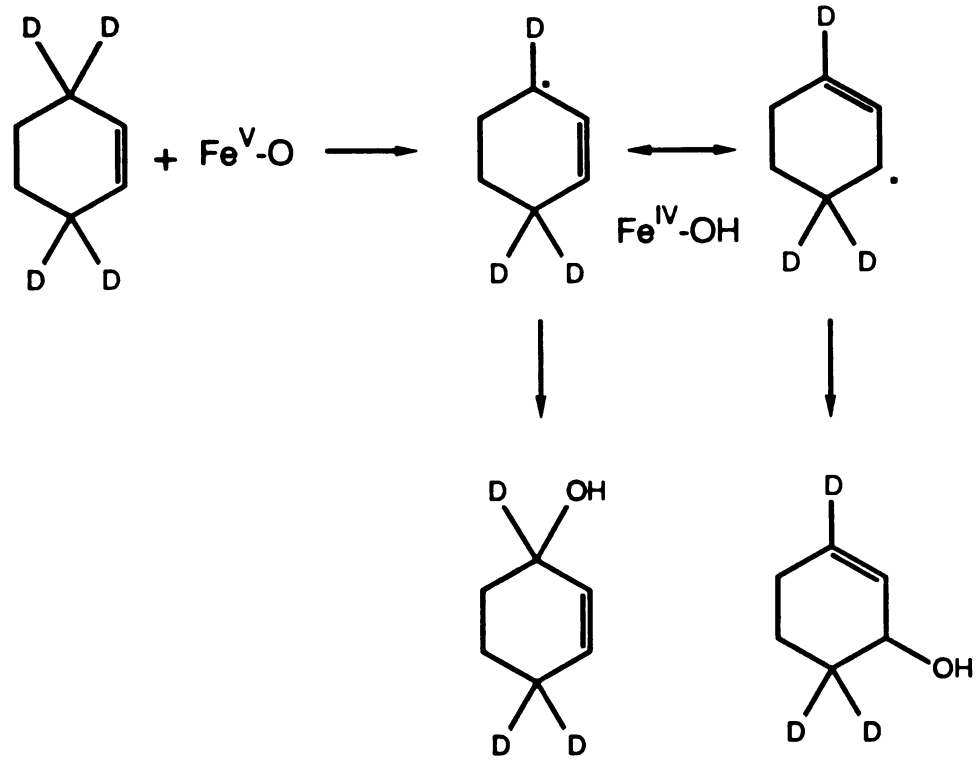

Figure 1.7.1.6 Radical mechanism, in which the radical is delocalized to the olefin, consistent with the loss of regiochemistry observed during hydroxylation of the allylic position.

allows structural rearrangement. The intermediate is believed to be an allylic radical generated by hydrogen atom abstraction by the iron-oxo species (Figure 1.7.1.6).

An interesting approach to establish a nonconcerted mechanism for carbon hydroxylation involves the oxidation of quadricyclane by cytochrome P-450 (Stearns and Ortiz de Montellano, 1985). Quadricyclane, with a very low oxidation potential ($E_{1/2}$ =0.92 V (Gassman and Yamaguchi, 1982)) due to the ring strain, has been shown to be oxidized by cytochrome P-450 to *endo*-bicyclo[3.1.0]-hex-2-ene-6-carboxaldehyde (catalytic turnover

dependent) and nortricyclanol (autooxidation) (Figure 1.7.1.7, paths a and b, respectively). Unlike normal hydroxylation, the proposed

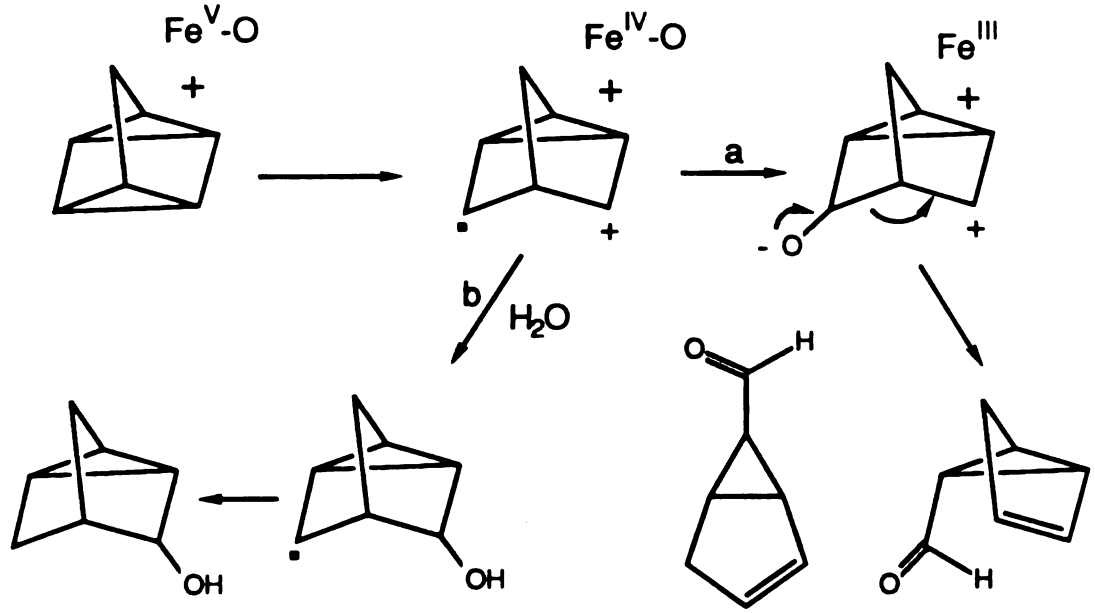

Figure 1.7.1.7 Proposed mechanism for oxidation of quadricyclane by cytochrome P-450.

mechanism of quadricyclane oxidation calls for cleavage of the carbon-carbon bond instead of carbon-hydrogen bond to form a radical cation. Trapping of the radical cation by the catalytic iron-oxo species forms the oxy-cation species which rearranges to the aldehyde. Nortricyclanol, the non-enzymatic product, is formed by addition of H_2O to the radical cation.

Aldehyde formation from quadricyclane as well as the large kinetic isotope effects and the scrambling of stereo- and regiochemistry are best explained by radical intermediates

generated during substrate hydroxylation reactions (Figures 1.7.1.4 and 1.7.1.6).

1.7.2 Olefin Epoxidation

The mechanism of olefin epoxidation by cytochrome P-450 remains vague and controversial. Cytochrome P-450-catalyzed epoxidations have been shown to maintain the stereochemistry of the olefins (Watabe and Akamatsu, 1974, Watabe et al., 1971, and Ortiz de Montellano et al., 1983). The retention of stereochemistry supports a concerted process but does not rule out the possibility of intermediates that recombine before bond rotation takes place (Maynert et al., 1970). A growing body of evidence supports a nonconcerted mechanism. A secondary isotope effect (k_H/k_D = 0.93) for epoxidation of olefins containing an internal deuterium but not terminal deuterium has been observed. This suggests an asymmetric addition of oxygen to the double bond (Hanzlik and Shearer, 1978). If formation of the two C-O bonds of the epoxide is concurrent, then the isotope effect might be expected to be similar for both the internal and terminal deuterium.

More direct evidence for the existence of an intermediate is provided by substrate rearrangement reactions and destruction of the heme (heme alkylation) during the oxidation of olefins. Cytochrome P-450-catalyzed oxidations of olefins occasionally produce not only epoxides but also the corresponding aldehydes or ketones. The oxidation of styrene and *trans*-1-phenylbutene yields minor amounts of phenylacetaldehyde and 1-phenyl-1- or 1-phenyl-

2-butanone, respectively (Figure 1.7.2.1) (Mansuy et al., 1984 and Liebler and Guengerich, 1983), although Ortiz de Montellano and colleagues did not observe the formation of phenylacetaldehyde in the oxidation of styrene (Ortiz de Montellano et al., 1987). The

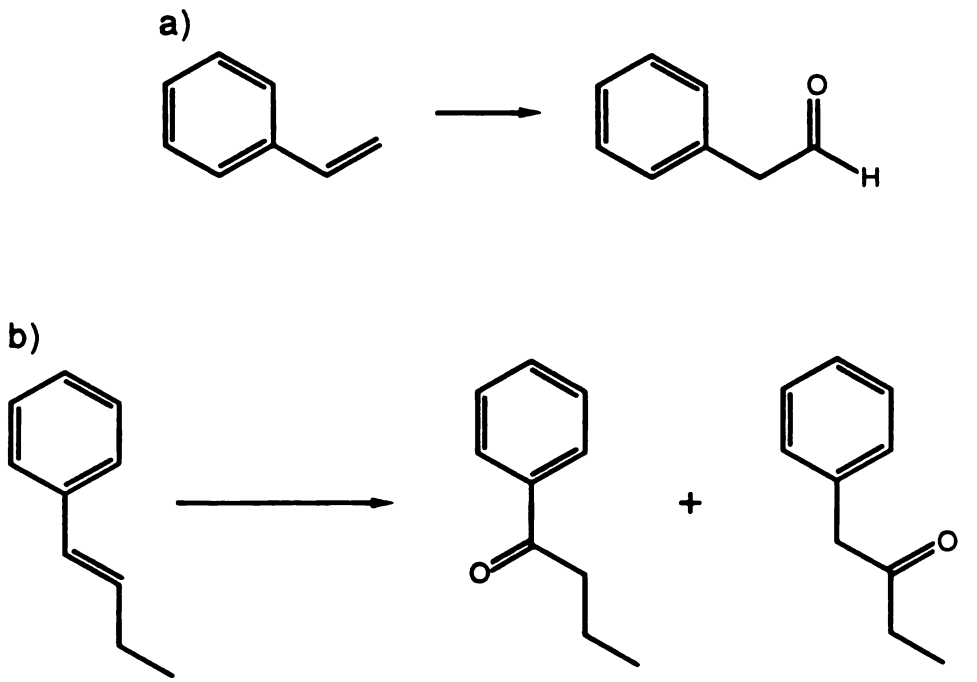

Figure 1.7.2.1 Rearrangement during epoxidation catalyzed by cytochrome P-450 of a) styrene and b) *trans*-1-phenylbutene.

aldehydes and ketones have been shown not to be rearranged products of the corresponding epoxide metabolites. The oxidation of trichloroethylene and 1,1-dichloroethylene similarly exhibited a migration of chlorine to yield, respectively, trichloroacetaldehyde and chloro- and dichloroacetic acids, none of which result from rearrangement of the epoxide metabolites (Figure 1.7.2.2) (Miller and

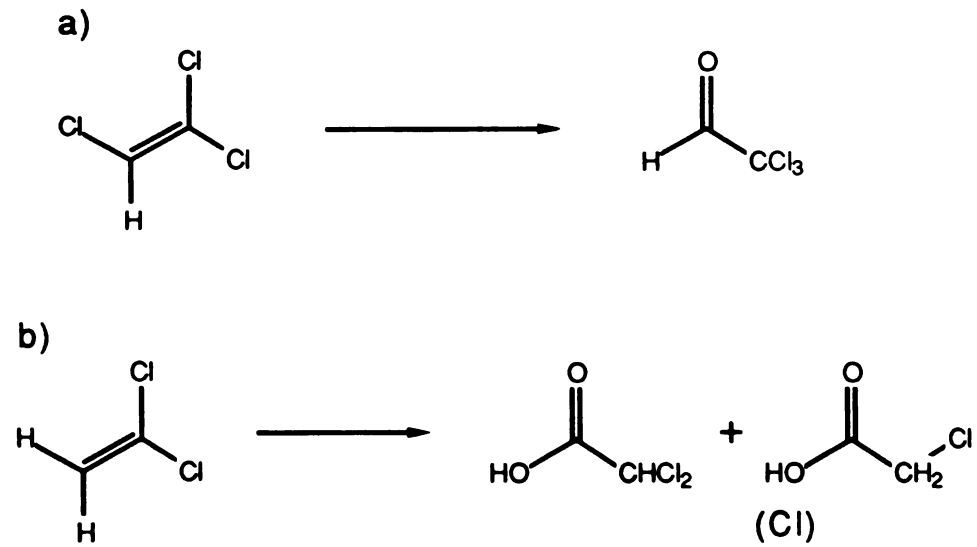

Figure 1.7.2.2 Migration of chloride during oxidation catalyzed by cytochrome P-450 of a) trichloroethylene and b) 1,1-dichloroethylene.

Guengerich, 1982, Miller and Guengerich, 1983, Liebler and Guengerich, 1983, and Henschler et al., 1979). These results indicate that the chlorine and hydrogen migrate prior to epoxide formation and thus suggest a stepwise epoxidation process.

Cytochrome P-450 oxidation of teminal olefins results in the formation of epoxides and the destruction of cytochrome P-450. Cytochrome P-450 is suicidally inactivated by terminal olefins due to alkylation of the prosthetic heme group (Ortiz de Montellano and Reich, 1986 and ref. therein) (Figure 1.7.2.3). Again, the fact that the epoxide metabolites are not responsible for heme alkylation suggests the presence of intermediates which proceed to epoxide formation or heme alkylation.

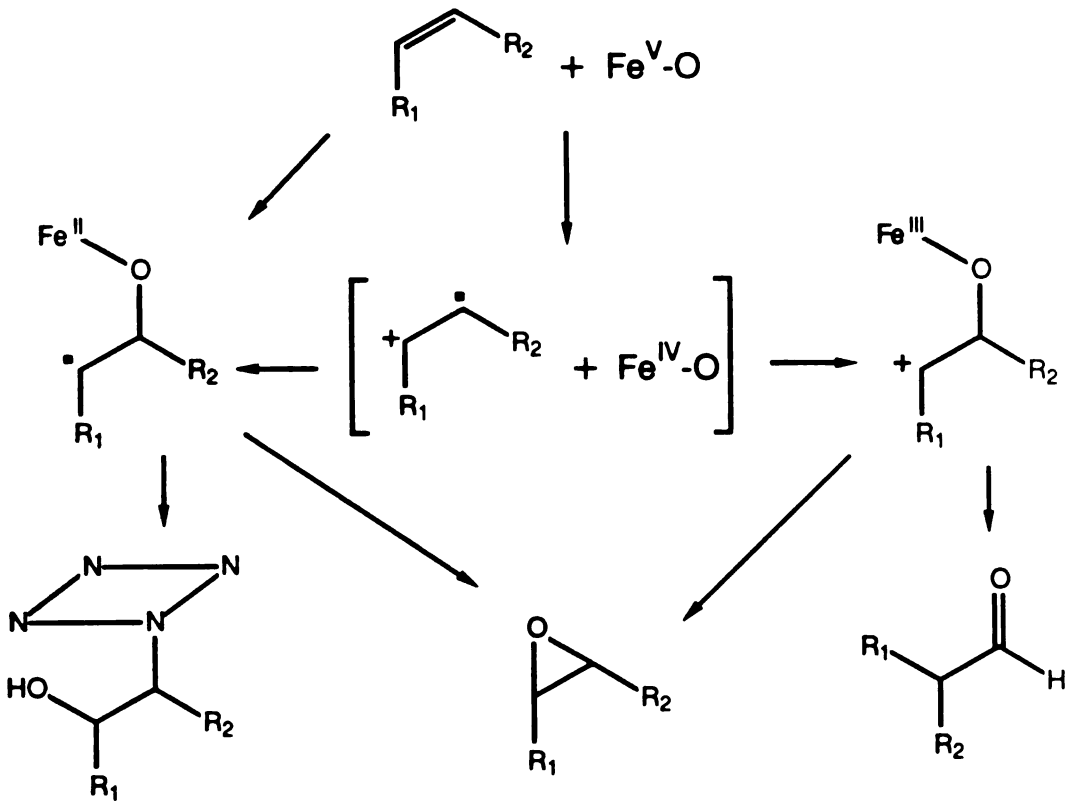

Figure 1.7.2.3 Proposed mechanisms for olefin oxidation by cytochrome P-450.

An attractive mechanism for olefin epoxidation which is consistent with the evidence is demonstrated in Figure 1.7.2.3 (Ortiz de Montellano, 1986). The iron-oxo species of cytochrome P-450 abstracts an electron from the double bond to form a radical cation and an iron-hydroxy radical (Fe^{IV}-O). Recombination of the radicals forms either an acyclic cation centered complex, which rearranges to form the carbonyl products and epoxides, or an acyclic radical centered complex, which closes to give the epoxide or is trapped by the pyrrole nitrogen of the heme to form the heme adduct. The

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1.7.3 Heteroatom

Heteroatoms a

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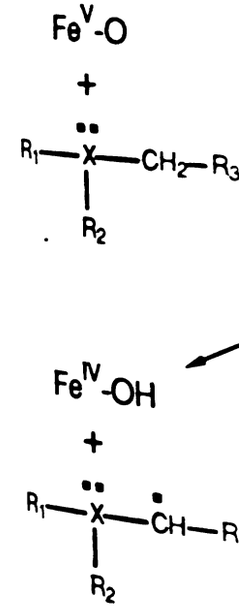

Figure 1.7.3.1 P
cytochrome P-45

oxygenation, the
heteroatom dealk
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acyclic radical centered complex maybe formed directly from the olefin and iron-oxo species.

1.7.3 Heteroatom Oxidation

Heteroatoms are oxidized by cytochrome P-450 to yield either heteroatom oxygenation or heteroatom dealkylation products by closely related processes (Figure 1.7.3.1). In heteroatom

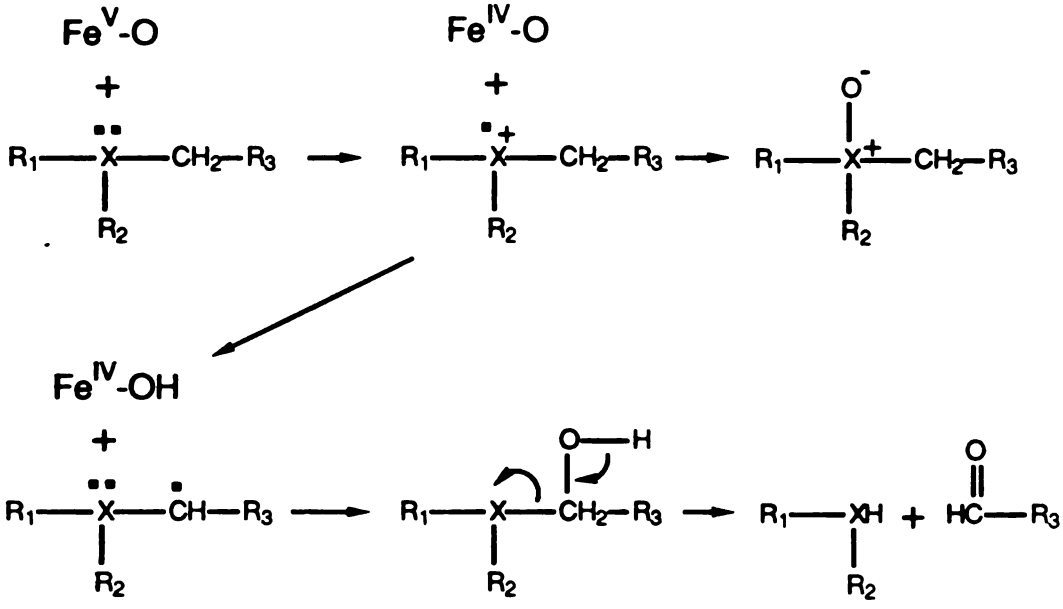


Figure 1.7.3.1 Proposed mechanism for heteroatom oxidation by cytochrome P-450.

oxygenation, the heteroatom itself is oxidized directly. In heteroatom dealkylation, the carbon adjacent to the heteroatom is ultimately hydroxylated to generate a carbinolamine (MaMahon and Sullivan, 1964 and McMahon, 1966) which falls apart to yield a

dealkylated amine and an aldehyde or ketone. The mechanisms of heteroatom oxidation are not as clear as the mechanism of carbon hydroxylation, although the radical mechanism illustrated in Figure 1.7.3.1 is generally accepted for heteroatom oxidation. The iron-oxo species of cytochrome P-450 abstracts an electron from the heteroatom to generate an iron-hydroxy radical (Fe^{IV}-O) and a heteroatom radical cation. The radicals either recombine to give heteroatom oxygenation or an α proton is removed from the heteroatom radical cation to give a carbon radical species. Recombination of the iron-hydroxy radical and carbon radical then yields the carbinolamine which falls apart to give the dealkylated products. The source of the oxygen of the carbinolamine and aldehyde has been determined by ^{18}O experiments to be the activated oxygen of cytochrome P-450 (Shea et al., 1982, Kedderis et al., 1980, and McMahon et al., 1969). Hydroxylations of heteroatom containing compounds have demonstrated a regioselectivity that favors the α over the β and γ carbons (Testa and Mihailova, 1978).

Another possible mechanism for heteroatom dealkylation is illustrated in Figure 1.7.3.2. The α carbon is directly hydroxylated by cytochrome P-450 rather than as a consequence of oxidation of the heteroatom. In the case of *O*-dealkylation this mechanism is probably accurate since *O*-oxygenation, unlike *N*- and *S*-oxygenation, is not known to be catalyzed by cytochrome P-450. The greater electronegativity of the oxygen apparently decreases the reactivity of the oxygen enough to prevent *O*-oxygenation (Ortiz de Montellano, 1986). Another piece of evidence which suggests that oxygen is not

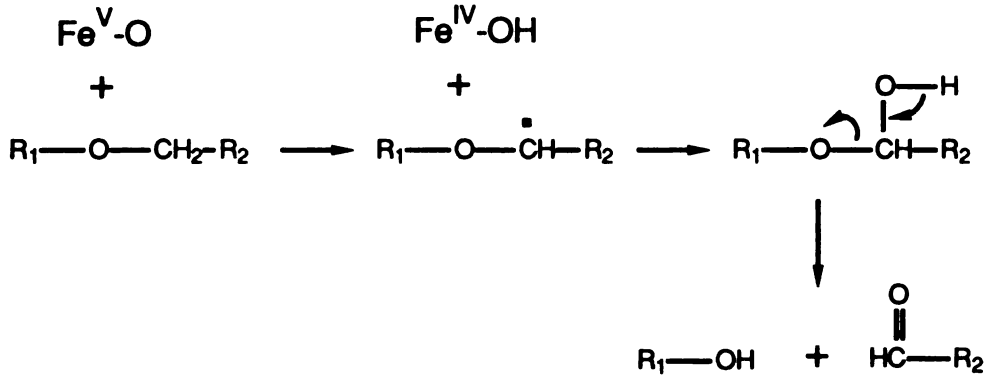


Figure 1.7.3.2 The alternate mechanism for heteroatom dealkylation in which the α-carbon is directly hydroxylated. This is the most likely mechanism for *O*-dealkylation.

directly oxidized by cytochrome P-450 is the high kinetic isotope effects (k_H/k_D = 10 - 13.5) observed for *O*-dealkylation reactions. Product analysis of the cytochrome P-450-catalyzed *O*-demethylation of *p*-trideuteriomethoxyanisole showed a ratio of 10:1 for *p*-trideuteriomethylphenol: *p*-methoxyphenol, giving k_H/k_D = 10 (Foster et al., 1974). The deuterium and tritium intrinsic kinetic isotope effects on V_m/K_m and on V_m for *O*-deethylation of 7-ethoxycoumarin yield a calculated k_H/k_D of 12.8 - 14 (Miwa et al., 1984). The decreased rate of *O*-deethylation of 7-ethoxycoumarin caused by deuterium labeling was compensated by increased formation of 7-ethoxy-6-hydroxycoumarin (metabolic switching). The rates of NADPH and O_2 consumption were not altered (Harada et al., 1984). This result supports the idea of irreversible formation of the activated oxygen. The high isotope effects, indicative of cleavage of a C-H bond in the rate determining step, are consistent with the hydrogen abstraction and radical recombination mechanism

associated with carbon hydroxylation. In contrast, the isotope effects associated with *N*-dealkylation, which are discussed in the following section, are relatively small. Recently, Hanzlik (Hall and Hanzlik, 1987 and Hall et al., 1989) have shown that the cytochrome P-450-catalyzed *N*-dealkylation of a series of amides exhibits large intramolecular kinetic isotope effects (k_H/k_D = 4 - 7). This result is consistent with the idea that highly electronegative heteroatoms favor direct hydrogen abstraction rather than electron abstraction.

As mentioned earlier, the kinetic isotope effects for *N*-dealkylation are relatively small (k_H/k_D = 1.3-3.1). Product analysis by mass spectrometry of the cytochrome P-450-catalyzed *N*-demethylation of 1-(*N*-methyl-*N*-trideuteriomethylamino)-3-phenylpropane resulted in a product ratio of 1.36 for 1-(*N*-trideuteriomethyl)-3-phenylpropane to 1-(*N*-methyl)-3-phenylpropane (Abdel-Monem 1975). Shono and colleagues (Shono et al., 1982) have measured the intramolecular isotope effects for the cytochrome P-450-catalyzed *N*-demethylations of *N*-methyl-*N*-trideuterioimipramine and *N*-methyl-*N*-trideuteriohydrocinnamide to be k_H/k_D = 1.64 and 1.75, respectively. These low product isotope effects are also observed by Miwa and colleagues (Miwa et al., 1983), who determined the product isotope effects for *N*-demethylation of *N*-methyl-*N*-trideuteriomethylaniline by cytochrome P-450b and c to be k_H/k_D = 1.78 and 1.61, respectively. The intramolecular kinetic isotope effects for *N*-demethylation catalyzed by cytochrome P-450, observed on V_{max} and V_{max}/K_m, were also small (k_H/k_D = 1.6 - 2.0) (Miwa et al., 1980). Small isotope

effects ($k_H/k_D < 3.6$) have been observed for the one-electron electrochemical (Shono et al., 1975, Smith and Mann, 1969, Portis et al., 1970, Masui and Sayo, 1971, and Lindsay-Smith and Masheder, 1976), photochemical (Dopp and Heufer, 1982 and Takami et al., 1974), and chemical (Hull et al., 1967, Lindsay-Smith and Mead, 1973, and Lewis and Ho, 1980) oxidation of amines, while high isotope effects (Wei and Stewart, 1966) have been observed for hydrogen abstraction. Hence, *N*-demethylation catalyzed by cytochrome P-450 must proceed through an aminium radical cation which deprotonates to give a nitrogen-conjugated carbon radical that recombines with the oxygen of cytochrome P-450 to yield the carbinolamine (Figure 1.7.3.1). An unresolved discrepancy exists between the cytochrome P-450-catalyzed *N*-dealkylation isotope effects and the large kinetic isotope effects ($k_H/k_D = 10$) obtained for the analogous hydroperoxide-dependent reactions catalyzed by hemoglobin and horseradish peroxidase (Miwa et al., 1983). The *N*-dealkylation reactions catalyzed by horseradish peroxidase almost certainly involve oxidation of nitrogen to the corresponding radical cation analogous to the mechanism proposed for cytochrome P-450 (Ortiz de Montellano, 1986).

Cyclopropyl amine has been a valuable probe in examining the formation of nitrogen radical cation intermediates. Hanzlik and colleagues (Hanzlik et al., 1979) have demonstrated that a series of *p*-substituted *N*-benzylcyclopropylamines are mechanism-based inhibitors of cytochrome P-450. The postulated mechanism for this inhibition is illustrated in Figure 1.7.3.3, in which the enzyme is

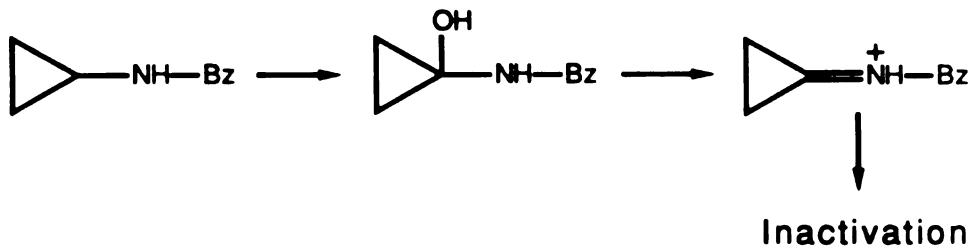


Figure 1.7.3.3 Initial mechanism proposed for cyclopropylamine inactivation of cytochrome P-450.

inactivated by the highly reactive cyclopropyliminium ion generated by elimination of H_2O from the cyclopropylcarbinolamine intermediate. The formation of iminium ions catalyzed by cytochrome P-450 oxidation of amines has precedent in the literature: e. g., oxidation of lidocaine (Nelson et al., 1973), methapyrilene (Ziegler et al., 1981), 1-benzylpyrroline (Ho and Castagnoli, 1980), phencyclidine (Ward et al., 1982), and (S)-nicotine (Murphy, 1975 and Nguyen et al., 1976).

According to the mechanism in Figure 1.7.3.3, *N*-benzyl-1-methylcyclopropylamine should not be a mechanism-based inhibitor of cytochrome P-450 since the methyl group prevents formation of the iminium ion due to unavailability of an α hydrogen. However, *N*-benzyl-1-methylcyclopropylamine has been demonstrated to be equally efficient as *N*-benzylcyclopropylamine in inactivating cytochrome P-450 (Macdonald et al., 1982 and Hanzlik and Tullman, 1982). In light of this observation, a revised mechanism, which includes an aminium radical cation intermediate, has been proposed by both Hanzlik and Tullman (1982) and Macdonald and colleagues

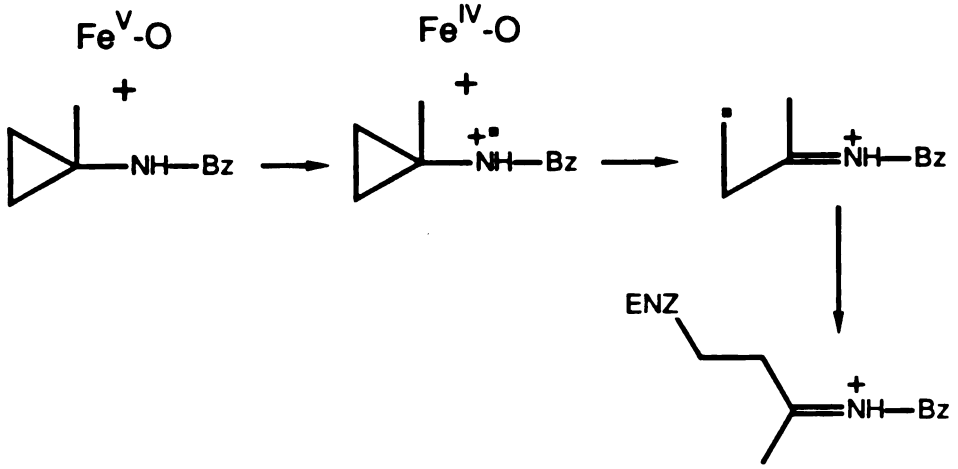


Figure 1.7.3.4 Mechanism proposed by Macdonald and Guengerich for inactivation of cytochrome P-450 by cyclopropylamine.

(Macdonald et al., 1982). Macdonald and colleagues have proposed that oxidation of cyclopropylamine to the aminium radical cation is followed by ring opening to generate the reactive group (Figure 1.7.3.4). Cyclopropyl amine radicals have been shown to rearrange very rapidly ($k > 10^8$ sec^{-1}) to the corresponding ring opened amine radicals (Maeda and Ingold, 1980). Such species, if generated in the active site, are likely to be very detrimental to the enzyme (Griller and Ingold, 1980). The idea of a ring opened terminal carbon-centered radical alkylating the protein is supported by the work of Silverman and colleagues, who have proposed a similar mechanism for the inactivation of monoamine oxidase by *N*-benzylcyclopropylamines (Silverman and Yamasaki, 1984, Silverman, 1984, and Vazquez and Silverman, 1985). Silverman and Yamasaki (1984) have shown that dialysis following the inactivation of monoamine oxidase by *N*-benzyl-1-methylcyclopropylamine results

in loss of the benzyl group while the methyl and cyclopropyl groups are retained by the inactivated protein-adduct. Loss of the benzyl group was prevented by treating the inactivated enzyme with cyanoborohydride, which suggests the presence of an imine linkage. These results are consistent with the expected enzyme-inactivator adduct from the proposed mechanism. Alkylation of the enzyme by the ring opened imine radical is also supported by release of cinnamaldehyde from *trans*-2-phenylcyclopropylamine inactivated monoamine oxidase (Silverman, 1983) (Figure 1.7.3.5). Monoamine oxidase is believed to oxidize amine to the radical cation. The release of cinnamaldehyde is consistent with the expected structure of the monoamine oxidase-inhibitor complex generated from the oxidation of *trans*-2-phenylcyclopropylamine to the cyclopropylamine radical cation - ring opening - protein alkylation mechanism (Figure 1.7.3.5).

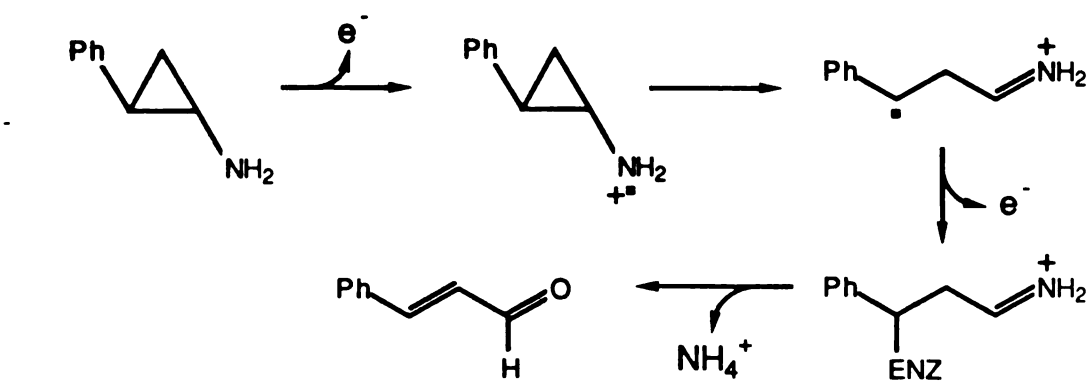


Figure 1.7.3.5 Inactivation of monoamine oxidase by *trans*-2-phenylcyclopropylamine and release of cinnamaldehyde from the inactivated enzyme.

The mechanism proposed by Hanzlik and Tullman is initially similar but differs in the species that inactivates the enzyme (Figure 1.7.3.6). The reactive species is a quaternized radical azetidinium ion which is generated by ring expansion. The difference in the mechanisms arises from the fact that Hanzlik and Tullman noticed that inactivation of cytochrome P-450 by [7-^{3}H]-*N*-benzylcyclopropylamine resulted in acid stable covalent linkage of tritium to the heme-free protein. The adduct in Figure 1.7.3.6 would

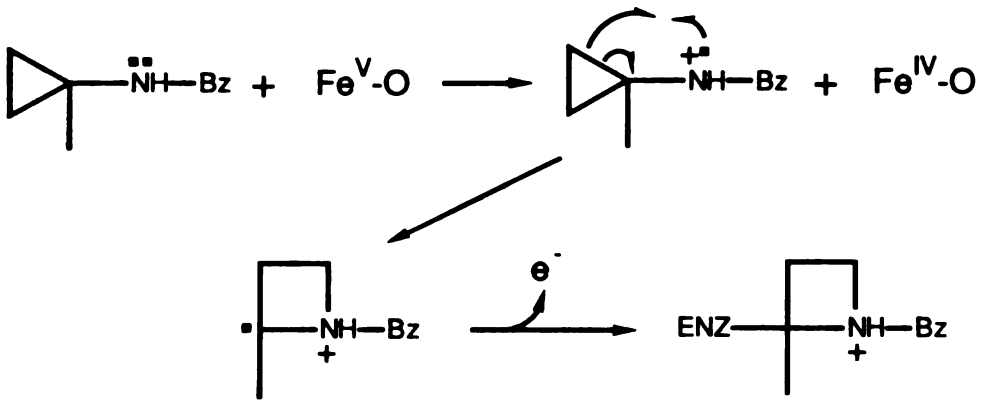


Figure 1.7.3.6 Mechanism proposed by Hanzlik for inactivation of cytochrome P-450 by cyclopropylamine.

be stable to acid treatment whereas the adduct in Figure 1.7.3.4 would not be because the iminium linkage would be hydrolyzed under acidic conditions.

The general mechanism of initial one-electron oxidation by cytochrome P-450 is bolstered by the demonstration that the rates of inactivation of cytochrome P-450 by a series of cyclopropyl- and

1-methylcyclopropyl amines, amides, ethers, alcohols, bromides, and iodides correlate with their one-electron oxidation potentials (Guengerich et al., 1984). Note that the oxidation of cyclopropyl ethers requires the formation of an oxygen radical cation intermediate. As mentioned previously, oxygenation of oxygen has not been observed, so that the oxygen radical cation species either must be very unstable or the above mentioned correlation must arise from the special properties of the cyclopropyl moiety.

The most direct evidence for a radical mechanism for the cytochrome P-450-catalyzed oxidation of heteroatoms has been provided by Ortiz de Montellano and colleagues, who used spin trapping techniques to monitor the presence of radical intermediates in the oxidation of 3,5-dicarbethoxy-2,6-dimethyl-4-ethyl-1,4-dihydropyridine (DDEP) (Augusto et al., 1982). DDEP has been shown to suicidally inactivate cytochrome P-450 with concomitant formation of both an abnormal hepatic pigment identified as *N*-ethylprotoporphyrin IX (Ortiz de Montellano et al., 1981) and ethyl radicals which could be spin trapped by α-(4-pyridyl-1-oxide) *N-tert*-butylnitrone (POBN) (Augusto et al., 1982). The POBN-ethyl adducts were purified and shown to be identical to the authentic POBN-ethyl adduct generated by chemical oxidation of ethylhydrazine with $CuCl_2$ in the presence of POBN. The proposed mechanism is illustrated in Figure 1.7.3.7. DDEP is one-electron oxidized to the radical cation that aromatizes by extruding the 4-ethyl group as a radical that either alkylates the heme, generating the hepatic pigment, or escapes the active site to be trapped by

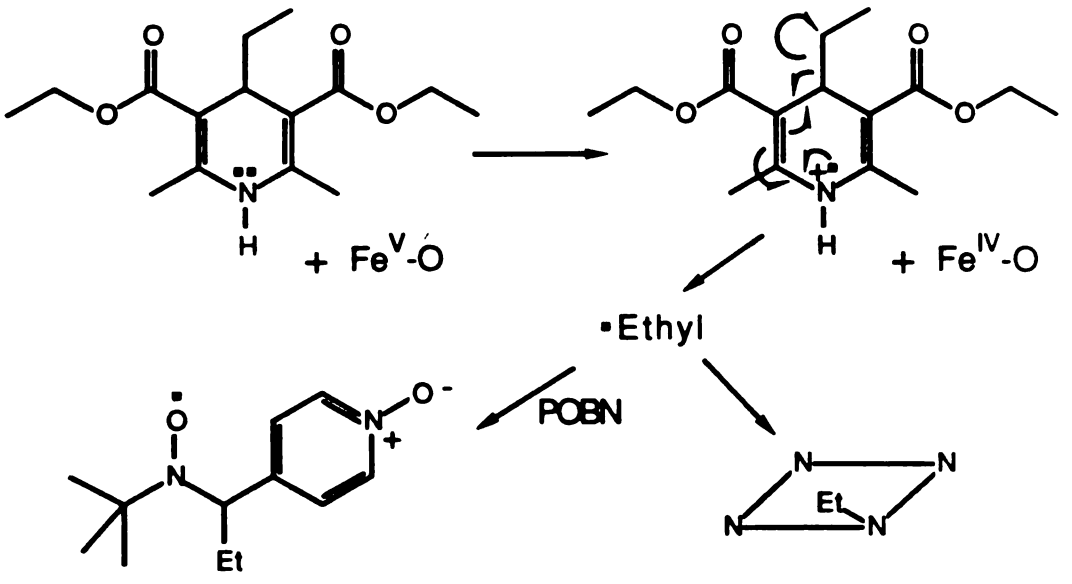


Figure 1.7.3.7 Proposed mechanism for the oxidation of DDEP by cytochrome P-450 to generate the ethyl radical.

POBN (Augusto et al., 1982).

Much of the focus on heteroatom oxidation has been on heteroatom dealkylation. Comparatively little information is available for heteroatom oxygenation. Many of the heteroatom oxygenations are catalyzed by the flavin-containing monooxygenase rather than cytochrome P-450 (Guengerich and Macdonald, 1984). Strong cases of *N*-oxygenation by cytochrome P-450 are represented by phentermine (Duncan and Cho, 1982), azoprocarbazine (Cummings et al., 1982), 2-aminofluorene (Frederick et al., 1982), and 2-(acetylamino)fluorene (Johnson et al., 1980 and Guengerich and Macdonald, 1984). Even less information is available for sulfur and phosphorous oxygenation. Examples of phosphorous oxygenation are given by 3-methylaminopropyldiphenylphosphine and

diphenylmethylphosphine, in which the phosphines are converted into the corresponding phosphine oxides (Wiley et al., 1972). The oxygenation of a series of sulfides and sulfoxides has been shown to be correlated with the one-electron oxidation potentials of the sulfides and sulfoxides (Watanabe et al., 1980 and Watanabe et al., 1982). Similar results were obtained for cytochrome P-450 and chemical (hydroxy radical) oxidation of alkyl sulfides (Watanabe et al., 1981). This suggests that sulfides are oxidized through a radical intermediate because hydroxy radical oxidations of sulfides have been shown to generate sulfinium radical cations. As for sulfur oxygenation, little is known about *S*-dealkylation. *S*-Dealkylations were observed with 6-methylthiopurine and its analogues as early as 1964 by Mazel and colleagues (Mazel et al., 1964, Henderson and Mazel, 1964a and b) but product analyses were incomplete. The acidity of the vicinal proton has been shown to correlate with the ratio of *S*-dealkylation to *S*-oxygenation (Watanabe et al., 1981), indicating that the acidity of the vicinal proton determines the extent of dealkylation. The mechanism of sulfur oxidation is believed to be similar to that for nitrogen oxidation, with the sulfenium radical cation as the intermediate (Figure 1.7.3.1).

The distinction between cytochrome P-450-catalyzed heteroatom oxygenation and dealkylation arises from the physical properties of the compound. Both processes are believed to be mediated by one-electron oxidation of the heteroatom. The heteroatom oxygenation occurs by oxygen rebound with the heteroatom radical cation and heteroatom dealkylation occurs by α

deprotonation. For nitrogen, the aminium radical species has been assumed to be extremely acidic. However, Nelsen (Nelsen and Ippoliti, 1986) has estimated a pK_a value, which was calculated from electrochemical and bond dissociation energy data, of 15 for the trialkylamine radical cation. This indicates that the deprotonation process requires the presence of a strong base. The Fe^{IV}-O species of cytochrome P-450 is probably the strong base that removes the α proton. For the enzymatic oxidation of nitrogen, α deprotonation has been shown to be faster than the oxygen rebound (Guengerich, 1984). In general, heteroatom dealkylation takes place over heteroatom oxygenation unless a) the radical is unusually stable, b) α protons are not available, or c) α protons are inaccessible for abstraction (Guengerich and Macdonald, 1984).

2.0 PROPOSAL

The results outlined in the introduction of this thesis overwhelmingly suggest a nonconcerted radical mechanism for substrate oxidation by the reactive iron-oxo species of cytochrome P-450. The intent of the work reported in this thesis was to further substantiate the involvement of radicals in the cytochrome P-450-catalyzed oxidation of nitrogenous compounds by using a series of 4-alkyl-1,4-dihydropyridines and 2,2-dialkyl-1,2-dihydroquinolines as mechanistic probes. A second purpose of the reported work was to decipher the detailed mechanism by which 4-alkyl-1,4-dihydropyridines are oxidized by cytochrome P-450.

3.0 DIHYDROPYRIDINE

3.1 Introduction

The effects of 4-alkyl-1,4-dihydropyridines on biological systems have been examined extensively in the past decade. Solomon and Figge (Solomon and Figge, 1959) first reported that oral administration of 3,5-dicarbethoxy-2,6-dimethyl-4-methyl-1,4-dihydropyridine (DDC) generates porphyria and causes red fluorescence of the gall bladder and liver under ultraviolet light. The porphyrinogenic properties of DDC have also been reported by De Matteis (De Matteis and Prior, 1962), Labbe (Onisawa and Labbe, 1963), Wada (Wada et al., 1968), Tephly (Tephly et al., 1971), Rifkind (Rifkind, 1979) and Marks (Cole et al., 1979). DDC mediated porphyria results from inhibition of ferrochelatase (Onisawa and Labbe, 1963, De Matteis et al., 1973, Tephly et al., 1979a, and Cole and Marks, 1984) and elevation of 5-aminolevulinic acid synthase (ALAS) activity (Grannick, 1966) (Figure 3.1.1). Ferrochelatase inserts the iron into protoporphyrin IX to generate heme. This is the last step in the heme biosynthetic pathway. 5-Aminolevulinic acid synthase (ALAS) is the first step in the heme biosynthetic pathway and is under feedback control by heme (Yamamoto et al., 1982). Porphyrins accumulate because they are not converted to heme by ferrochelatase and are synthesized at an increased rate due to the increase in ALAS activity.

The porphyrinogenic properties of DDC are observed *in vivo* but not *in vitro* (Onisawa and Labbe, 1963, Tephly et al., 1979b, and

al., 1981), and also treatment of rats or mice with the 4-ethyl analogue of DDC (DDEP) results in the formation of *N*-ethylprotoporphyrin IX (*N*-ethylPPIX) (Ortiz de Montellano et al., 1981b and De Matteis et al., 1981, and Augusto et al., 1982). These results are supported by the observation that the 4-desmethyl analogue of DDC lacks the inhibitory properties of ferrochelatase (Cole and Marks, 1980) and the fact that chemical oxidation of 4-alkyl-1,4-dihydropyridines results in elimination of the 4-alkyl substituents (Loev and Snader, 1965).

Administration of DDC or DDEP has been shown to result in decreased levels of cytochrome P-450 *in vivo* (Wada et al., 1968 and Waterfield et al., 1968) and *in vitro* (Ortiz de Montellano et al., 1981b, Augusto et al., 1982, and Coffman et al., 1982). Further examination has demonstrated that the dihydropyridines are mechanism-based inhibitors of cytochrome P-450 for which the decrease in cytochrome P-450 level occurs concomitantly with the formation of *N*-alkylprotoporphyrin IX (*N*-alkylPPIX) (Ortiz de Montellano et al., 1981 and Augusto et al., 1982). The mechanism of *N*-alkylprotoporphyrin IX formation is discussed in Section 3.2.

For each *N*-alkylPPIX, there exist four regioisomers, each alkylated at a different pyrrole nitrogen (Figure 3.1.2). The four *N*-methylPPIX isomers have been synthesized, purified, and identified (Kunze and Ortiz de Montellano, 1981) and shown to be equally potent as inhibitors of ferrochelatase (Ortiz de Montellano et al, 1980). However, only the N_A- and N_B-ethylPPIX isomers (alkylation at the pyrrole nitrogens of rings A and B, respectively) exhibited activities

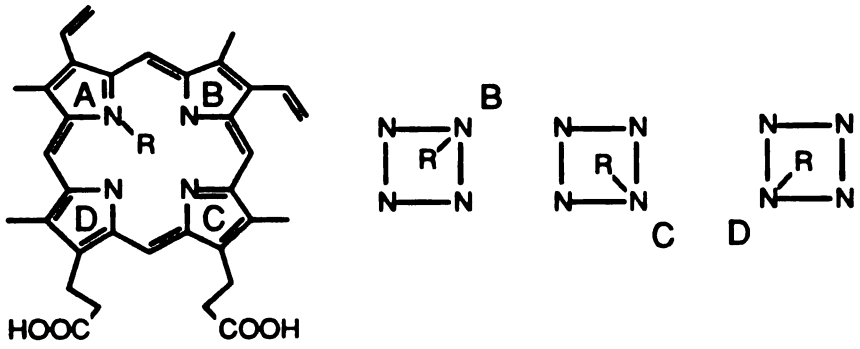
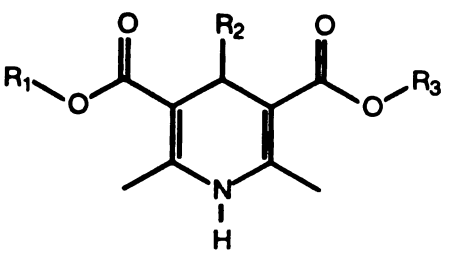


Figure 3.1.2 The four regioisomers of *N*-alkylprotoporphyrin IX.

comparable to those of the *N*-methylPPIX isomers. The N_C- and N_D-ethylPPIX isomers were 30-100 fold less potent as ferrochelatase inhibitors (Ortiz de Montellano et al., 1981). The 4-*n*-propyl- (De Matteis et al., 1985) 4-*n*-butyl-, 4-*n*-pentyl-, and 4-*n*-hexylprotoporphyrin IX isomers (McCluskey et al., 1988) gave results similar to those obtained with the *N*-ethylPPIX isomers. These results indicate that the location and size of the alkyl group is very important in determining the ferrochelatase inhibitory activity. From the relative activities of the *N*-alkylPPIX ferrochelatase inhibitors, the ferrochelatase active site has been proposed to tightly accommodate ring C and D of the porphyrin and to be positively charged to interact with the propionic side chains of the porphyrin (Ortiz de Montellano et al., 1985). The proposed active site accommodates methyls on the N_C- and N_D-pyrrole nitrogens but not larger alkyl groups. Since the N_A- and N_B-alkylPPIX with alkyl groups larger than methyl still strongly inhibit ferrochelatase the active site region covering the N_A- and N_B-pyrrole nitrogens is believed to be spacious or on the edge of the heme binding site.

Whereas the 4-alkyl-1,4-dihydropyridines cause porphyria, the 4-aryl-1,4-dihydropyridines (Figure 3.1.3) have been shown to possess strong vasodilating and negative inotropic properties (Vater et al., 1972, Loev et al., 1974, Rodenkirchen et al., 1979, and Dargie et al., 1981). The dihydropyridines prevent the entrance of calcium into the cell by blocking a subset of the calcium channels (Fleckenstein, 1977, Janis and Triggle, 1983, and Lee and Tsien, 1983). The process that regulates the contraction of vascular smooth muscle is mediated by calcium ions, so that calcium ions play a vital role in cardiac contractility (Kretsinger, 1976 and Braunwald, 1982). Figure 3.1.4 illustrates the function of Ca^{+2} in vascular smooth muscle. The intracellular concentration of Ca^{+2} is very low ($\leq 10^{-7}$ M) but increases up to 10^{-5} M upon contraction (Kretsinger, 1976). When the Ca^{+2} concentration in the cell reaches approximately 10^{-6} M, then Ca^{+2} binds to an intracellular Ca^{+2} receptor called calmodulin (Janis and Triggle, 1983). The calmodulin-Ca^{+2} complex activates myosin kinase which in turn activates myosin by phosphorylating it (Epstein, 1982). The phosphorylated myosin interacts with actin to bring about contraction. The entrance of Ca^{+2} into the cell is regulated by three mechanisms: nonspecific leakage (Baker and Glitsch, 1975) and potential (PDC) and receptor (RDC) dependent channels (Bolton, 1979 and Meisheri et al., 1981). The PDC and RDC are activated by membrane-depolarization and specific agonist-receptor interactions, respectively. It is not known whether PDC and RDC are two distinct channels or a single channel in which the agonist-receptor association triggers the potential difference (Janis and



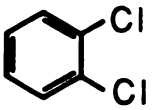
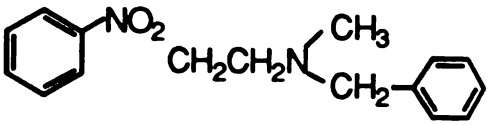
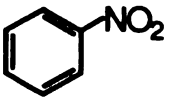
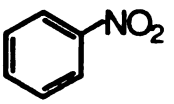
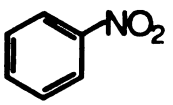
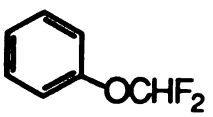
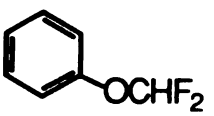
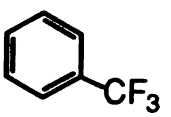
	R_1	R_2	R_3
felodipine	CH_3		CH_2CH_3
nicardipine	CH_3		
nifedipine	CH_3		CH_3
nilvadipine	CH_3		$CH(CH_3)_2$
nimodipine	$(CH_3)_2CH$		$CH_2CH_2OCH_3$
nitrendipine	CH_3		CH_2CH_3
ryodipine	CH_2CH_3		CH_2CH_3
SKF 24260	CH_2CH_3		CH_2CH_3

Figure 3.1.3 Structures of the dihydropyridine calcium channel antagonists.

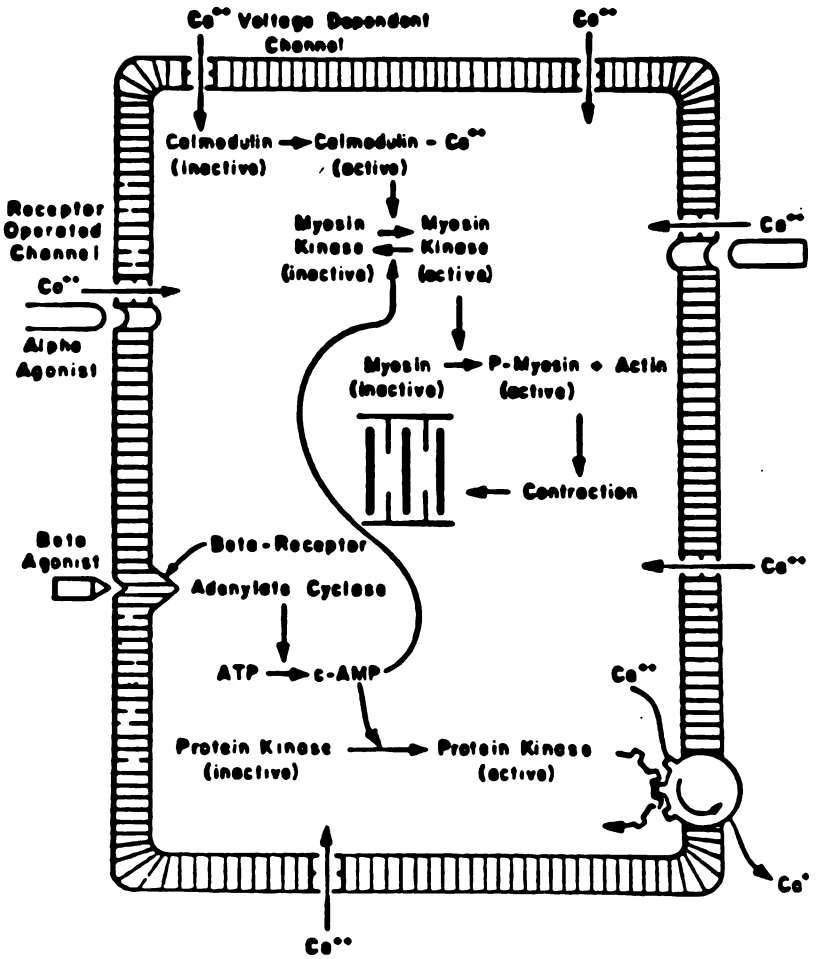
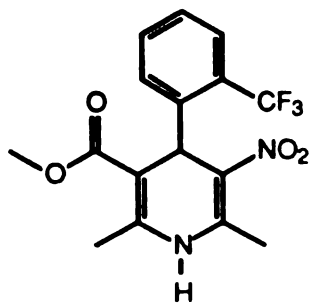
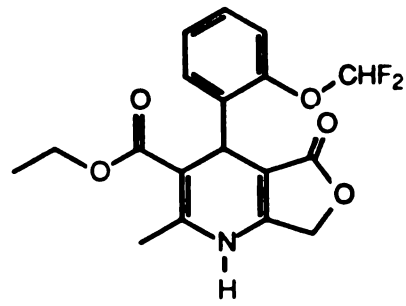


Figure 3.1.4 Diagram of the role of Ca^{++} in vascular smooth muscle (Braunwald, 1982).

Triggle, 1983). The dihydropyridines exert calcium channel blockage through specific dihydropyridine membrane-bound receptors (Bellemann et al., 1981, Gould et al., 1982, Ehlert et al., 1982, Glossmann et al., 1982, Bolger et al., 1983, and Bellemann et al., 1983) and they do not bind to other known receptors (Janis and



Bay K 8644



CGP 28 392

Figure 3.1.5 Structures of dihydropyridine calcium channel agonists.

Triggle, 1983 and Bolger et al., 1983). Minor alterations in the side chain of the dihydropyridine antagonists results in dihydropyridine calcium channel agonists (Figure 3.1.5) (Schramm et al., 1983a, Schramm et al., 1985b, and Su et al., 1984).

The dihydropyridine calcium channel blockers, for the most part, are very well absorbed following an oral dose and are extensively metabolized and excreted by a first pass effect (Higuchi et al., 1977, Weidolf et al., 1984, Parinov et al., 1985, Inoue et al., 1985, Rush et al., 1986, Hoffmann and Andersson, 1987, Terakawa et al., 1987, and Tokuma et al., 1988). The metabolism of the various dihydropyridines has been shown to be very similar by numerous groups: nifedipine (Medenwald et al., 1972 and Duhm et al., 1972), nitrendipine (Meyer et al., 1983a and Kann et al., 1984), nicardipine (Higuchi et al., 1977, Higuchi and Shiobara, 1980, and Rush et al., 1984), ryodipine (Parinov et al., 1985, Inoue et al., 1985, and Midgley et al., 1985), nimodipine (Meyer et al., 1983b), felodipine (Weidolf et al., 1984, Baarnhielm et al., 1984, Baarnhielm et al., 1986, Hoffmann

and Andersson, 1987), SKF 24260 (Walkenstein et al., 1973 and Parker and Weinstock, 1973), and nilvadipine (Niwa et al., 1988a, Niwa et al., 1988b, Terashita et al., 1987). The general metabolic scheme, illustrated in Figure 3.1.6, involves: 1) oxidation of the dihydropyridine to the corresponding pyridine which is still not hydrophilic enough to be excreted, 2) hydrolysis of one of the ester groups to generate the carboxylic acid, and 3) hydroxylation of the methyl group neighboring the carboxylic acid moiety to yield an acid-alcohol that closes to form a lactone. The lactone can also be formed by hydroxylation of the methyl group prior to ester hydrolysis (Figure 3.1.6). Removal of the ester side chain has also been shown to occur by *O*-dealkylation catalyzed by cytochrome P-450 (Guengerich, 1987 and Guengerich et al., 1988). The carboxylic acid and acid-alcohol (lactone) metabolites are found in the urine. The metabolism of the dihydropyridines is complete since the parent dihydropyridine drugs are not detected in the urine. The oxidation of dihydropyridines occurs very rapidly (Baarnhielm et al., 1984) and renders the drugs inactive. The dihydropyridine ring system is essential for calcium channel agonist or antagonist activity (Triggle, 1982). The aromatization of the dihydropyridines has been reported to be mediated by cytochrome P-450 (Baarnhielm et al., 1984 and Guengerich et al., 1988). The exact metabolic pathway for each of the dihydropyridines varies slightly from the general metabolic pathway depending on the structure of the dihydropyridine. For nicardipine, two parallel routes of metabolism take place: one following the general dihydropyridine pathway and the other following the non-oxidized dihydropyridine pathway

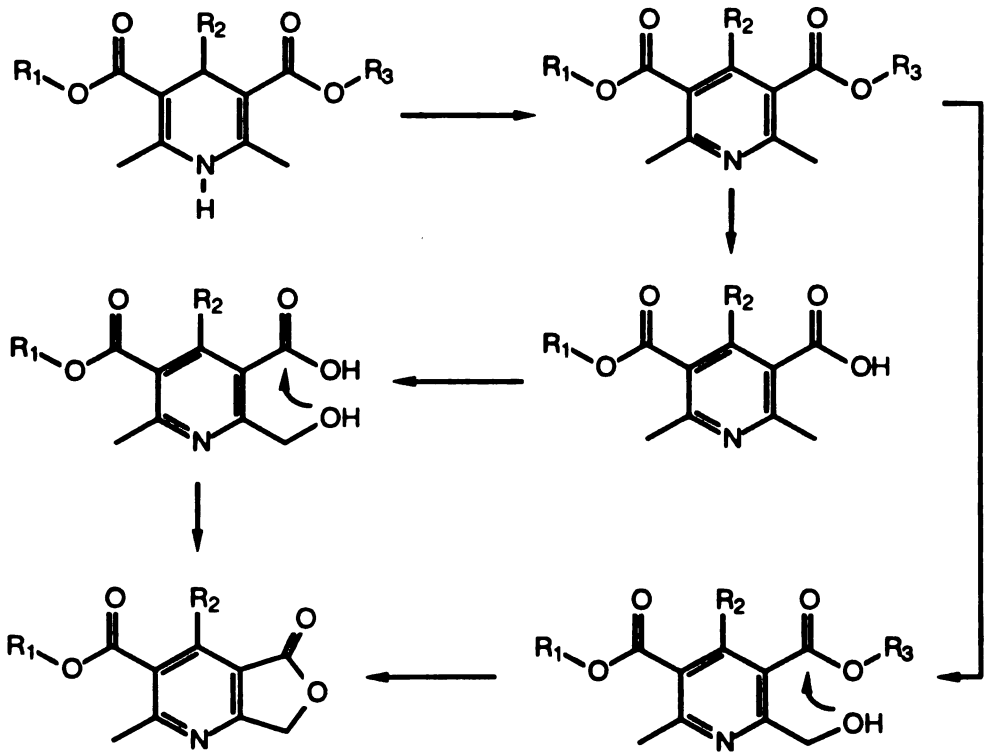
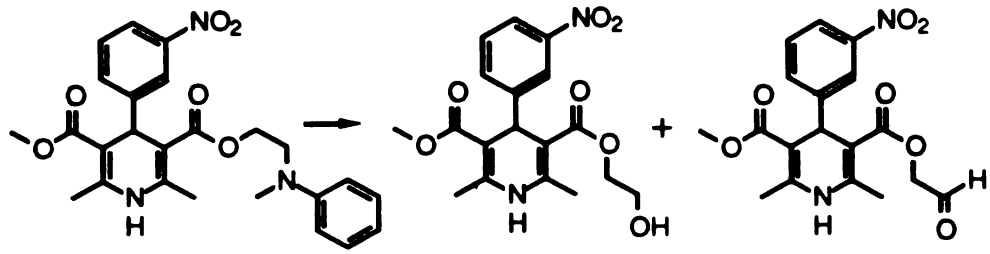


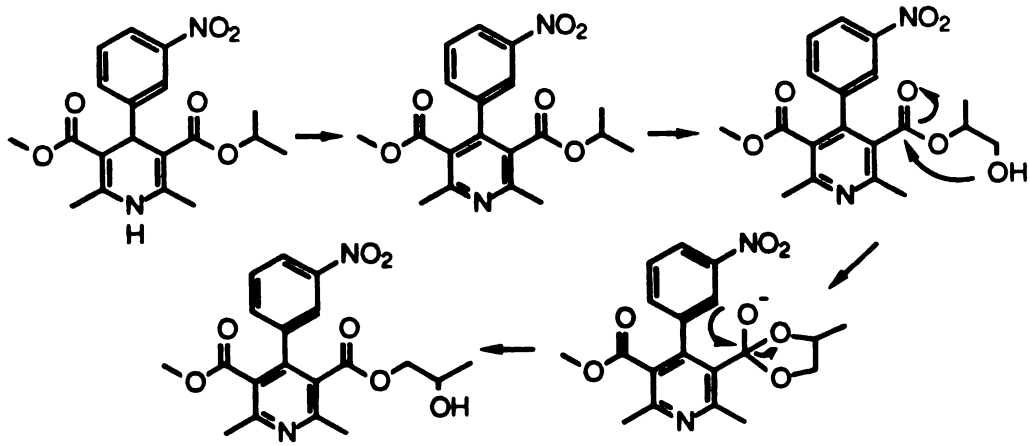
Figure 3.1.6 General pathway for the metabolism of the dihydropyridine calcium channel antagonists.

illustrated in Figure 3.1.7a. Nicardipine, instead of being dehydrogenated, can undergo an *N*-dealkylation to generate the ester-alcohol metabolite which is detected in the urine (Rush et al., 1986). Hydroxylation of the ester side chain of nilvadipine (Terashita et al., 1987) (Figure 3.1.7b) and *O*-demethylation of the ester-ether side chain of nimodipine have been observed (Meyer et al., 1983b) (Figure 3.1.7c). For ryodipine, generation of a 4-phenyl group lactone by displacement of the difluoromethoxy moiety has been observed (Figure 3.1.7d). In general, however, the metabolism of dihydropyridine calcium blockers is, for the most part, similar.

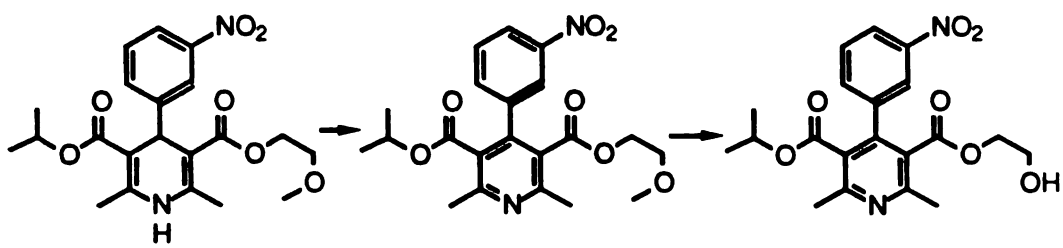
a) nicardipine



b) nilvadipine



c) nimodipine



d) ryodipine

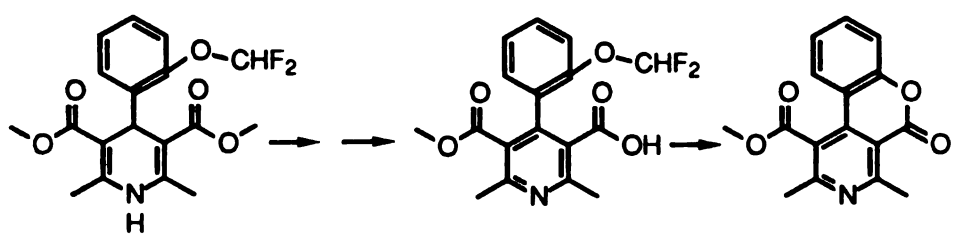


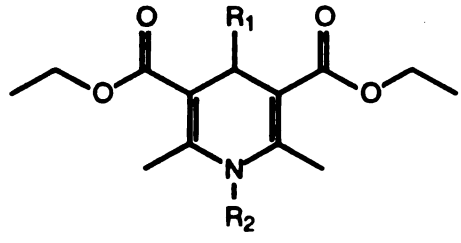
Figure 3.1.7 Novel metabolism of specific dihydropyridine calcium channel antagonists.

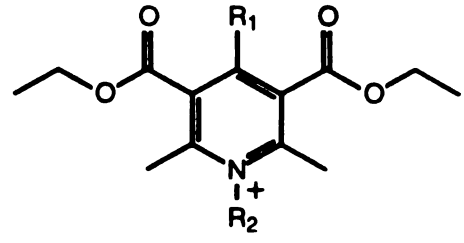
3.2 3,5-Dicarbethoxy-2,6-dimethyl-4-alkyl-1,4-dihydropyridines

4-Alkyl-1,4-dihydropyridines have been shown to be mechanism-based inhibitors of cytochrome P-450 (Ortiz de Montellano et al., 1981b and Augusto et al., 1982). The enzyme loss (destruction of the cytochrome P-450 chromophore) is coupled to the formation of *N*-alkylPPIX, which has been purified and characterized by absorption, NMR, and mass spectroscopy (Ortiz de Montellano et al., 1981b). The source of the alkyl group has been demonstrated to be the 4-substituent of the dihydropyridine. A radical mechanism for transfer of the 4-alkyl group from the dihydropyridine to one of the pyrrole nitrogens of the heme has been proposed by Ortiz de Montellano and colleagues (Augusto et al., 1982) (Figure 1.7.3.7), who observed the formation of spin trappable alkyl radicals. The iron-oxo species of cytochrome P-450 is believed to abstract one-electron from a molecular orbital of DDEP that includes the lone pair of electrons on the nitrogen to generate a radical cation intermediate that aromatizes by ejecting the 4-ethyl moiety as a free radical. The ethyl radicals either alkylate a pyrrole nitrogen of the heme or leave the active site and are trapped by α-(pyridyl-1-oxide) *N-tert*-butylnitrone (POBN).

Destruction of the Cytochrome P-450 Chromophore A series of 4-alkyl analogues (Table 3.2.1) of DDEP was examined for its ability to destroy the cytochrome P-450 chromophore, which was quantitated by CO-reduced (sample) vs. CO (reference) difference spectroscopy (Estabrook et al., 1972). At the desired time points, an aliquot was removed from the incubation mixture to measure the

Table 3.2.1 The structures and synthetic yields of dihydropyridines, pyridines, and pyridiniums. The pyridiniums where R_2 = H are referred to as pyridines (deprotonated form).





Dihydropyridine	R_1	R_2	Yield (%)
DDC	CH_3	H	- -
DDEP	CH_2CH_3	H	55
DDIP	$CH(CH_3)_2$	H	24
N-MethylDDEP	CH_2CH_3	CH_3	53
N-MethylDDIP	$CH(CH_3)_2$	CH_3	55
N-EthylDDEP	CH_2CH_3	CH_2CH_3	50
N-EthylDDIP	$CH(CH_3)_2$	CH_2CH_3	29
N-PhenylDDEP	CH_2CH_3	C_6H_6	1.2
N-PhenylDDIP	$CH(CH_3)_2$	C_6H_6	1.0

Pyridinium	R_1	R_2	Yield (%)
4-Dealkylpyridine	H	H	89
4-Methylpyridine	CH_3	H	81
4-Ethylpyridine	CH_2CH_3	H	62
4-Isopropylpyridine	$CH(CH_3)_2$	H	- -
N-Methyl-4-dealkyl-pyridinium	H	CH_3	62
N-Methyl-4-ethyl-pyridinium	CH_2CH_3	CH_3	42
N-Ethyl-4-dealkyl-pyridinium	H	CH_2CH_3	38
N-Ethyl-4-ethyl-pyridinium	CH_2CH_3	CH_2CH_3	25
N-Phenyl-4-dealkyl-pyridinium	H	C_6H_6	57
N-Phenyl-4-ethyl-pyridinium	CH_2CH_3	C_6H_6	60

cytochrome P-450 chromophore. The dihydropyridines were synthesized as described by Loev (Loev et al., 1974). The graph in Figure 3.2.1 displays the cytochrome P-450 chromophore destruction by three dihydropyridines: the 4-methyl (DDC), 4-ethyl (DDEP), and 4-isopropyl (DDIP) analogues. As reported previously (Augusto et al., 1982), incubation of DDC with hepatic microsomes and NADPH did not result in significant loss of the cytochrome P-450 chromophore. DDIP produced greater destruction of the cytochrome P-450 chromophore than DDEP. This suggests that the ability of the dihydropyridines to destroy the cytochrome P-450 chromophore

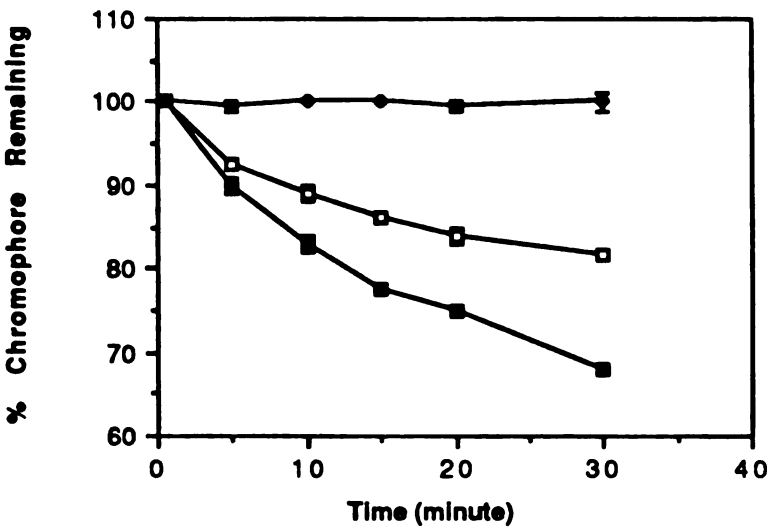


Figure 3.2.1 Destruction of the microsomal cytochrome P-450 chromophore by DDC (diamond), DDEP (open square), and DDIP (close square). The microsomes are from rats pretreated with phenobarbital. The error bars represent the maximum deviation. For the points without error bars, the errors are not greater than the % covered by the point markers.

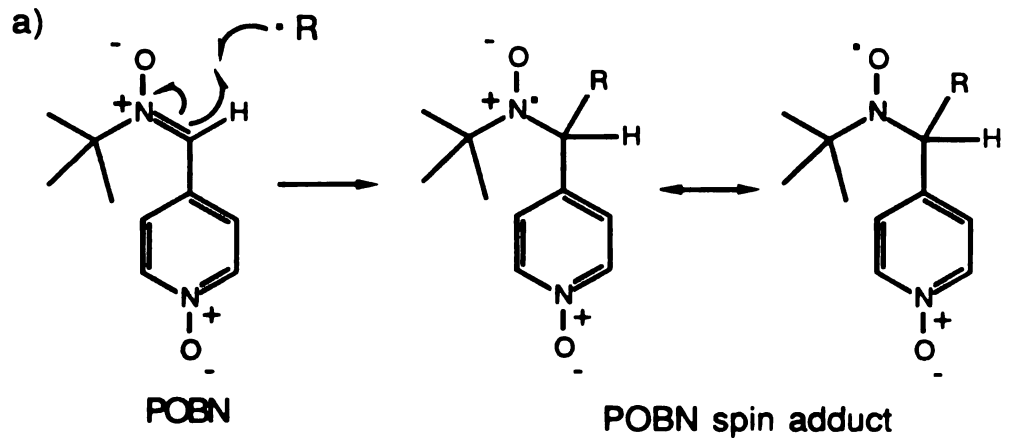
depends on the relative stability of the 4-alkyl substituents. This is supported by the finding that the 4-phenyl analogue, which would have to eliminate the relatively unstable phenyl radical, did not destroy the cytochrome P-450 chromophore (Table 3.2.2). In contrast, the 4-benzyl analogue, which eliminates the relatively stable benzyl radical, produced a great amount of cytochrome P-450 chromophore destruction. The dihydropyridines with primary alkyl groups (*n*-propyl and *n*-butyl) at the 4-position, like DDEP, destroyed less cytochrome P-450 chromophore than did DDIP or the 4-benzyl analogue (Table 3.2.2). The cytochrome P-450 chromophore destruction by the dihydropyridines was turnover dependent, as indicated by NADPH- and time-dependence. The control incubations

Table 3.2.2 Percentage of destruction of microsomal cytochrome P-450 after 30 minutes of incubation with 4-alkyl-1,4-dihydropyridines.

Dihydropyridine 4-substitutient	% Cytochrome P-450 chromophore loss
-NADPH (control)	0
-substrate (control)	0
methyl (DDC)	0
phenyl	0
ethyl (DDEP)	20 ± 0
n-propyl	27 ± 1
n-butyl	25 ± 1
isopropyl (DDIP)	33 ± 1
benzyl	43 ± 0

lacking either the substrate or NADPH did not result in loss of the cytochrome P-450 chromophore. The relative stability of the radicals expected from elimination of the 4-alkyl group can be roughly correlated with the ability of these dihydropyridines to destroy the cytochrome P-450 chromophore (Augusto et al., 1982). The desmethyl DDC analogue, which does not have an alkyl group at the 4-position, also failed to destroy the cytochrome P-450 chromophore. This result supports the proposal that loss of the cytochrome P-450 chromophore is due to alkylation of the heme moiety. Since desmethyl DDC possesses only hydrogen moieties on the 4-position, it is not expected to destroy the chromophore because transfer of the hydrogen from the 4-position to the pyrrole nitrogen will not irreversibly alter the heme moiety.

Formation of Spin Adducts The 4-ethyl group of DDEP has previously been shown to be ejected and trapped by POBN during the oxidation of DDEP by cytochrome P-450 (Augusto et al., 1982). Spin trapping agents convert unstable radicals into the relatively more stable nitroxide radicals, as illustrated in Figure 3.2.2a (Iwamura and Inamoto, 1967, Iwamura and Inamoto, 1970, and Janzen, 1971). The stabilities of the POBN-adduct nitroxide radicals depend on the nature of the trapped radical. For alkyl carbon radicals the spin adducts are stable enough to be purified and characterized. The EPR spectra of POBN-adducts typically have six lines resulting from splitting by the nitrogen (triplet, α_N = 15.7 G) and α-proton (doublet, α_H = 2.7 G) (Figure 3.2.2b).



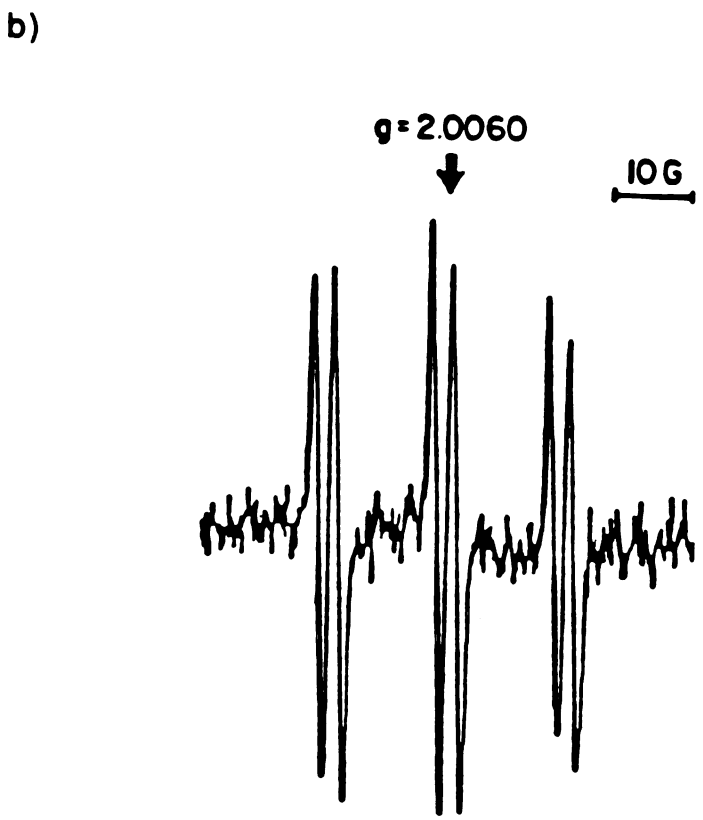


Figure 3.2.2 a) Reaction of POBN with radicals to generate relatively more stable radicals (spin adducts). b) Typical EPR spectrum of POBN spin adduct (triplet, α_N = 15.7 G and doublet, α_H = 2.7 G).

The spin trap studies were extended to the same series of dihydropyridines as the cytochrome P-450 chromophore destruction assays. The dihydropyridines were incubated with cytochrome P-450 in the presence of POBN and NADPH. Aliquots were removed from the incubation mixture at the desired time points and the EPR spectra were taken. The dihydropyridines (DDC, the 4-phenyl analogue, and 4-desmethyl DDC) which did not destroy the cytochrome P-450 chromophore also did not generate EPR signals. The strongest EPR signal was observed with DDIP followed by DDEP and then the 4-*n*-propyl analogue (Figure 3.2.3). The EPR signal

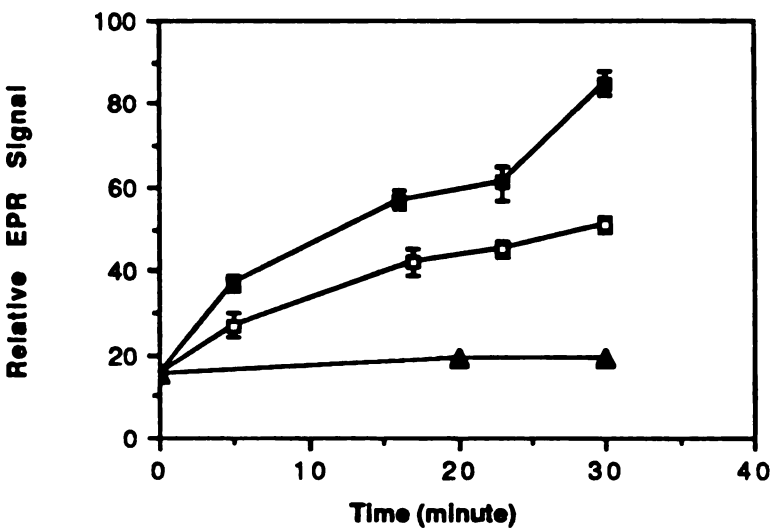


Figure 3.2.3 Relative EPR signals generated by microsomal cytochrome P-450 incubations with DDIP (close square), DDEP (close square), and the 4-*n*-propyl analogue (triangle) in the presence of POBN and NADPH. The microsomes are made from rats pretreated with phenobarbital. The error bars represent the maximum deviation. For the points with error bars, the error is not greater than the area covered by the point markers.

produced by the 4-*n*-propyl analogue was barely above the noise level. Control experiments using the inhibitors of cytochrome P-450, SKF525A and carbon monoxide (CO), proved that generation of the radicals is cytochrome P-450 mediated. Incubation of DDEP and DDIP with hepatic microsomes in the presence of SKF525A suppressed the EPR signal generated by DDEP completely and decreased by 40 % the EPR signal generated by DDIP. Similar results were obtained when the incubations were conducted under an atmosphere of 20 % O_2 and 80 % CO. CO binds to the reduced form of cytochrome P-450 and thus inhibits the enzyme. Furthermore, the generation of the EPR signals, like the chromophore destruction, is turnover-dependent since it is NADPH- and time-dependent. Surprisingly, the 4-benzyl- and 4-*n*-butyl analogues did not generate detectable EPR signals. It is possible that the benzyl and *n*-butyl radicals are either generated more slowly or quenched before completely escaping from the active site.

Metabolite Profile Three dihydropyridines: DDC, DDEP, and DDIP, representing the least and greatest generation of the EPR signal and destruction of the cytochrome P-450 chromophore, were selected to conduct metabolite studies. The dihydropyridines were incubated with microsomal cytochrome P-450 and NADPH and the metabolites were extracted into CH_2Cl_2. The samples were analyzed using a silica high pressure liquid chromatography column and 25 % THF in hexane as the mobile phase. The metabolite profiles of the three dihydropyridines are demonstrated in Figure 3.2.4. The large peaks at approximately 8.5 min are the parent dihydropyridines. The

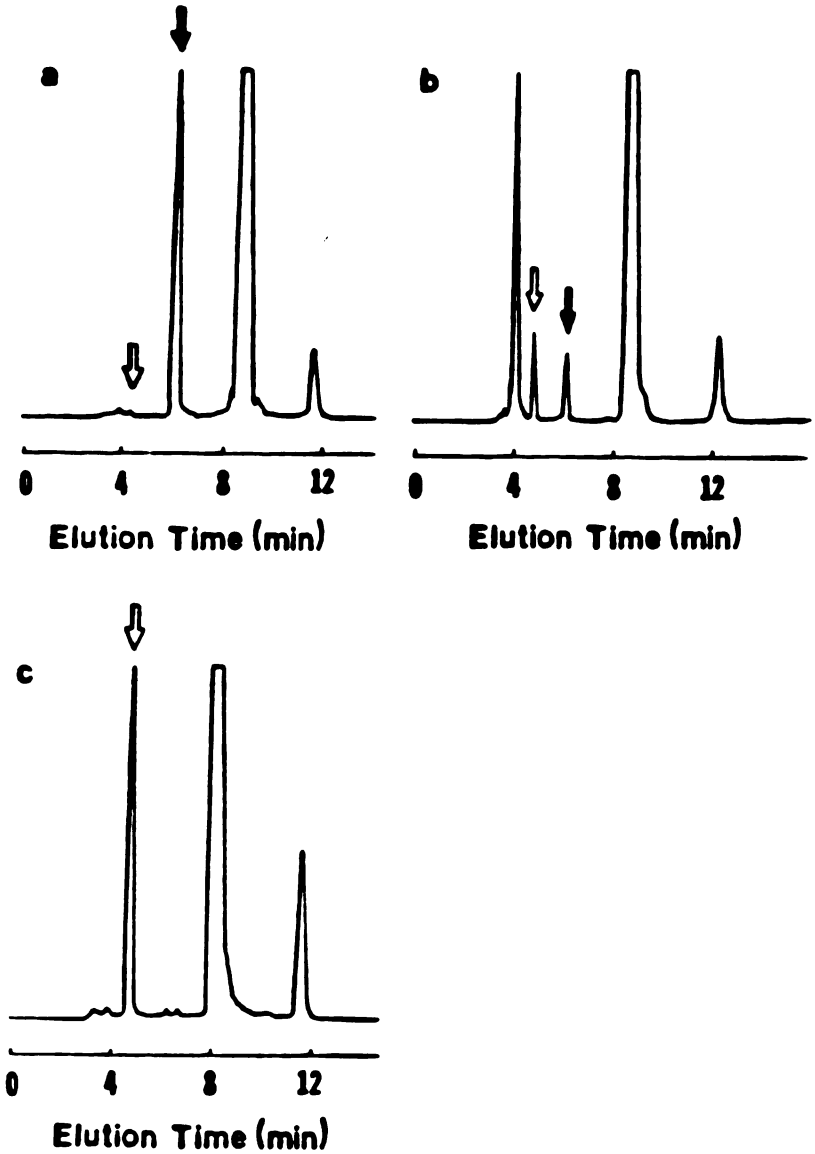


Figure 3.2.4 HPLC profiles of the pyridine metabolites from microsomal cytochrome P-450 incubations of (a) DDC, (b) DDEP, and (c) DDIP. The open arrows indicate the 4-dealkylpyridines and the closed arrows the 4-alkylpyridines. The pyridines were separated on a Whatman silica column using 25 % THF in hexane as the mobile phase.

peaks indicated by the opened and closed arrows are the corresponding pyridines which have lost and retained, respectively,

the 4-alkyl group (Figure 3.2.5). The metabolites were identified by their co-elution with synthetic authentic standards. The pyridine standards were synthesized by oxidizing the corresponding dihydropyridines with $NaNO_2$. The oxidation of DDC and DDEP with $NaNO_2$ produced in 3,5-dicarbethoxy-2,4,6-trimethylpyridine and 3,5-dicarbethoxy-2,6-dimethyl-4-ethylpyridine, respectively. However, oxidation of DDIP with $NaNO_2$ yielded 3,5-dicarbethoxy-2,6-dimethylpyridine and the pyridine with the retention of the 4-isopropyl group was not produced. Thus, the metabolite peak for the 3,5-dicarbethoxy-2,6-dimethyl-4-isopropylpyridine was assigned on the basis of its absorption spectrum. DDC and DDEP were not metabolized in the absence of NADPH, but DDIP did aromatize, albeit at a reduced rate, even without NADPH. Table 3.2.3 lists the metabolites and their yield.

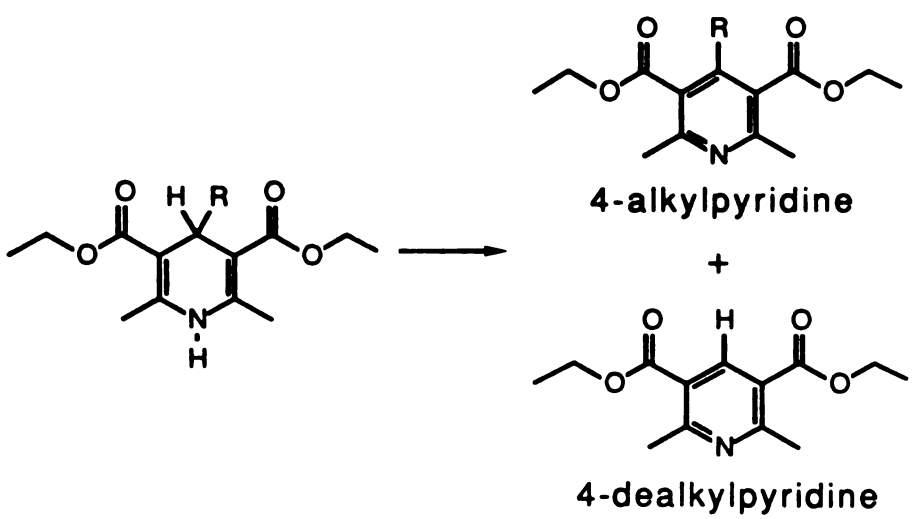


Figure 3.2.5 Oxidation of dihydropyridines to the 4-alkyl- and 4-dealkylpyridines by cytochrome P-450.

Table 3.2.3 Pyridine metabolite formation from the microsomal cytochrome P-450 oxidation of DDC, DDEP, and DDIP. The 4-alkyl- and 4-dealkylpyridine metabolites are 3,5-dicarbethoxy-2,6-dimethyl-4-alkylpyridine and 3,5-dicarbethoxy-2,6-dimethylpyridine, respectively.

Dihydropyridines	Pyridine Metabolites	
	4-alkyl	4-dealkyl
	(nmole/nmole cytochrome P-450/minute)	
DDC	5.25 ± 0.55 (99 %)	(1 %) [a]
DDEP	0.36 ± 0.01 (43 %)	0.48 ± 0.01 (57 %)
DDIP	(6 %) [b]	1.25 ± 0.31 (94 %)

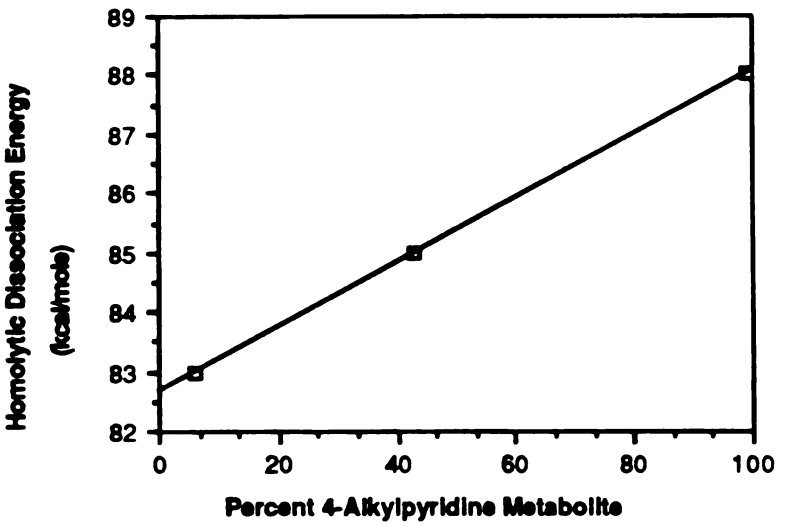
[a] Only the percentage of the 4-demethylpyridine metabolite is reported because accurate values of the yield can not be determined due to meager production of the 4-demethylpyridine metabolite from the oxidation of DDC. [b] Only the percentage of the 4-dealkylpyridine metabolite is reported because accurate values of the yield can not be determined due to meager production of the 4-dealkylpyridine metabolite from the oxidation of DDIP.

Cytochrome P-450 metabolizes dihydropyridines to the corresponding pyridines, as seen with the dihydropyridine calcium channel antagonists. DDEP is oxidized to both the 4-deethyl- and 4-ethylpyridines in almost equal amounts. The presence of the 4-dealkylated pyridine metabolite supports the proposed mechanism for the oxidation of DDEP by cytochrome P-450 (Figure 1.7.3.7). DDC is oxidized predominantly to the pyridine with the 4-methyl group intact, whereas DDIP is oxidized predominantly to the dealkylated pyridine. It is obvious that the nature of the 4-alkyl group

determines whether the 4-hydrogen or 4-alkyl group is lost by cytochrome P-450-catalyzed oxidation of the dihydropyridines. Retention of the 4-methyl group of DDC is consistent with the absence of cytochrome P-450 chromophore destruction and EPR signal generation. Loss of the 4-isopropyl moiety of DDIP upon cytochrome P-450-catalyzed oxidation is consistent both with the observation of chromophore destruction and EPR signal generation and with the fact that oxidation of DDIP by $NaNO_2$ yields only the 4-dealkylated product.

The percent of 4-alkylated pyridines, using the sum of 4-alkylated + 4-dealkylated pyridines as 100 %, is directly proportional to the homolytic dissociation bond energies (Kerr, 1966) of the methyl, ethyl, and isopropyl groups (Figure 3.2.6a). This supports the inference that the 4-alkyl moiety is being extruded as a free radical. The plot indicates that the lower the energy required to break the carbon-carbon bond, the greater the production of the 4-dealkylated pyridine metabolite. Unfortunately, the percent of the 4-alkylated pyridine metabolites is also directly proportional to the heterolytic dissociation bond energies (Egger and Cocks, 1973) of the 4-alkyl groups (Figure 3.2.6b). Since the homolytic and heterolytic dissociation bond energies are correlated with each other the distinction between the two processes cannot be made on the basis of these relationships, although the homolytic process is favored. Similar correlations are seen with the percent of the 4-dealkylated pyridine and the amount of cytochrome P-450

a)



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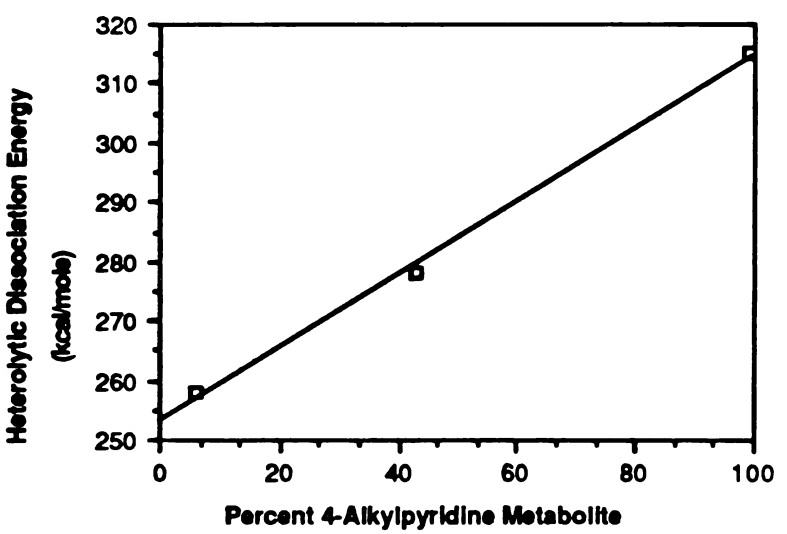
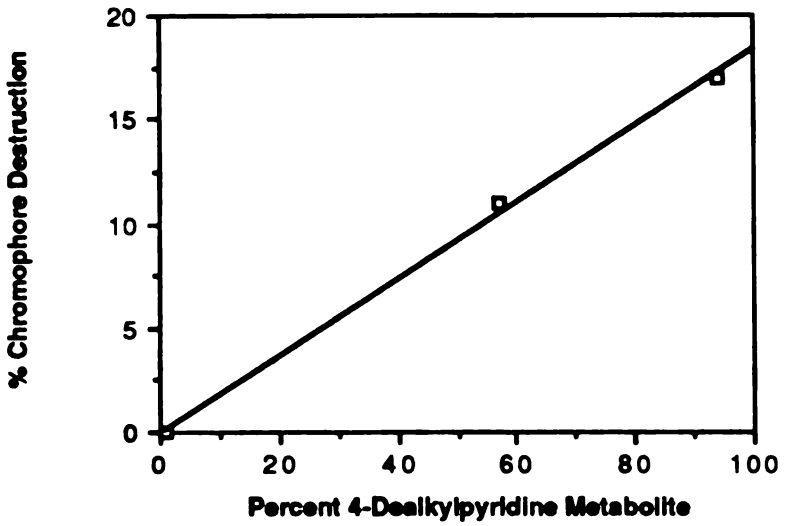


Figure 3.2.6 a) Plot of homolytic and b) heterolytic dissociation energies of the methyl, ethyl, and isopropyl moieties vs. the % 4-alkylpyridine metabolites from DDC, DDEP, and DDIP. The pyridine metabolite values are % of total pyridine metabolites.

a)



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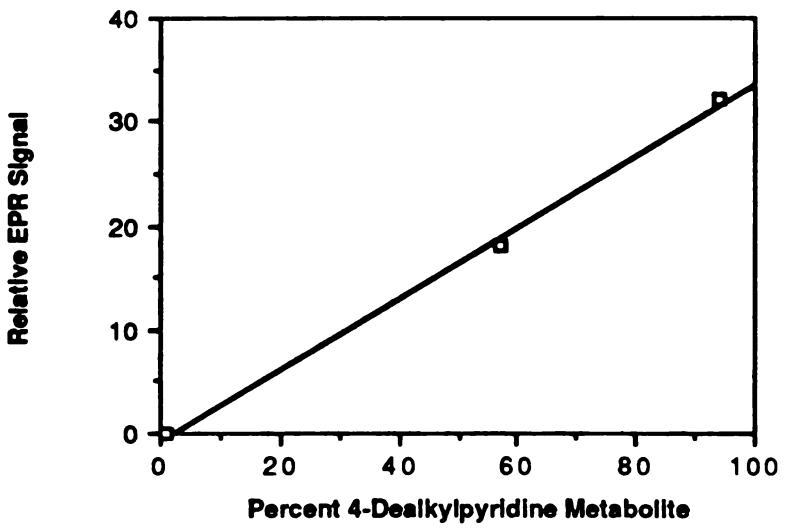


Figure 3.2.7 Plot of (a) the cytochrome P-450 chromophore destruction and (b) relative EPR signal after 10 min of microsomal incubation with DDC, DDEP, and DDIP vs. the percent 4-dealkylpyridine metabolite from the oxidation of DDC, DDEP, and DDIP by cytochrome P-450.

chromophore destruction (Figure 3.2.7a) and EPR signal generation (Figure 3.2.7b).

The excellent correlations of the ratio of the 4-dealkylated to 4-alkylated pyridine metabolites with the stabilities of the methyl, ethyl, and isopropyl groups, destruction of the cytochrome P-450 chromophore, and EPR signal generation provide strong support for the proposed radical cation-alkyl radical extrusion mechanism for the oxidation of dihydropyridines by cytochrome P-450 (Figure 1.7.3.7). The reason for the inconsistency of the 4-benzyl and 4-*n*-butyl analogues in generating EPR signals is not evident.

Relationship Between the Dihydropyridine Oxidation Potentials and Loss of the 4-Alkyl Group The relevancy of the oxidation potentials of the dihydropyridines was examined in light of the fact that the stabilities of the 4-alkyl groups as radicals correlate with the extent to which they are eliminated from the dihydropyridine ring system. The oxidation potentials of the dihydropyridines were measured by cyclic voltammetry in acetonitrile using a saturated calomel reference electrode and a freshly polished platinum working electrode. Table 3.2.4 lists the oxidation potentials of the dihydropyridines. All of the oxidation potentials were measured under identical conditions to insure their internal consistency. The absolute values of the oxidation potentials differ slightly from those reported in the literature (Stradyn' et al., 1972 and Skala et al., 1977) but they are internally consistent. A typical cyclic voltammogram for a dihydropyridine (DDC) is shown in Figure 3.2.8. The cyclic voltammogram exhibits oxidation of the dihydropyridine

Table 3.2.4 Oxidation potentials of the dihydropyridines.

Dihydropyridine	$E_{1/2}$	Ep
DDC [a]	1.06 ± 0.01	1.16 ± 0.02
DDEP [a]	1.02 ± 0.02	1.11 ± 0.01
DDIP [a]	0.97 ± 0.02	1.09 ± 0.02
DDC [b]	0.92	1.00
DDC [c]	0.90	
DDEP [c]	0.92	

[a] Experimentally determined. [b] From Stradyn' et al., 1972. [c] From Skala et al., 1977.

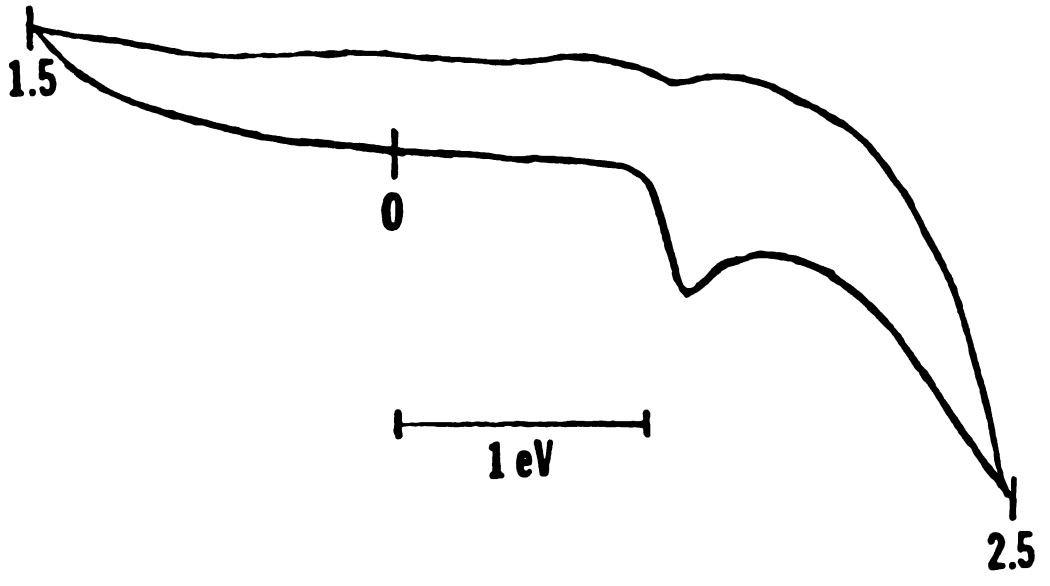


Figure 3.2.8 Cyclic voltammogram of DDC. The experimental conditions are in the experimental section (Section 5.0).

but not reduction, indicating that the oxidation is not reversible. This is expected since the oxidized pyridine is not likely to be reduced to the dihydropyridine and loss of the alkyl group is irreversible.

The oxidation potentials of the dihydropyridines increase as the radical stabilities of the 4-alkyl groups decrease (Figure 3.2.9). This correlation suggests that the oxidation potentials of the dihydropyridines are influenced by the 4-alkyl moiety. The oxidation potentials for DDC, DDEP, and DDIP show an excellent inverse correlation with the degree of 4-alkyl group loss during the cytochrome P-450-catalyzed oxidation of the dihydropyridines (Figure 3.2.10). The 4-alkyl groups thus influence both the oxidation

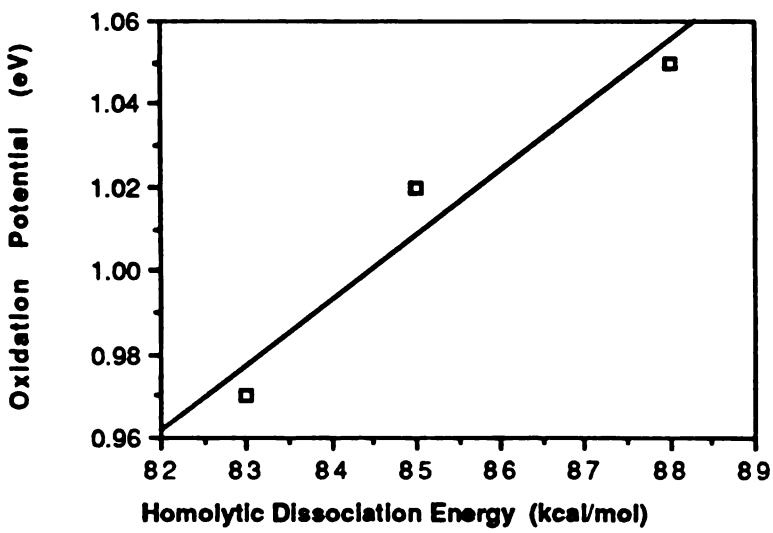


Figure 3.2.9 Plot of the oxidation potentials of DDC, DDEP, and DDIP vs. the homolytic dissociation energies of the methyl, ethyl, and isopropyl moieties. The oxidation potentials were measured by cyclic voltammetry.

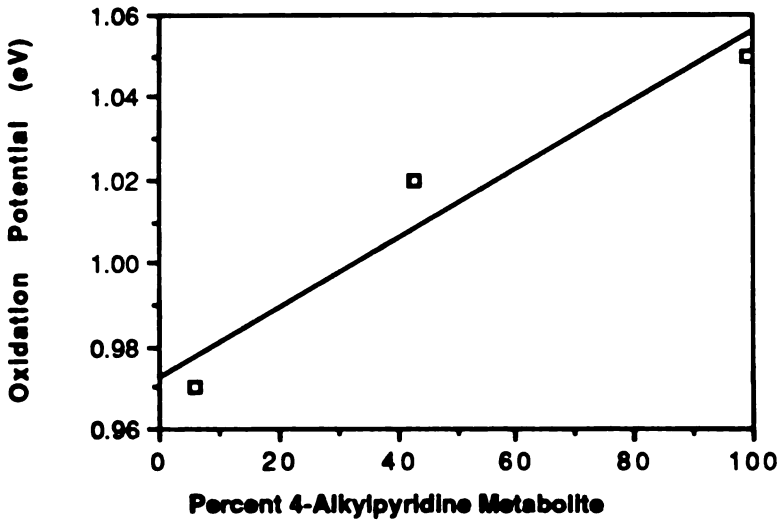


Figure 3.2.10 Plot of the oxidation potentials of DDC, DDEP, and DDIP vs. the % 4-alkylpyridine metabolites. The values for the pyridine metabolites are % of total metabolites.

potentials and the product ratios. One possible explanation for these observations is that the carbon-carbon bond between C-4 of the dihydropyridine ring and 4-alkyl group is broken in concert with initial abstraction of the electron from the nitrogen by the iron-oxo moiety of cytochrome P-450. However, this mechanism is eliminated by the results from the oxidation of *N*-alkylDDEP discussed in Section 3.3.0. Another possible explanation is that the three-dimensional structure of the dihydropyridines may be altered by the 4-alkyl moiety, resulting in an indirect relationship between the oxidation potentials and the stabilities of the 4-alkyl groups as radicals. As the size of the 4-alkyl group increases, the steric interactions between the 4-alkyl group and the adjacent carbethoxy side chains may cause the dihydropyridine ring to take on more of a

boat conformation and may twist the carbethoxy side chains to a greater extent out of the plane of the dihydropyridine ring. Both of these conformational changes should result in lowering of the oxidation potential. The boat conformation of the dihydropyridine ring and twisted carbethoxy side chains should decrease the conjugation of the lone pair of nitrogen electrons with the carbon-carbon double bonds and carbonyl moieties of the ester side chains and thus decrease the oxidation potential. Precedents for these ideas are found in the literature. Crystallographic structures (Fossheim et al., 1982, Triggle et al., 1980, and Langs and Triggle, 1985) and theoretical calculations of the conformational energies (Mahmoudian and Richards, 1986) of dihydropyridine calcium channel antagonists have shown that the dihydropyridine rings exist in a boat conformation and that the degree of ring puckering is influenced by the 4-substituent. The crystallographic studies have also shown that the 4-substituent influences the angle between the carbonyl moieties of the ester side chains and the dihydropyridine ring (Fossheim et al., 1982). The proposal that the 4-substituents influence the availibility of the nitrogen lone pair of electrons is partly supported by the shift of the λ_{max} in the electronic absorption spectra of the dihydropyridines (Table 3.2.5).

Isotope Effects on the 4-Position Cytochrome P-450 oxidizes dihydropyridines to the corresponding 4-alkyl- and 4-dealkylpyridines. The formation of the 4-dealkylpyridine metabolites is proposed to proceed through cytochrome P-450-catalyzed oxidation of the dihydropyridine to the corresponding

Table 3.2.5 λ_{max} of the dihydropyridines.

Dihydropyridines 4-substituent	λ_{max} nm
methyl	351
ethyl	350
isopropyl	344
n-propyl	348
n-butyl	349
phenyl	356
benzyl	353

nitrogen radical cation that aromatizes by ejecting the 4-alkyl moiety. However, the mechanism for generation of the 4-alkylpyridine metabolites has not been fully elucidated even though it is obvious that it arises by loss of the 4-hydrogen moiety. The mechanism of the loss of the 4-hydrogen is discussed here. Since the 4-alkyl group is eliminated as a radical, the 4-hydrogen might also be extruded as a hydrogen radical, although this is unlikely because the hydrogen radical is extremely unstable.

The mechanism for the loss of 4-hydrogen was examined by deuterium isotope effects at the 4-position. A deuterium isotope effect on the formation of 4-alkyl- and 4-dealkylpyridines should arise if loss of the 4-alkyl moiety competes directly with loss of the 4-hydrogen. Since deuterium substitution makes breakage of the

carbon-deuterium bond more difficult, it should enhance elimination of the 4-alkyl group. For this purpose, [4-^{2}H]-DDC and [4-^{2}H]-DDEP were synthesized by Dr. Neil E. Jacobsen. For the synthesis of the two compounds, [1-^{2}H]-acetaldehyde and [1-^{2}H]-propionaldehyde were used as the starting materials along with ethyl acetoacetate and ammonium carbonate. The [1-^{2}H]-acetaldehyde was obtained from Aldrich Chemical Co. (St. Louis) and [1-^{2}H]-propionaldehyde was synthesized. The synthetic scheme for [4-^{2}H]-DDEP is illustrated in Figure 3.2.11. [1-^{2}H]-Propionaldehyde

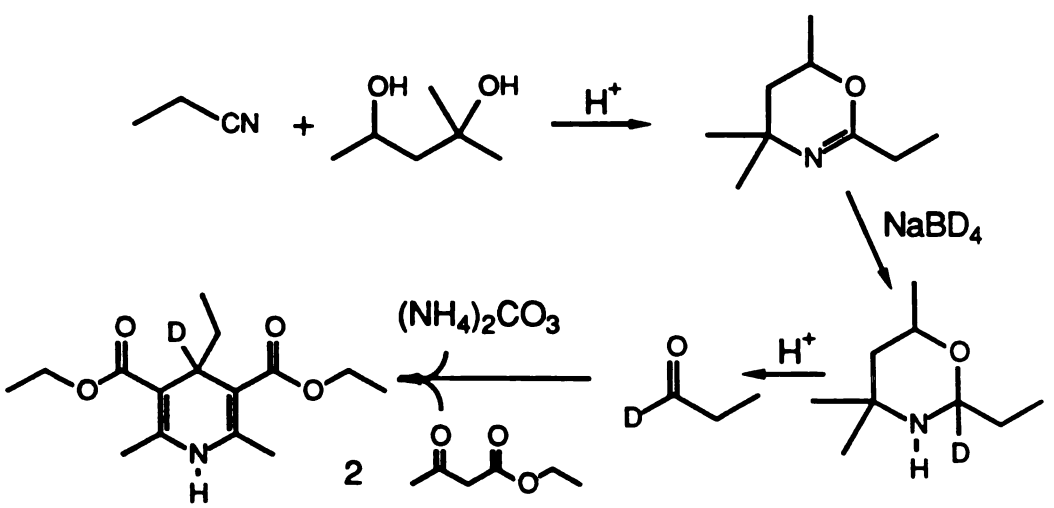


Figure 3.2.11 Synthetic scheme for [4-^{2}H]-DDEP.

was obtained by acidic hydrolysis of [2-^{2}H]-2-ethyl-4,4,6-trimethyltetrahydro-1,3-oxazine in H_2O (Meyers et al., 1967). The [1-^{2}H]-propionaldehyde was not isolated prior to its use. The ethyl acetoacetate and ammonium carbonate were added directly to the reaction mixture to synthesize [4-^{2}H]-DDEP. [2-^{2}H]-2-Ethyl-4,4,6-

trimethyltetrahydro-1,3-oxazine was synthesized by NaB^2H_4 reduction of 2-ethyl-4,4,6-trimethyldihydro-1,3-oxazine which was prepared by cyclizing 2,4-dihydroxy-2-methylpentane with propionitrile (Tillman and Ritter, 1957).

The effects of deuterium substitution at C-4 of DDC and DDEP were studied by three techniques: cytochrome P-450 chromophore destruction, EPR spin trapping, and metabolite profile. As mentioned previously, [4-^{1}H]-DDC did not significantly destroy the cytochrome P-450 chromophore in the presence of NADPH. The same result was obtained in incubations of cytochrome P-450 with [4-^{2}H]-DDC. The deuterium substitution at the 4-position did not have any effect on

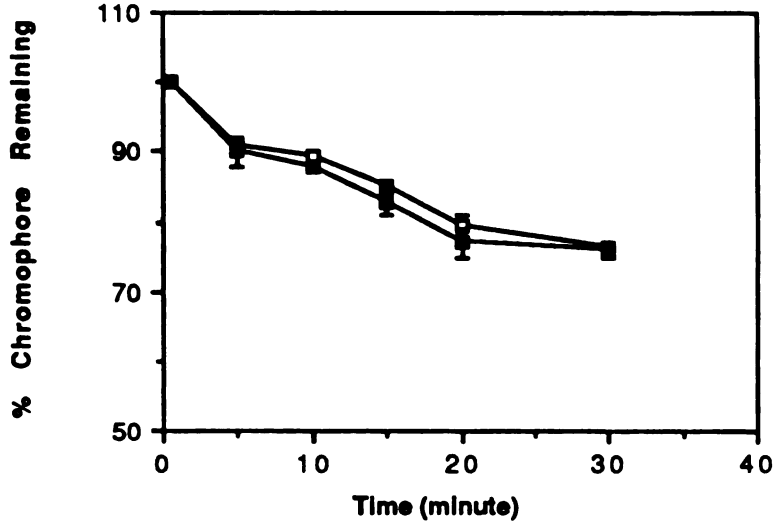


Figure 3.2.12 Destruction of the microsomal cytochrome P-450 chromophore by [4-^{1}H]-DDEP (open squares) and [4-^{2}H]-DDEP (closed squares). The microsomes were made from rats pretreated with phenobarbital. The error bars represent the maximum deviation. For the points without error bars, the errors are not greater than the % covered by the point markers.

the ability of DDC to destroy the cytochrome P-450 chromophore. Likewise, deuterium substitution at the 4-position of DDEP did not alter the rate of cytochrome P-450 chromophore loss (Figure 3.2.12). Both the [4-^{1}H]- and [4-^{2}H]-DDEP exhibited the same time dependence and extent of cytochrome P-450 chromophore destruction.

The EPR spin trapping studies with [4-^{1}H]- and [4-^{2}H]-DDC and [4-^{1}H]- and [4-^{2}H]-DDEP yield the same conclusions as the chromophore destruction studies. Incubation of [4-^{1}H]- and [4-^{2}H]-DDEP with cytochrome P-450 in the presence of NADPH and POBN resulted in the same level of radical production (Figure 3.2.13). As

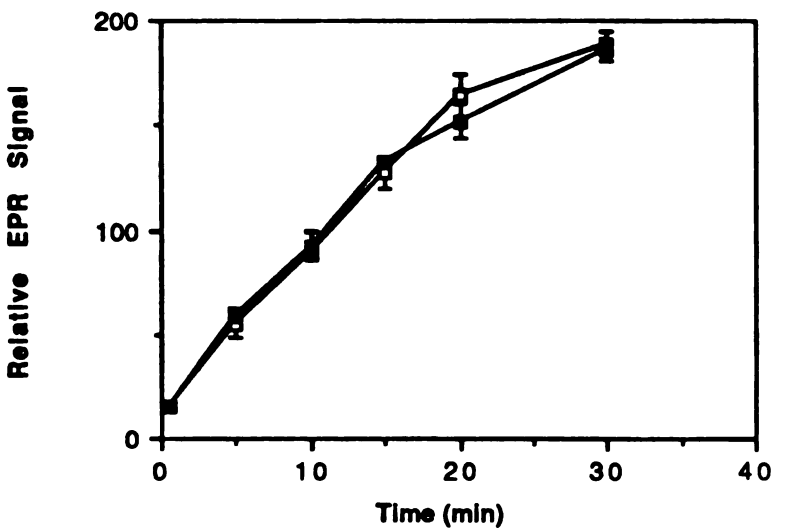


Figure 3.2.13 Relative generation of EPR signals in microsomal cytochrome P-450 incubations with [4-^{1}H]-DDEP (open squares) and [4-^{2}H]-DDEP (closed squares) in the presence of POBN and NADPH. The microsomes were made from rats pretreated with phenobarbital. The error bars represent the maximum deviation. For the points without error bars, the errors are not greater than the % covered by the point markers.

mentioned before, DDC did not generate an EPR signal when incubated with cytochrome P-450, NADPH, and POBN. [4-^{2}H]-DDC was used to possibly increase the elimination of the methyl radical but no EPR signal was detected. Thus, deuterium substitution at the 4-position of DDC and DDEP did not significantly alter the ability of DDC and DDEP to generate spin-trappable radicals.

The metabolite profiles for [4-^{1}H]- and [4-^{2}H]-DDC and [4-^{1}H]- and [4-^{2}H]-DDEP were determined as mentioned before. The deuterium isotope effects on the ratios of the 4-alkylated and 4-dealkylated pyridine metabolites generated by cytochrome P-450

Table 3.2.6 Pyridine metabolite formation from the microsomal cytochrome P-450-catalyzed oxidation of 4-deuterated and 4-undeuterated DDC and DDEP. The 4-alkyl- and 4-dealkylpyridine metabolites are 3,5-dicarbethoxy-2,6-dimethyl-4-alkylpyridine and 3,5-dicarbethoxy-2,6-dimethylpyridine, respectively.

Dihydropyridine	Pyridine Metabolites	
	4-alkyl	4-dealkyl
	(nmole/nmole cytochrome P-450/minute)	
[4-^{1}H]-DDC	5.25 ± 0.55 (99 %)	(1 %) [a]
[4-^{2}H]-DDC	4.25 ± 0.28 (99 %)	(1 %) [a]
[4-^{1}H]-DDEP	0.36 ± 0.01 (43 %)	0.48 ± 0.01 (57 %)
[4-^{2}H]-DDEP	0.35 ± 0.01 (41 %)	0.50 ± 0.01 (59 %)

[a] Only the percentage of the 4-demethylpyridine metabolite is reported because accurate values of the yield can not be determined due to meager production of the 4-demethylpyridine metabolite from DDC.

were determined for DDC and DDEP. Table 3.2.6 lists the metabolite yields for deuterated and undeuterated DDC and DDEP. Deuterium substitution at the 4-position of DDEP did not significantly alter the ratio of the 4-alkylated and 4-dealkylated pyridine metabolites. As presented previously, oxidation of DDC resulted in complete retention of the 4-methyl moiety. Deuteration at the 4-position did not increase the loss of the methyl moiety of DDC.

All three studies suggest that deuterium substitution at the 4-position does not effect the oxidation of the dihydropyridines by cytochrome P-450. The lack of deuterium isotope effects indicates that elimination of the 4-alkyl moiety from the initial nitrogen radical cation is not in any way in direct competition with loss of the 4-hydrogen. This eliminates the possibility that the 4-hydrogen comes off directly as a radical or a proton from the radical cation, since both of these processes should compete with elimination of the alkyl radical from the radical cation. Guengerich (Guengerich and Bocker, 1988) proposed the mechanism, illustrated in Figure 3.2.14 to explain a 4-position kinetic isotope effect (k_H/k_D = 2.9) on the oxidation of 3,5-dicarbmethoxy-2,6-dimethyl-4-phenyl-1,4-dihydropyridine by cytochrome P-450h. The kinetic isotope effect was only observed with the reconstituted purified cytochrome P-450 and not with microsomal cytochrome P-450. Guengerich proposed that the 4-hydrogen is removed from the radical cation as a proton followed by loss of an electron to generate the 4-phenylpyridine metabolite. An alternate mechanism, in which the 4-hydrogen is removed as a proton from a nitrogen cation intermediate

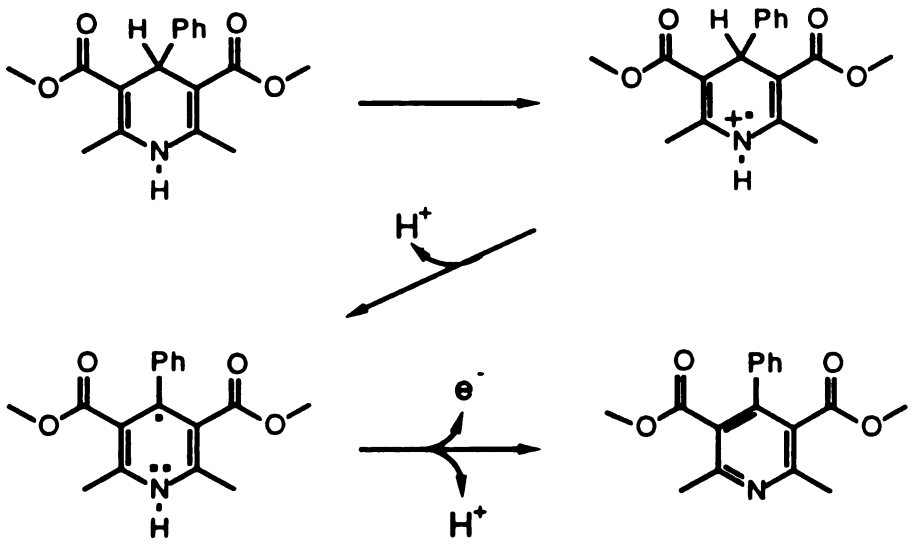


Figure 3.2.14 Mechanism of dihydropyridine oxidation involving 4-deprotonation of the radical cation.

rather than from the nitrogen radical cation (Figure 3.2.15), provides a possible explanation for the lack of product isotope effects with 4-deuterium substitution. From the initial radical cation a proton is lost from the nitrogen to generate the neutral radical species which loses an electron to form the cationic species. Aromatization of the cationic species by removal of the 4-hydrogen as a proton generates the 4-alkylpyridine metabolite. In this mechanism, the pathway to loss of the 4-hydrogen is controlled by deprotonation of the nitrogen. Since cleavage of the carbon-hydrogen bond at the 4-position does not compete directly with extrusion of the 4-alkyl radical, the reaction should not be sensitive to deuterium substitution.

The kinetic isotope effect observed by Guengerich (Guengerich et al., 1988) can be explained since the 4-phenyl analogue, used by

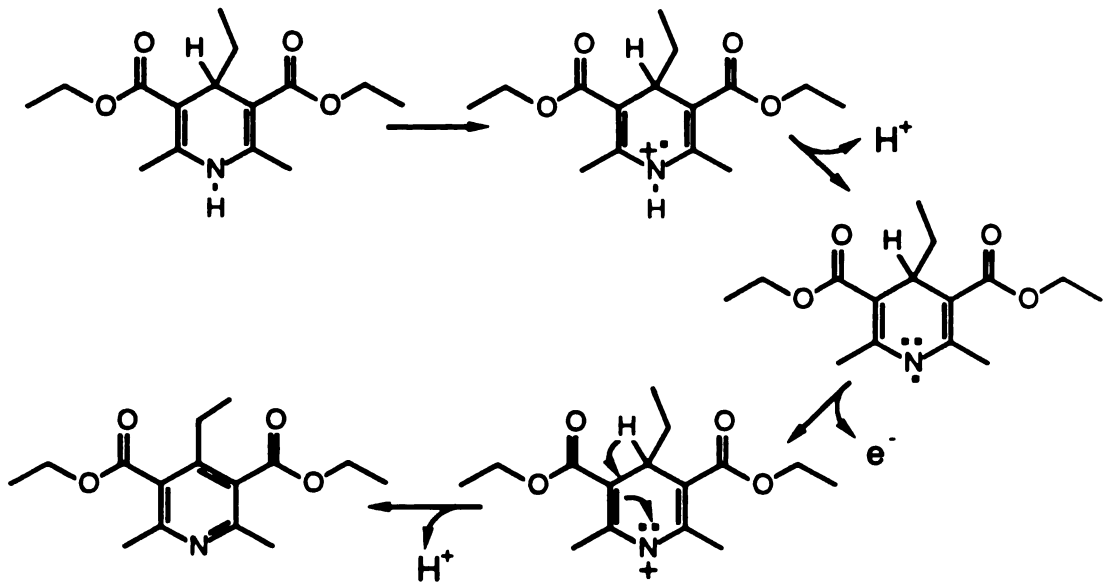
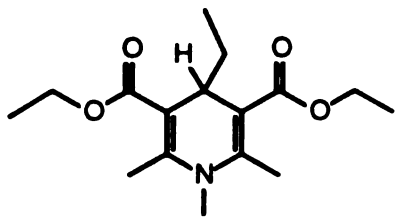


Figure 3.2.15 A mechanism for 4-alkylpyridine formation that explains the absence of product isotope effects from the 4-position.

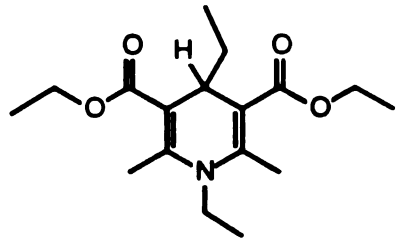
these authors, is only oxidized to the 4-phenylpyridine metabolite. The 4-dephenylpyridine metabolite is not produced. Therefore, the observed isotope effect is a kinetic isotope effect on formation of 4-phenylpyridine and not a product isotope effect on the partition between loss of the 4-substituent or 4-hydrogen moiety, as in the case of DDEP.

3.3 3,5-Dicarbethoxy-2,6-dimethyl-4-alkyl-*N*-alkyl-1,4-dihydropyridines

In an attempt to put the alternative mechanism (Figure 3.2.15) for loss of the 4-hydrogen to the test, the *N*-methyl and *N*-ethyl analogues of DDEP (Figure 3.3.1) were synthesized. The *N*-alkylDDEP analogues were synthesized as reported previously by Ortiz de



N-methylDDEP



N-ethylDDEP

Figure 3.3.1 Structure of *N*-methylDDEP and *N*-ethylDDEP.

Montellano (Ortiz de Montellano et al., 1981b) by condensing ethyl acetoacetate, alkylamine hydrochloride, and propionaldehyde in pyridine. The placement of an alkyl moiety on the nitrogen of the dihydropyridine ring should prevent formation of the 4-alkylated pyridine if nitrogen deprotonation is required for 4-alkylpyridine production. If the mechanism proposed in Figure 3.2.15 is correct, then incubation of *N*-alkylDDEP with cytochrome P-450 should result in exclusive formation of the 4-dealkylated product. In this case, the aromatized product should have the *N*-alkylpyridinium rather than pyridine structure.

The pyridinium standards were synthesized by a procedure modified from that of Sugiyama and colleagues (Sugiyama et al., 1964). The *N*-alkyldihydropyridines were oxidized to the pyridinium ions in ethanol with nitric acid (Figure 3.3.2). The *N*-methyl- and *N*-ethyl-4-ethylpyridiniums were obtained by oxidation of *N*-methyl- and *N*-ethylDDEP, respectively, whereas the 4-dealkylated *N*-methyl- and *N*-ethylpyridiniums were obtained by oxidation of *N*-methyl- and *N*-ethylDDIP, respectively. The pyridinium products

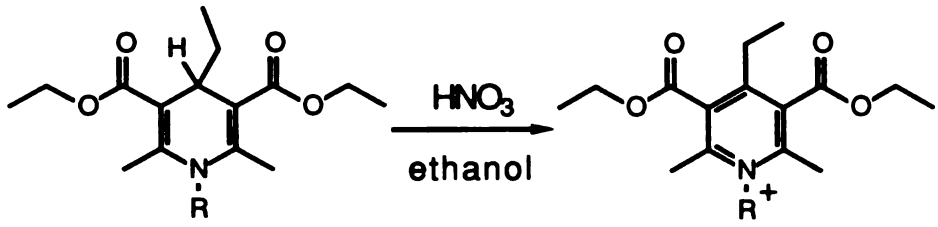


Figure 3.3.2 Oxidation of *N*-alkylDDEP to the corresponding *N*-alkylpyridinium nitrate by HNO_3.

were characterized by NMR and liquid secondary ion mass spectrometry (LSIMS). The LSIMS yield the correct *m/z* values for of the organic part of the pyridinium products. The NMR spectra of the 4-ethyl- and 4-deethylpyridiniums are characterized by a downfield shift of the 2,6-dimethyl singlet due to aromatization of the ring (Figure 3.3.3). In addition, the NMR spectrum of 4-deethylpyridinium exhibited a peak at approximately 9 ppm due to the C-4 aromatic hydrogen. The isopropyl moiety is lost in the oxidation as seen in the oxidation of DDIP itself. Prior to the oxidation procedure, unsuccessful attempts were made to synthesize the *N*-alkylpyridiniums by alkylation of the corresponding pyridines. The use of iodoalkanes as the alkylating agents did not produce the *N*-alkylpyridiniums when the reactions were monitored by TLC. If the pyridiniums were generated, they should have remained at the origin but no spots were detected at the origin. The use of dialkylsulfates, more reactive alkylating agents than iodoalkanes, was more fruitful; products resembling pyridinium ions were detected in the NMR spectra of the resulting black and messy crude reaction mixtures.

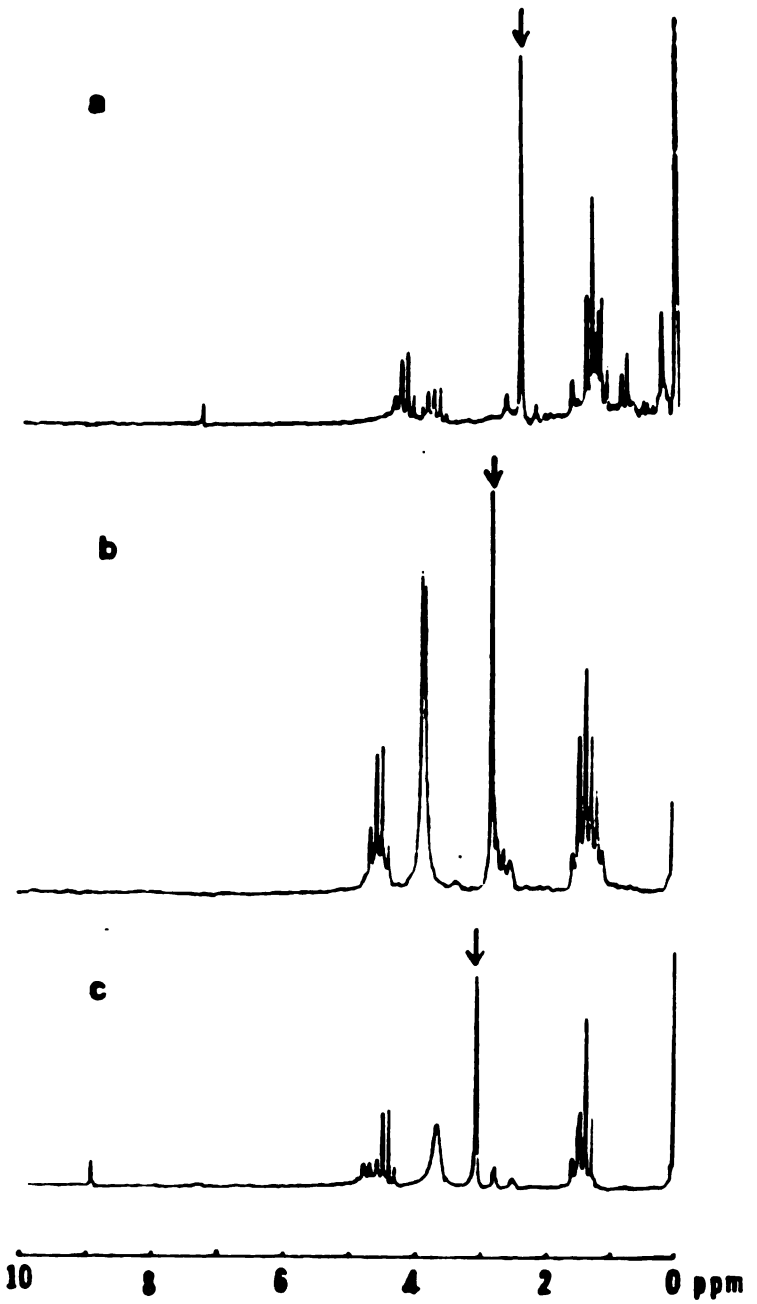


Figure 3.3.3 NMR spectra of *N*-ethylDDEP (a) and the corresponding 4-ethyl- and 4-deethylpyridinium nitrates (b and c, respectively). The arrows indicate the 2,6-dimethyl peaks. The large peak approximately at 4 ppm for spectra (b) and (c) is water.

However, this method was abandoned because of difficulties with purification of the products and because the oxidation of dihydropyridines with HNO_3 proves to be much simpler and cleaner.

Analysis of the *N*-alkylated pyridinium metabolites was conducted by ion pair reverse phase (C_{18} column) high pressure liquid chromatography. The ion pair chromatography was performed using methanesulfonic acid as the ion pairing agent. The metabolites were monitored at 280 nm using 50 % methanol in H_2O as the eluting solvent. The ion pairing technique was necessary because without it the pyridiniums eluted off the column as multiple peaks rather than as a clean single peak. The pyridinium compounds presumably pick up trace counter ions present in the system. When the mobile phase is swamped with one counter ion (methanesulfonic acid), the pyridinium compounds elute as single peaks.

Incubations of *N*-methyl- and *N*-ethylDDEP with microsomal cytochrome P-450 and NADPH were extracted with CH_2Cl_2 and both the aqueous and organic layers were analyzed for pyridinium products. The organic layer was also analyzed for non-ionic metabolites. In the case of the aqueous layer, the H_2O was removed by lyophilization and the residue was extracted with methanol. The methanol was removed and the sample was dissolved in the HPLC solvent for analysis. The organic layer was split into two fractions and the CH_2Cl_2 was removed from both fractions. The residues were dissolved in the appropiate HPLC solvents for analysis of pyridinium (reverse phase) and non-ionic metabolites (normal phase).

Analysis of the extracts from the cytochrome P-450-catalyzed oxidations of *N*-methyl- and *N*-ethylDDEP did not show the presence of *N*-methyl- or *N*-ethyl pyridinium products in either the aqueous or organic layers (Figure 3.3.4), indicating that cytochrome P-450 does not aromatize the *N*-alkyldihydropyridines to the corresponding pyridinium ions. However, examination of the non-ionic metabolites

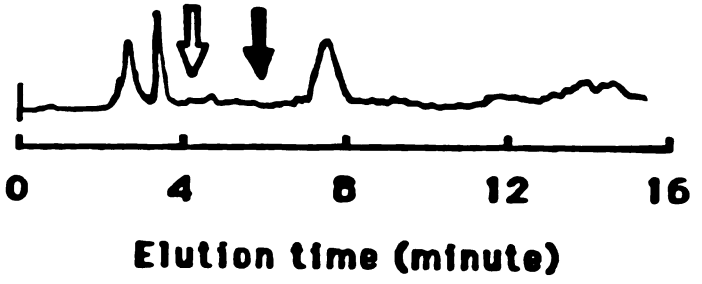


Figure 3.3.4 HPLC profile of the pyridinium metabolites from incubations of *N*-methylDDEP with microsomal cytochrome P-450. The open and closed arrows indicate the elution times where the 4-deethyl- and 4-ethylpyridinium metabolites, respectively, should have eluted. All other peaks are not turnover-dependent.

revealed the presence of a significant amount of DDEP as the major metabolite (Figure 3.3.5). Trace amounts of the 4-alkyl- and 4-dealkylpyridine metabolites were also found. Cytochrome P-450 thus oxidizes the *N*-alkyldihydropyridines via simple *N*-dealkylation rather than aromatization. The fact that *N*-dealkylation overwhelmingly dominates aromatization is very interesting because the radical cation necessary for aromatization of the dihydropyridine ring is exactly the same radical cation that is required for the *N*-dealkylation process (Figure 3.3.6). From the

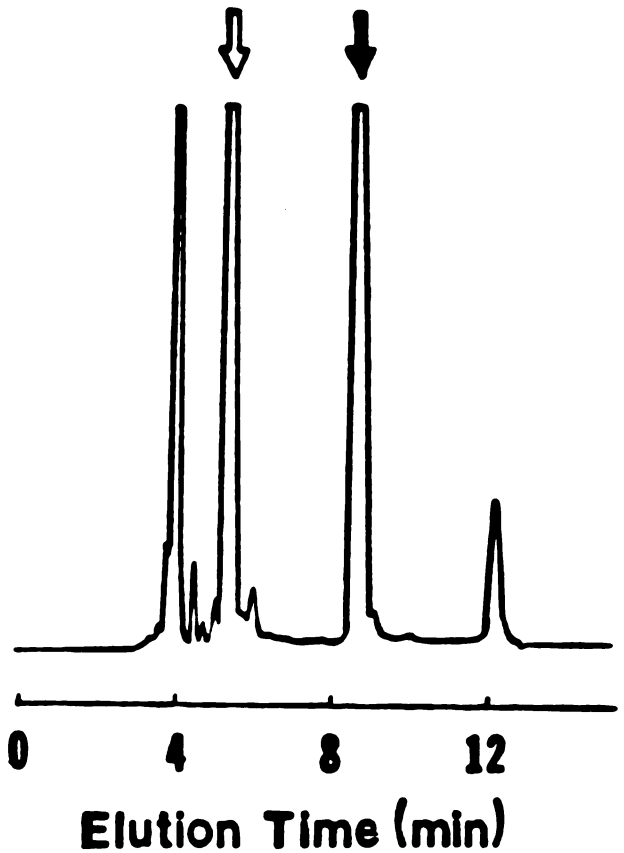


Figure 3.3.5 HPLC profile of the non-ionic products formed in the incubation of *N*-ethylDDEP with microsomal cytochrome P-450. The open and closed arrows indicate *N*-ethylDDEP and DDEP, respectively. All other peaks are not turnover-dependent.

radical cation, deprotonation of the α proton of the *N*-alkyl group leading to *N*-dealkylation must be very much favored over elimination of the 4-alkyl or 4-hydrogen moieties leading to aromatization. The absence of 4-dealkylpyridinium products clearly establishes that elimination of the 4-alkyl group is not concerted with abstraction of an electron from the nitrogen by cytochrome P-450.

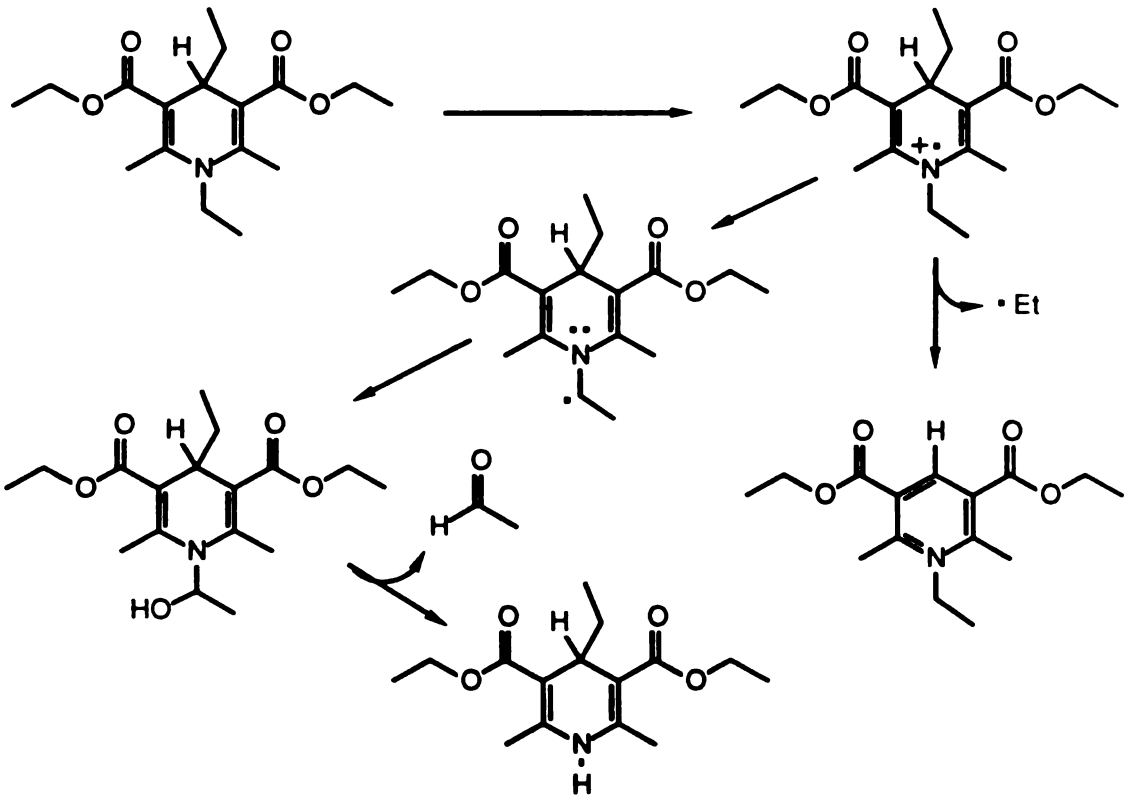


Figure 3.3.6 Pathways for aromatization and *N*-dealkylation of the radical cation.

The presence of the 4-ethyl- and 4-deethylpyridine (non-ionic) metabolites is explained by secondary metabolism of the initial DDEP metabolite. This explanation is consistent with the results obtained from cytochrome P-450 chromophore destruction and EPR spin trapping studies. Both experiments, as shown in Figures 3.3.7 and 3.3.8, exhibited slower release of the ethyl group from *N*-alkylDDEP than DDEP . Both *N*-methyl- and *N*-ethylDDEP destroyed the cytochrome P-450 chromophore and generated trappable alkyl radicals but at a slower rate than DDEP itself. *N*-AlkylDDEP must undergo *N*-dealkylation to DDEP prior to release of the 4-ethyl group

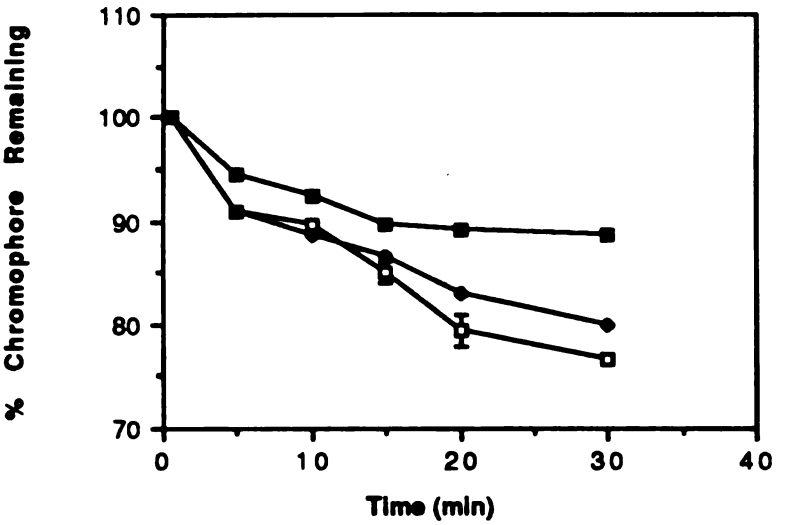


Figure 3.3.7 Destruction of the cytochrome P-450 chromophore in microsomal incubations with DDEP (open squares), *N*-methylDDEP (closed squares), and *N*-ethylDDEP (diamonds). The microsomes were made from rats pretreated with phenobarbital. The error bars represent the maximum deviation. For the points without error bars, the errors are not greater than the area covered by the point markers.

in a second oxidation reaction, thus resulting in a decreased rate of ethyl radical generation.

In sum, the use of *N*-alkyl groups to test the proposal that nitrogen deprotonation precedes 4-deprotonation failed due to overwhelming interference by the *N*-dealkylation process.

3.4 3,5-Dicarbethoxy-2,6-dimethyl-4-ethyl-*N*-phenyl-1,4-dihydropyridine

Since the *N*-dealkylation reaction dominates the cytochrome P-450-catalyzed oxidation of *N*-alkylDDEP, *N*-phenylDDEP was

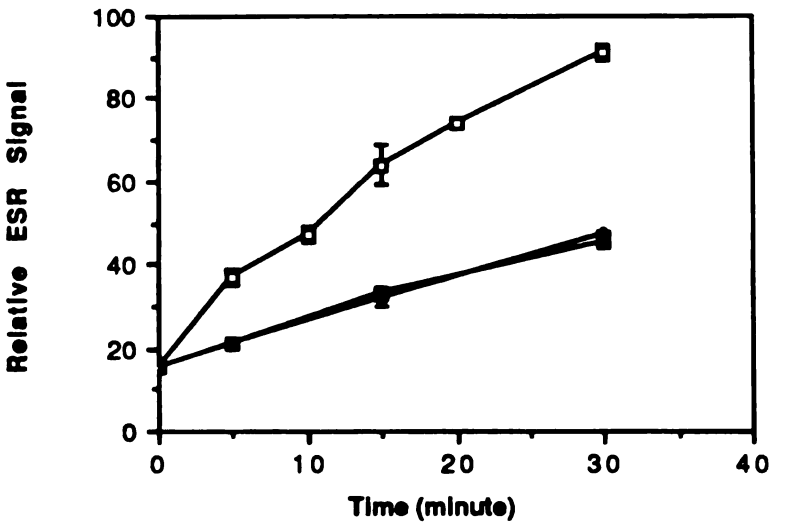


Figure 3.3.8 Relative EPR signals generated in microsomal incubations with DDEP (open squares), *N*-methylDDEP (closed squares), and *N*-ethylDDEP (diamonds). The microsomes were made from rats pretreated with phenobarbital. The error bars represent the maximum deviation. For the points without error bars, the errors are not greater than the area covered by the point markers.

synthesized to probe the mechanism by which 4-hydrogen is lost from dihydropyridines. *N*-phenylDDEP was synthesized by cyclization of ethyl acetoacetate, propionaldehyde, and aniline in ethanol. The reaction gave a meager 1.2 % yield of product. *N*-PhenylDDEP was purified by repetitive flash chromatography from a mixture of partially condensed and dead end products. The *N*-phenylDDEP was characterized by elemental analysis, NMR and mass spectrometry. The details of the synthesis are reported in Section 6.0. The *N*-phenyl moiety should void both the possibility of *N*-dealkylation and loss of the proton from the nitrogen if the mechanism in Figure 3.2.15 is correct. Only the 4-dealkylated

pyridinium metabolite should be formed. The 4-ethyl- and 4-deethyl-*N*-phenylpyridinium standards were synthesized by oxidation of *N*-phenylDDEP and *N*-phenylDDIP with nitric acid. The two pyridinium products were characterized by NMR and LSIMS as mentioned for the *N*-alkylpyridinium compounds. Again, the LSIMS gave the correct *m/z* values for the organic part of the pyridinium ions and down field shifts were observed for the 2,6-dimethyl peaks in the NMR spectra. The 4-deethylpyridinium ion possessed the aromatic 4-H proton at 9 ppm whereas the 4-ethylpyridinium ion did not.

Incubations of *N*-phenylDDEP with cytochrome P-450 were extracted with CH_2Cl_2 and the aqueous and organic layers were prepared and analyzed as described for the incubations with *N*-alkylDDEP. The mobile phases for both the normal and reverse phase analyses were changed slightly. For the normal phase, the solvent was 20 % rather than 25 % THF in hexane and for the reverse phase the solvent was 60 % rather than 50 % methanol in H_2O. The non-ionic metabolite analyses showed the presence of a metabolite that is more polar than the substrate (Figure 3.4.1). The new metabolite has not been identified but is believed to be a phenyl-hydroxylated derivative of *N*-phenylDDEP because absorption spectroscopy shows the dihydropyridine ring system is maintained. The absorption spectrum of the non-ionic metabolite exhibits λ_{max} 235 and 345 nm which resemble the absorption spectra of the dihydropyridines. Contrary to expectations, analyses of the ionic products showed the presence of a 1:1 ratio of both the 4-ethyl- and 4-deethyl pyridinium

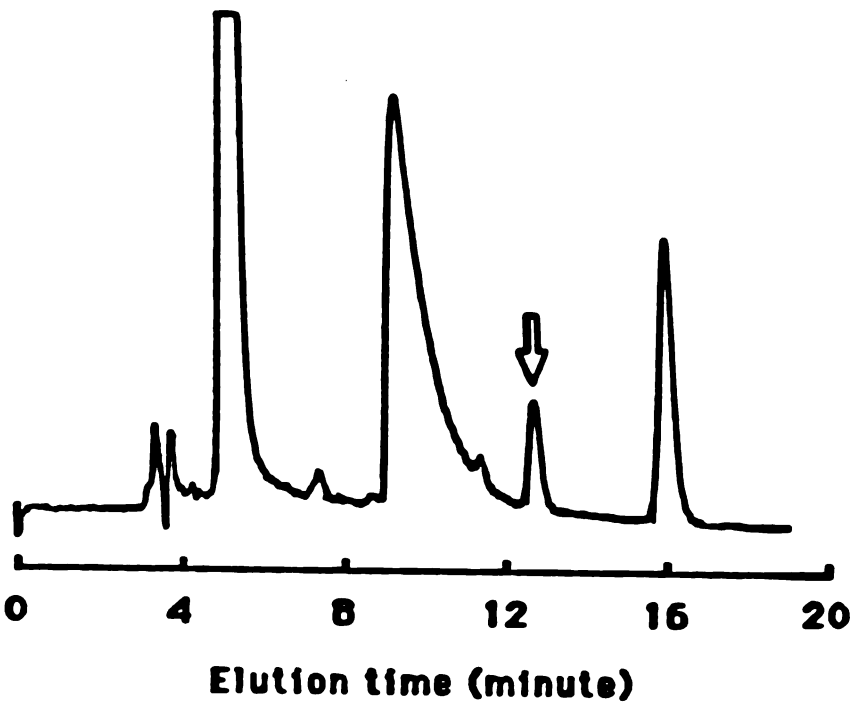


Figure 3.4.1 HPLC profile of the non-ionic metabolites formed in the microsomal oxidation of *N*-phenylDDEP. The open arrow indicates the only non-ionic metabolite. The large peak at approximately 4.5 min is *N*-phenylDDEP and the peak at 16 min is the internal standard. All other peaks are not turnover-dependent.

metabolites (Table 3.4.1 and Figures 3.4.2 and 3.4.3). These results indicate that nitrogen deprotonation is not required for loss of the 4-hydrogen to generate the 4-alkylpyridinium metabolite. However, further analysis of the oxidation of *N*-phenylDDEP indicates that the mechanism proposed for DDEP oxidation may not apply to *N*-phenylDDEP. In contrast to DDEP, *N*-phenylDDEP does not destroy the

Table 3.4.1 Pyridinium metabolite formation from the microsomal cytochrome P-450-catalyzed oxidation of [4-^{1}H]- and [4-^{2}H]-*N*-phenylDDEP. The 4-ethyl- and 4-deethylpyridiniums are 3,5-dicarbethoxy-2,6-dimethyl-4-ethyl-*N*-phenylpyridinium and 3,5-dicarbethoxy-2,6-dimethyl-*N*-phenylpyridinium, respectively.

Dihydropyridine	Pyridininum Metabolites	
	4-ethyl	4-deethyl
	(nmole/nmole cytochrome P-450/minute)	
[4-^{1}H]-*N*-phenylDDEP	0.081 ± 0.005 (51 %)	0.078 ± 0.005 (49 %)
[4-^{2}H]-*N*-phenylDDEP	0.012 ± 0.001 (14 %)	0.073 ± 0.004 (86 %)

Figure 3.4.2 HPLC profile of the *N*-phenylpyridinium metabolites from microsomal cytochrome P-450-catalyzed oxidations of *N*-phenylDDEP. The open and closed arrows indicate the 4-deethyl- and 4-ethylpyridinium metabolites, respectively.

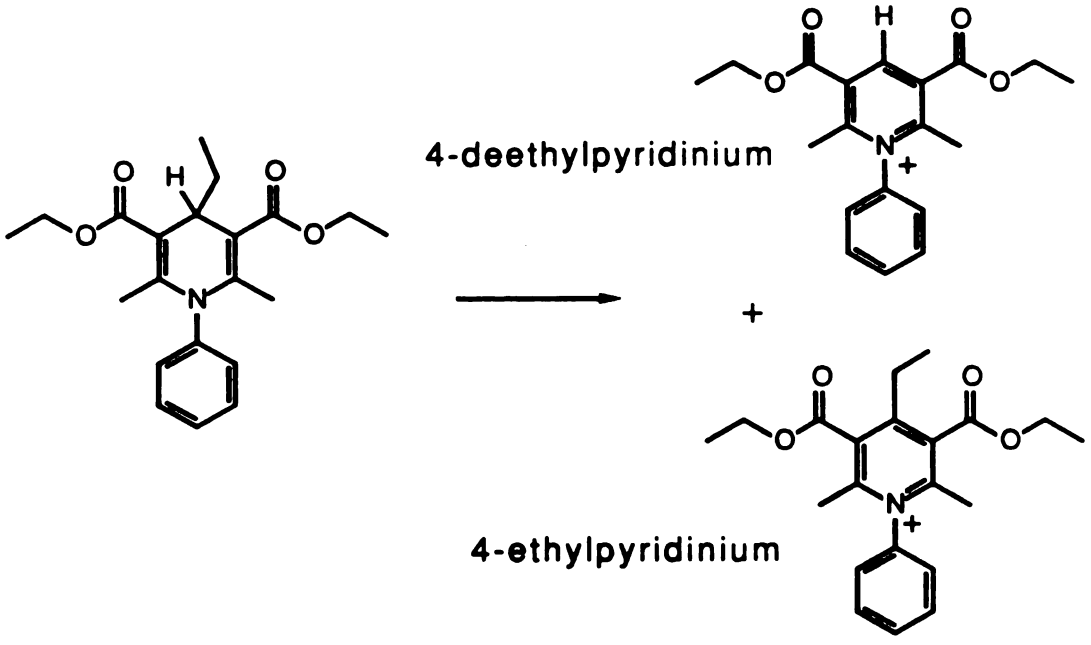


Figure 3.4.3 Oxidation of *N*-phenylDDEP to the 4-deethyl- and 4-ethyl-*N*-phenylpyridininum metabolites.

cytochrome P-450 chromophore or generate POBN-trappable radicals. These observations, by themselves, are not convincing evidence that *N*-phenylDDEP behaves differently from DDEP since DDC also does not destroy the cytochrome P-450 chromophore or generate spin trappable radicals. The applicability of the mechanism for DDEP oxidation (Figure 3.2.15) to *N*-phenylDDEP oxidation, however, was also tested by deuterium substitution at the 4-position.

[4-^{2}H]-*N*-phenylDDEP was synthesized by condensation of ethyl acetoacetate, aniline, and [1-^{2}H]-propionaldehyde in ethanol and was purified as mentioned previously for the unlabelled compound. The [1-^{2}H]-propionaldehyde was synthesized by acidic hydrolysis of [2-

^{2}H]-2-ethyl-4,4,6-trimethyltetrahydro-1,3-oxazine. For [4-^{2}H]-DDEP, [1-^{2}H]-propionaldehyde was not isolated from the hydrolysis reaction mixture but for the synthesis of [4-^{2}H]-*N*-phenylDDEP, purification of [1-^{2}H]-propionaldehyde was necessary due to the meager yield of *N*-phenylDDEP. The [1-^{2}H]-propionaldehyde was isolated by direct distillation from the reaction mixture. The NMR spectrum of the distilled [1-^{2}H]-propionaldehyde lacked the aldehyde proton at 9 ppm indicating the incorporation of deuterium. The NMR spectrum of [4-^{2}H]-*N*-phenylDDEP showed no trace of the 4-H triplet at 3.94 ppm and its mass spectrum exhibited a molecular ion at *m/z* 358, in agreement with quantitative incorporation of deuterium into the 4-position.

Analysis of the metabolites from incubations of [4-^{2}H]-*N*-phenylDDEP with cytochrome P-450 showed that formation of the 4-deethyl-*N*-phenylpyridinium product exceeds that of the 4-ethyl-*N*-phenylpyridinium metabolite (Table 3.4.1). Deuterium substitution thus decreased the formation of the 4-ethylpyridinium metabolite, indicating a direct competition between elimination of the 4-ethyl and 4-hydrogen. A product isotope effect of $k_H/k_D = 6$ was calculated from the 4-deethyl- to 4-ethyl-*N*-phenylpyridinium product ratios. The large product isotope effect suggests that substitution of a phenyl group on the nitrogen alters the mechanism from one that is insensitive to deuterium substitution at the 4-position to one that is sensitive. The presence of the phenyl group presumably alters the electronic nature of the nitrogen and/or the steric relationship between the substrate and the active site of the

enzyme. Two possible mechanisms rationalize the direct competition between extrusion of the 4-ethyl and 4-hydrogen. The first mechanism is direct competition for elimination of the ethyl or hydrogen radicals from the radical cation intermediate (Figure 3.4.4). The second mechanism requires the competition between

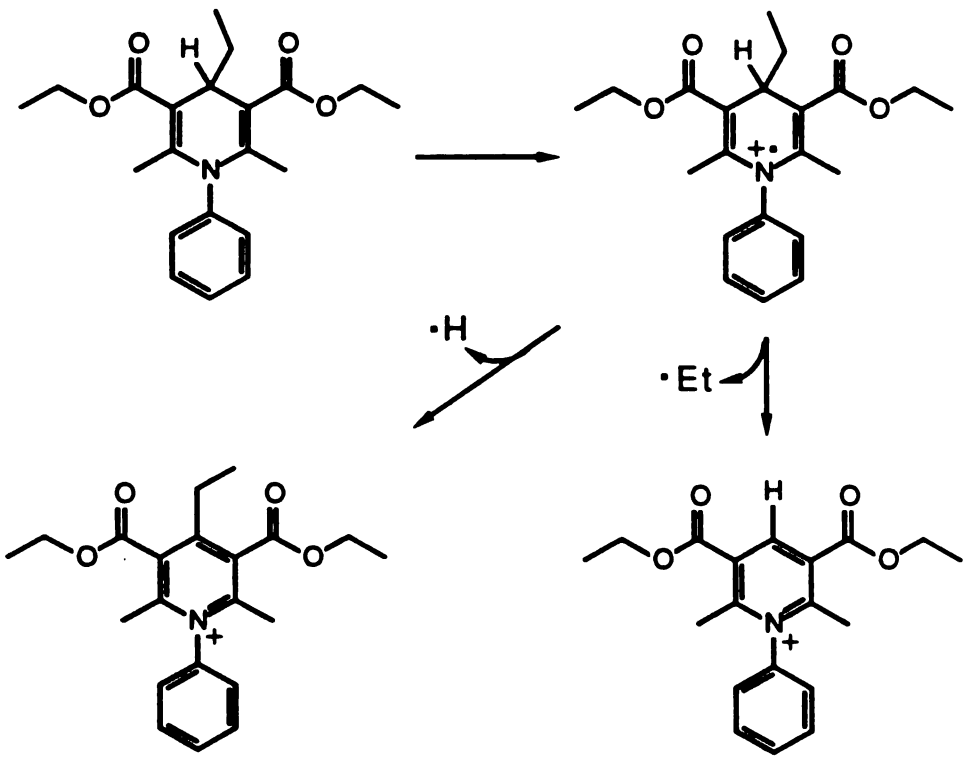


Figure 3.4.4 Competition between release of the 4-H or 4-ethyl from the radical cation intermediate.

abstraction of an electron from the nitrogen of the parent substrate, which leads exclusively to loss of the 4-alkyl moiety (Figure 3.4.5, path a), and abstraction of the 4-hydrogen by the iron-oxo species of cytochrome P-450, which leads predominantly to loss of the 4-

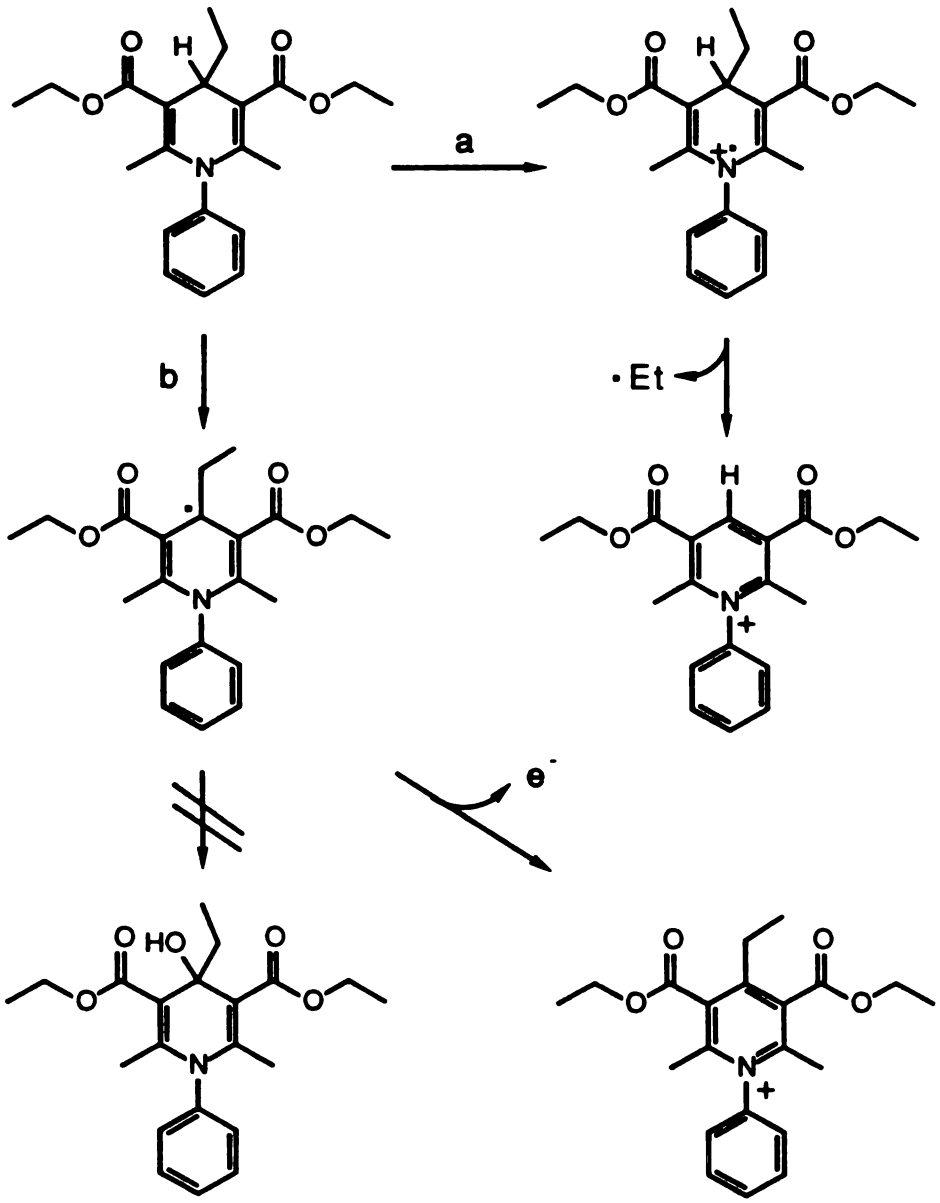


Figure 3.4.5 A possible mechanism for oxidation of *N*-phenylDDEP that explains the isotope effect from the 4-position.

hydrogen (Figure 3.4.5, path b). For the latter mechanism the radical recombination between Fe^{IV}-OH and C-4 radical, resembling the carbon hydroxylation mechanism, does not take place because formation of 4-hydroxy-*N*-phenylDDEP is not observed (Figure 3.4.5).

The new non-ionic metabolite, which is more polar than *N*-phenylDDEP, does not co-elute with authentic 4-hydroxy-*N*-phenylDDEP. The 4-hydroxy-*N*-phenylDDEP standard was synthesized by addition of hydroxide anion to the 4-ethyl-*N*-phenylpyridinium ion (Figure 3.4.6). The 4-ethyl-*N*-phenylpyridinium salt was dissolved

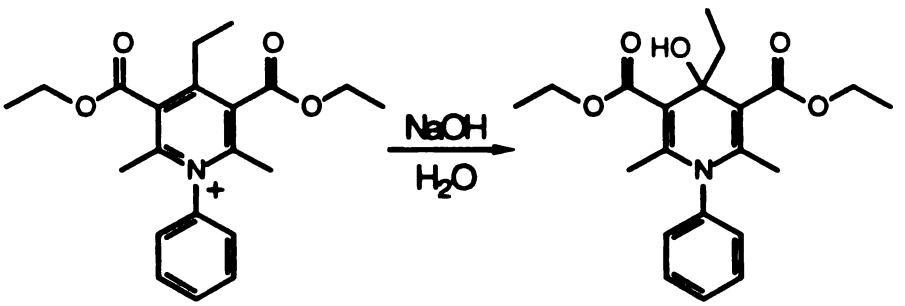


Figure 3.4.6 Synthesis of 4-hydroxy-N-phenylDDEP.

in H_2O and the pH was adjusted to 10 with NaOH. The product was characterized by NMR spectroscopy. The major differences between *N*-phenylDDEP and 4-hydroxy-*N*-phenylDDEP are the absence of the 4-H at 3.94 ppm and a downfield shift of the 4-$C\underline{H}_2CH_3$ quartet due to the presence of the 4-hydroxyl moiety.

Attempts to test the nitrogen deprotonation mechanism (Figure 3.2.15) by substitution of the nitrogen with methyl, ethyl, and phenyl groups thus failed due either to domination of *N*-dealkyation or to a change in the mechanism of the dihydropyridine oxidation. To minimize the perturbation caused by the nitrogen substituent, the synthesis of *N-tert*-butylDDEP was attempted

without success. The *tert*-butyl group would minimize the electronic effect without being susceptible to *N*-dealkylation. The condensation of ethyl acetoacetate with propionaldehyde and *tert*-butylamine hydrochloride in pyridine did not produce *N-tert*-butylDDEP. Synthesis of the dihydropyidines requires formation of four carbon-carbon bonds and the steric interactions are probably too great for all four condensations to occur in a one-pot reaction. However, a stepwise synthetic approach, in which the carbon-carbon bonds were formed one at a time, also failed. *tert*-Butylamine condensed with one molecule of ethyl acetoacetate to form the secondary amine but condensation with a second molecule of ethyl acetoacetate to form the tertiary amine apparently did not occur.

3.5 Fate of the 4-Alkyl Moiety of the Dihydropyridines

Oxidation of DDEP with cytochrome P-450 has been shown to result in alkylation of the heme moiety by an ethyl radical released from the 4-position. The ethyl radical can leave the active site of the enzyme and be trapped by POBN. It is also possible that the radical cation intermediate leaves the active site and the ethyl radical is eliminated outside of the active site. The fate of the ethyl radical in the absence of POBN has not been determined. In this section, the fate of the 4-alkyl group is discussed. For this study, 3,5-dicarbethoxy-2,6-dimethyl-4-(2-phenylethyl)-1,4-dihydropyridine (DDPEP) was used instead of DDEP because the higher boiling points of the metabolites resulting from the 4-phenylethyl moiety make them easier to handle and analyze than the

lower boiling metabolites resulting from the 4-ethyl moiety of DDEP.

The oxidation of phenelzine by cytochrome P-450 has been reported to generate phenylethyl radicals (Ortiz de Montellano et al., 1983) and has been shown more recently to yield phenylethyl alcohol, phenylacetaldehyde, ethylbenzene, benzyl alcohol, and benzaldehyde (Watanabe and Ortiz de Montellano, 1987). The phenylethyl radical has been proposed to be the common intermediate for the observed metabolites. The same set of metabolites is expected if oxidation of DDPEP eliminates the phenylethyl radical. Incubations of DDPEP with microsomal cytochrome P-450 were extracted with CH_2Cl_2 and the organic layers were analyzed by capillary gas chromatography (GC) for the presence of phenylethyl derivatives. The samples were divided into two equal fractions and one of the fractions was treated with bis(trimethylsilyl)trifluoroacetamide (BSTFA) for the analysis of the alcohols because the alcoholic metabolites required derivatization for GC analysis. Analysis of both fractions showed the formation of phenylethyl alcohol only; no other metabolites were detected (Figures 3.5.1 and 3.5.2). Control incubations lacking NADPH did not generate phenylethyl alcohol. Control incubations containing authentic phenylacetaldehyde, benzaldehyde, and ethylbenzene at 50 % of the concentration of phenylethyl alcohol showed that these compounds are detectable, confirming the absence of these metabolites. The absence of metabolites other than phenylethyl alcohol suggests that the phenylethyl group may not be

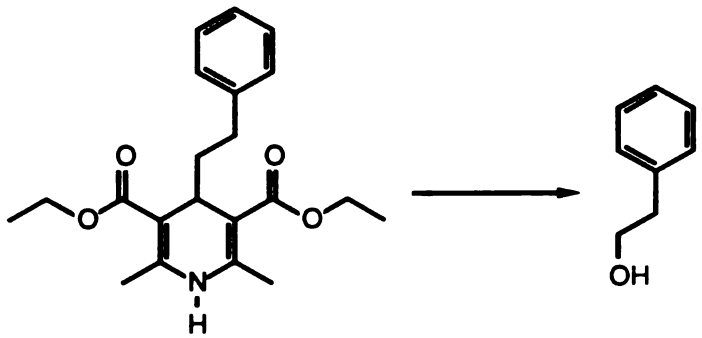


Figure 3.5.1 Oxidation of DDPEP by microsomal cytochrome P-450 to yield phenylethyl alcohol.

extruded as a radical. However, an incubation conducted under an $^{18}O_2$ atmosphere showed that 58 and 42 % of phenylethyl alcohol metabolites contained ^{18}O and ^{16}O, respectively. The percent of ^{18}O-phenylethyl alcohol was determined by gas chromatography/mass spectrometry of the BSTFA derivatized phenylethyl alcohol metabolite. The percentage was calculated from the major fragment (loss of a methyl group of the trimethylsilyl moiety) instead of the molecular ion because the molecular ion peak was only 2 % of the major fragment peak. Formation of ^{18}O-phenylethyl alcohol could result from radical recombination of the phenylethyl radical and Fe^{IV}-O of cytochrome P-450 or from trapping of phenylethyl radical by $^{18}O_2$ followed by reduction of the resulting phenylethylhydroperoxide. The two processes cannot be distinguished without further study. Nevertheless, both processes require the formation of the phenylethyl radical. Formation of the ^{16}O-phenylethyl alcohol can be explained by secondary oxidation of the phenylethyl radical to the phenylethyl cation followed by

Figure 3.5.2 Gas chromatograms of the metabolites from the microsomal cytochrome P-450-catalyzed oxidation of DDPEP: (a) +NADPH and (b) -NADPH. The arrow indicates the phenylethyl alcohol peak. All the other peaks are not turnover-dependent.

addition of a water molecule or by incomplete removal of $^{16}O_2$ from the incubation flask. The formation of both ^{18}O- and ^{16}O-phenylethyl alcohols has also been observed with the phenylethyl radical generated by reduction of 4-phenylethyl-1,4-peroxyquinol by

cytochrome P-450 under an $^{18}O_2$ atmosphere (Yumibe and Thompson, 1989).

Oxidation of DDPEP by cytochrome P-450 did not significantly destroy the cytochrome P-450 chromophore (Figure 3.5.3) or generate an EPR signal in the presence of POBN. The low chromophore destruction and the absence of an EPR signal might be due to the relatively low flux of phenylethyl radicals indicated by the finding of approximately a 12 fold lower production of the 4-dealkylpyridine metabolite than is observed with DDIP (Tables 3.2.3 and 3.5.1). Formation of phenylethyl alcohol as the only metabolite of the 4-phenylethyl group of DDPEP is supported by the finding that

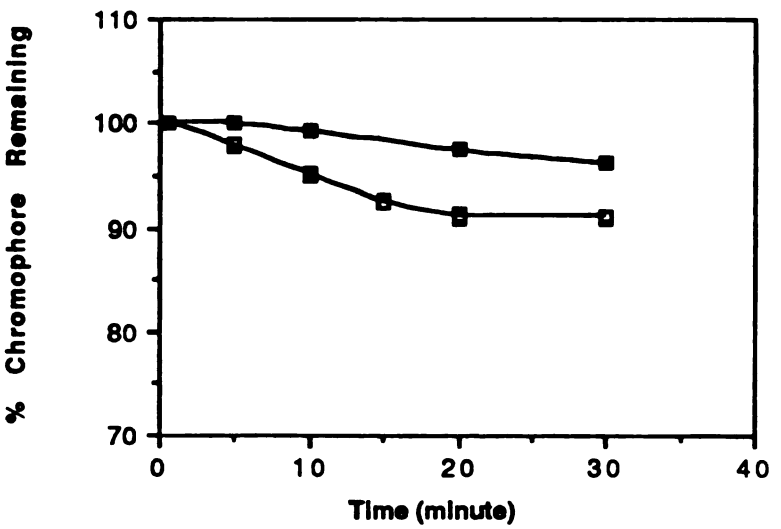


Figure 3.5.3 Destruction of the microsomal cytochrome P-450 chromophore by DDPEP (open squares). The closed squares represent the control incubation lacking DDPEP. The error bars represent the maximum deviation. For the points without error bars, the errors are not greater than the % covered by the point markers.

Table 3.5.1 Production of phenylethyl alcohol and 3,5-dicarbethoxy-2,6-dimethylpyridine from DDPEP oxidation by hepatic microsomal cytochrome P-450 from rats pretreated with phenobarbital.

Product	Yield (nmole/nmole cytochrome P-450/minute)
4-dealkylpyridine	0.09 ± 0.01
phenylethyl alcohol	0.11 ± 0.01

approximately equal amounts of 4-dealkylated pyridine and phenylethyl alcohol are produced (Table 3.5.1).

3.6 Oxidation of DDEP by Purified Cytochrome P-450

Cytochromes P-450 exist as multiple forms, each being slightly different with respect with to its spectral, immunochemical, physical, and catalytic properties (Waxman, 1986). Two purified cytochromes P-450 (b and c) have been tested for dihydropyridine oxidase activity. Only cytochrome P-450c was active in oxidizing DDEP. These findings are supported by recent studies which suggest that rat liver cytochromes P-450c, h, and p are involved in oxidation of the dihydropyridines (Tephly et al., 1986, Guengerich et al., 1986, Correia et al., 1987, and Riddick et al., 1988). Immunochemical quantification of various microsomal cytochromes P-450 from DDEP treated rats have shown that levels of cytochromes P-450c, h, and p are decreased (Tephly et al., 1986). This finding is in agreement with the catalytic studies. Treatment of microsomes made from rats pretreated with 3-

methylcholanthrene with DDEP resulted in loss of 7-ethoxyresorufin *O*-deethylase activity, which is relatively specific for cytochrome P-450c (Riddick et al., 1988). Furthermore, DDEP treatment of microsomes made from rats pretreated with dexamethasone resulted in loss of erythromycin *N*-demethylase and 2β- and 6β-testosterone hydroxylase activities, which are thought to be specific for cytochrome P-450p (Correia, et al., 1987). More directly, reconstituted pure cytochromes P-450h and p have been shown to oxidize the dihydropyridines (Guengerich et al., 1986). In addition, Guengerich and colleagues have purified cytochrome P-450$_{NF}$, which oxidizes the nifedipine (4-aryldihydropyridine) from human livers (Guengerich et al., 1986). Cytochrome P-450$_{NF}$ has been shown to be similar to rat liver cytochrome P-450p (77 % sequence identity) (Beaune et al., 1986).

Cytochromes P-450b and c were purified by the methods of Waxman and Walsh (Waxman and Walsh, 1982) and Levin and colleagues (Ryan et al., 1979), respectively. The cytochrome P-450 reductase was purified by the procedure of Yasukochi and Masters (Yasukochi and Masters, 1976). The flow diagrams for the purifications of cytochrome P-450b, c, and cytochrome P-450 reductase are illustrated in Figure 3.6.1. Cytochrome P-450c was purified by Dr. David R. Dutton.

Analysis of the incubations of DDEP with purified, reconstituted cytochrome P-450b showed no trace of the pyridine metabolites whereas purifed, reconstituted cytochrome P-450c metabolized DDEP to the corresponding 4-alkyl- and 4-

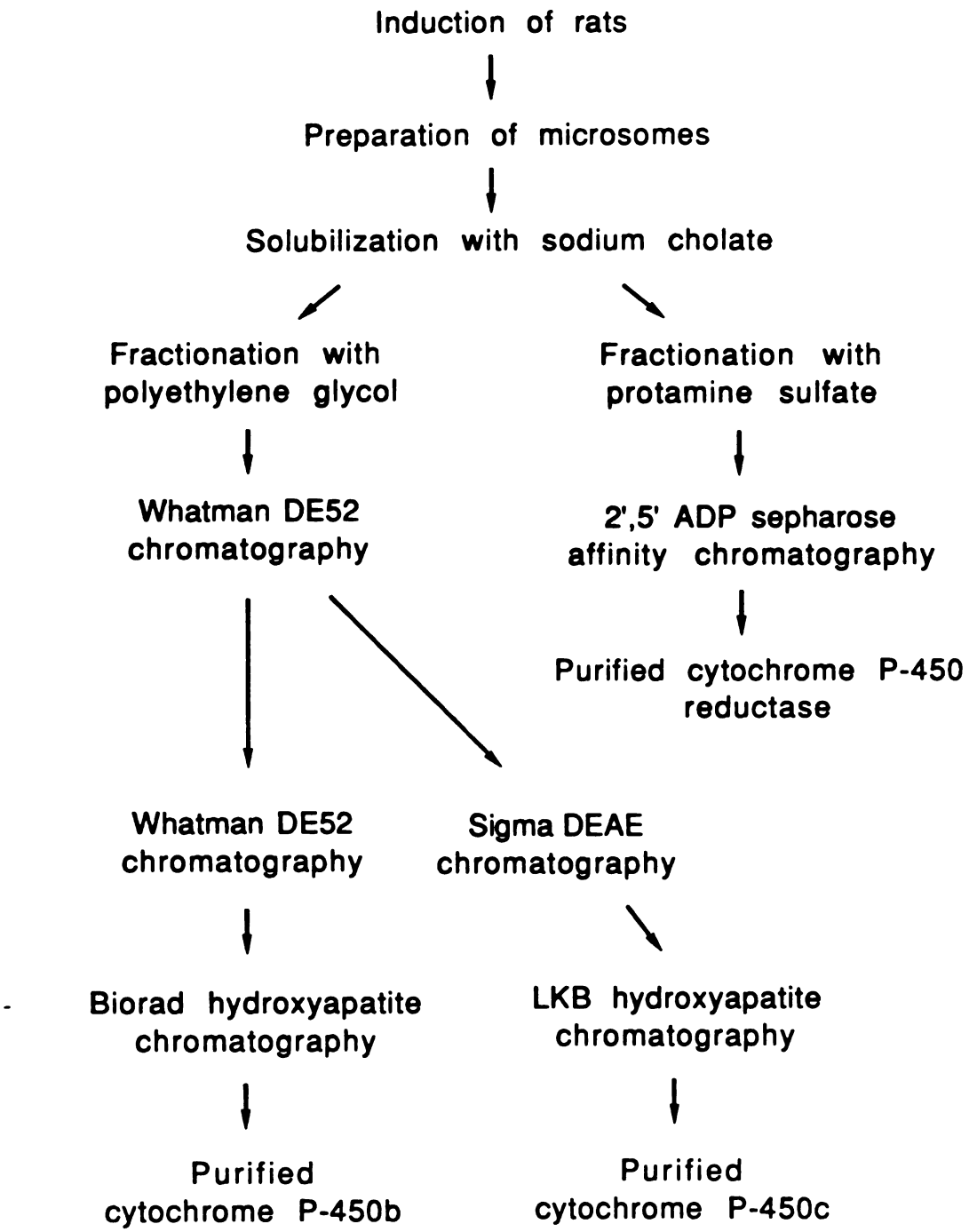


Figure 3.6.1 Flow diagram for the purification of cytochrome P-450b and c and cytochrome P-450 reductase.

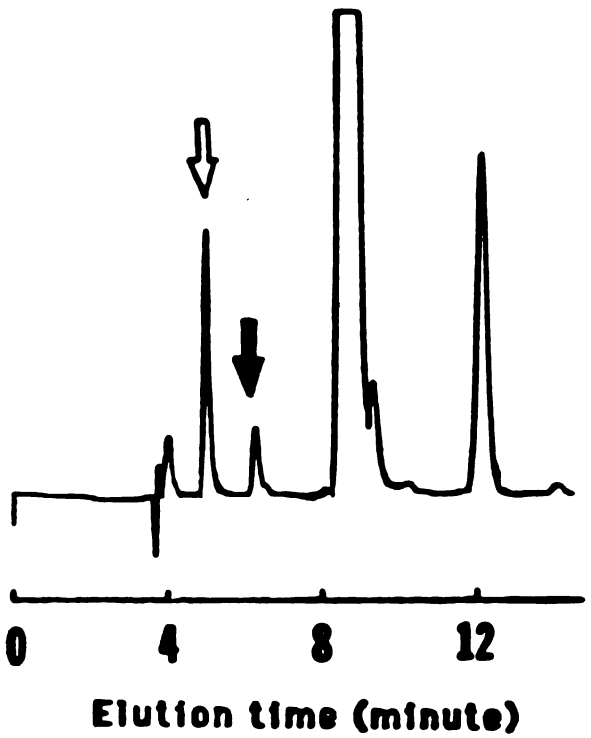


Figure 3.6.2 HPLC profile of the metabolites from oxidation of DDEP by pure cytochrome P-450c. The open and closed arrows indicate the 4-deethyl- and 4-ethylpyridine metabolites, respectively. The large peak at 8.5 minutes is DDEP. All the other peaks are not turnover-dependent.

deethylpyridines (Figure 3.6.2). DDEP also did not destroy the chromophore of pure cytochrome P-450b whereas the chromophore of pure cytochrome P-450c was lost in a time-dependent fashion (Figure 3.6.3). Formation of the pyridine metabolites and destruction of the cytochrome P-450c chromophore were NADPH dependent. The NADPH dependent loss of catalytic activity (7-ethoxyresorufin *O*-deethylase) caused by DDEP was also observed with cytochrome P-450c (Figure 3.6.4). The discrepancy between

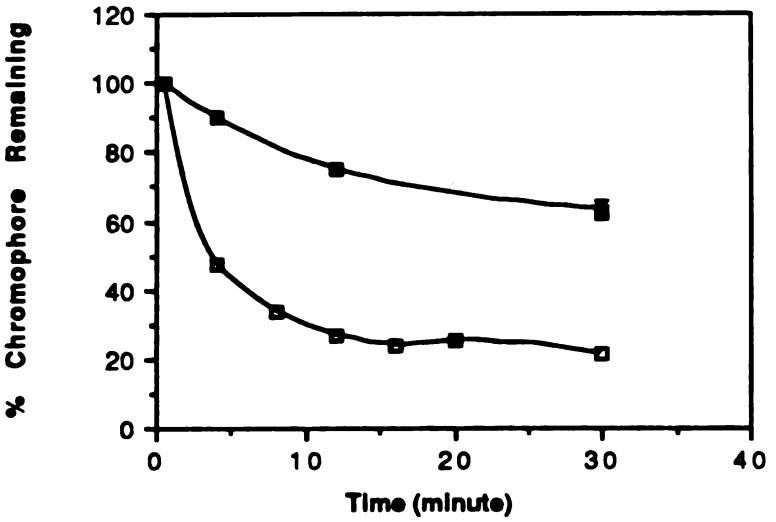


Figure 3.6.3 Destruction of the pure cytochrome P-450 chromophore during the oxidation of DDEP by cytochrome P-450c: +NADPH (open squares) and -NADPH (closed squares). The error bars indicate the maximum deviation. For the points with error bars, the error is not greater than the % covered by the point markers.

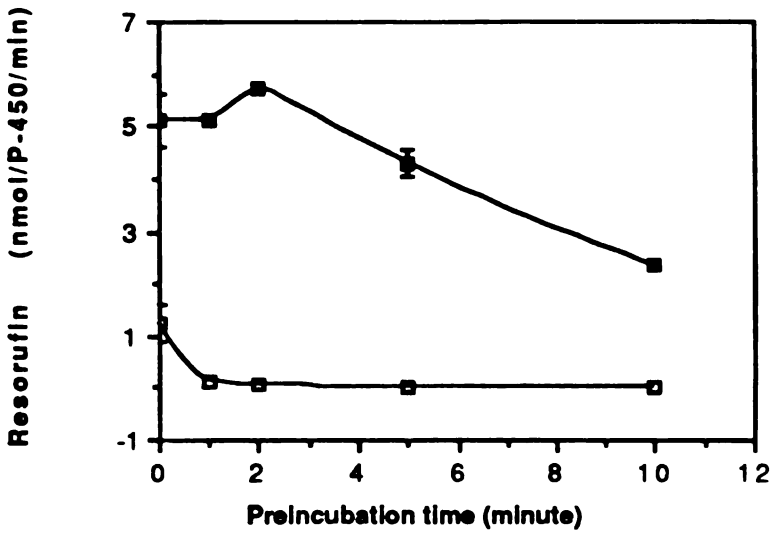


Figure 3.6.4 7-Ethoxyresorufin *O*-deethylase activity of cytochrome P-450c after preincubation with (open squares) DDEP and NADPH and (closed squares) NADPH alone. The error bars represent the maximum deviation. For the points without error bars, the error is not greater than the area covered by the point markers.

the -DDEP and +DDEP incubations indicates that DDEP competitively inhibits the 7-ethoxyresorufin *O*-deethylase activity of cytochrome P-450c. A discrepancy also exists between the control incubation and the incubation containing NADPH and DDEP in the case of the 7-ethoxycoumarin *O*-deethylase activity of cytochrome P-450b (Figure 3.6.5), suggesting that DDEP is a competitive inhibitor of cytochrome P-450b. However, it is possible that the inhibitory property of DDEP is a result of DDEP binding to a site other than the active site. The finding that DDEP is not turned over by cytochrome P-450b is consistent with the findings of Marks and colleagues

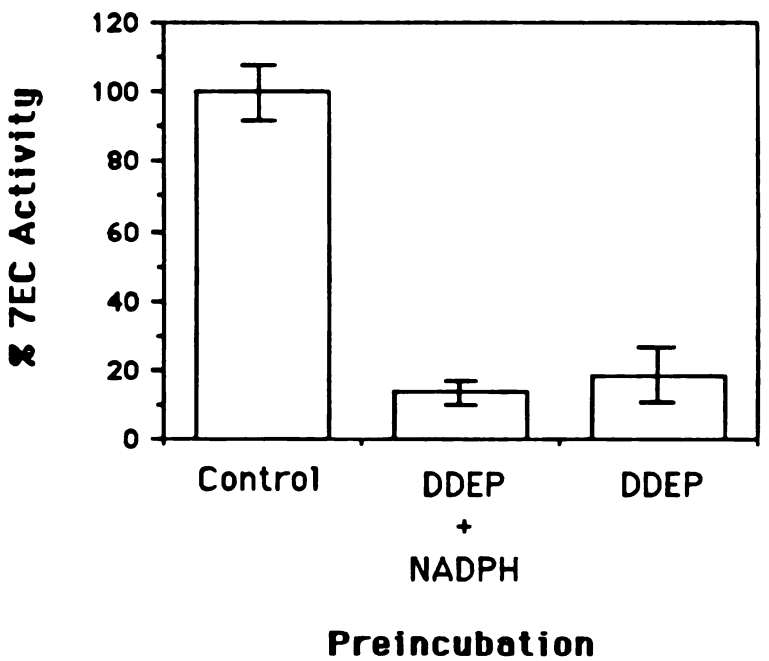


Figure 3.6.5 7-Ethoxycoumarin *O*-deethylase activities of cytochrome P-450b after 10 min preincubation with DDEP. The activities are represented as % of control. The error bars represent the maximum deviation.

(Riddick et al., 1988), who reported that the 7-pentoxyresorufin *O*-deethylase activity (thought to be specific for cytochrome P-450b) of liver microsomes from phenobarbital pretreated rats is not altered by DDEP.

EPR spin trapping studies were not possible with the reconstituted pure enzymes because cytochrome P-450 reductase in the presence of NADPH generates a significant amount of radicals even in the absence of cytochrome P-450. One possible explanation is that pure cytochrome P-450 reductase initiates lipid peroxidation to produce radicals that are trapped by POBN.

3.7 Chemical Oxidation of the Dihydropyridines

In order to define the mechanism of dihydropyridine oxidation and to determine how the enzymatic oxidation relates to the chemical oxidation, ferricyanide oxidations of dihydropyridines were studied. Potassium ferricyanide was chosen as the oxidant because it has been shown to oxidize 1,4-dihydropyridines to the corresponding pyridines (Powell et al., 1984) and is known to be a one-electron oxidant.

The products from the reaction of DDEP with potassium ferricyanide were analyzed as mentioned previously for the enzymatic metabolites. The chemical oxidation of DDC and DDIP by ferricyanide resulted in exclusive formation of the 4-methylpyridine and 4-deisopropylpyridine products, respectively, and the chemical oxidation of DDEP by ferricyanide produced both the 4-alkyl- and 4-dealkylpyridines (Figure 3.7.1). These results are consistent with

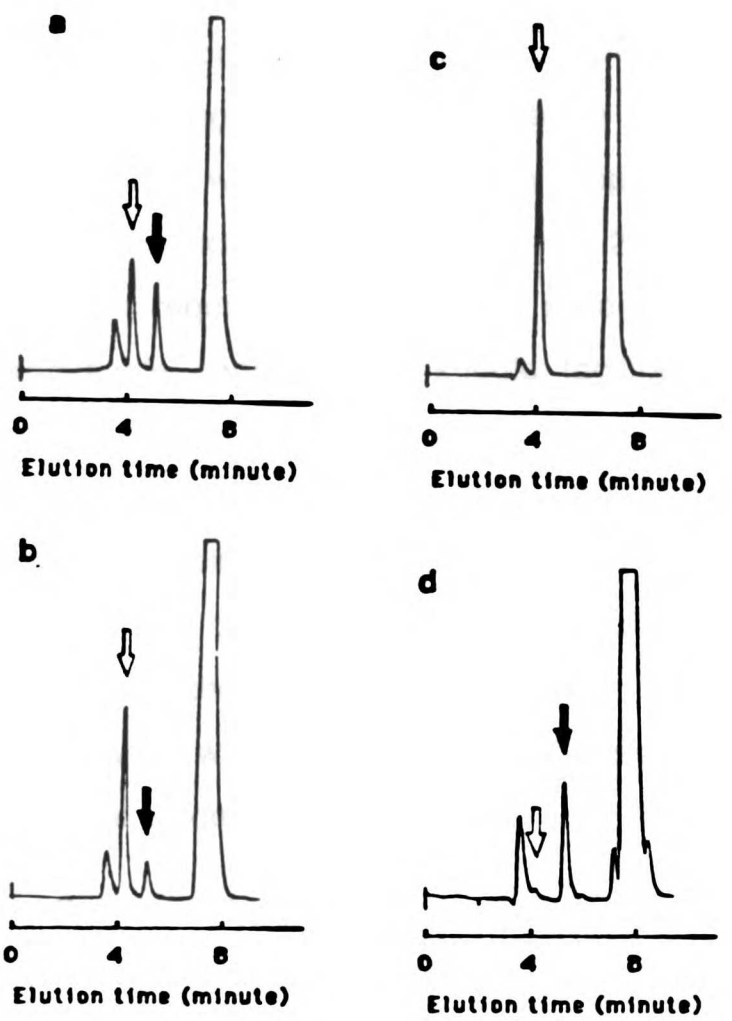
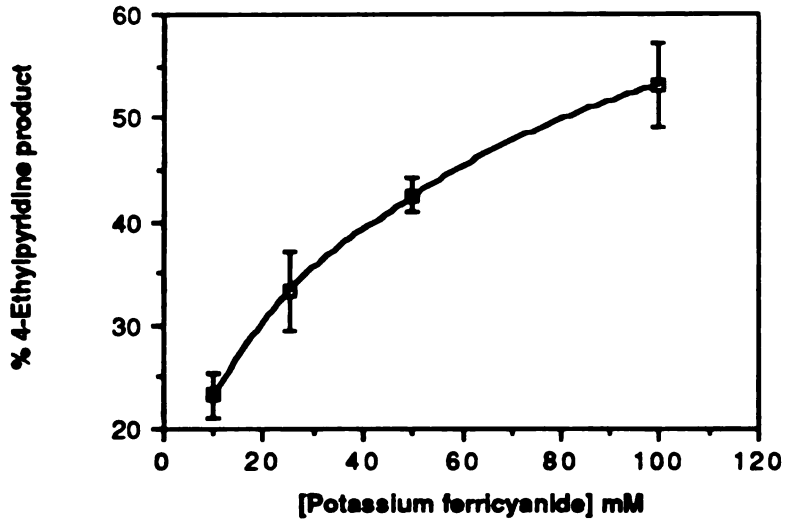


Figure 3.7.1 HPLC profiles of potassium ferricyanide oxidations of (a) DDEP, (b) [4-^{2}H]-DDEP, (c) DDIP, and (d) DDC. The open and closed arrows identify the 4-dealkyl- and 4-alkylpyridine products. The large peak at approximately 8 minutes is the substrate. All the other peaks are not turnover-dependent.

the results from the enzymatic oxidation. For DDEP the ratio of the products formed upon oxidation with ferricyanide depends on the concentration of ferricyanide and the pH (Figure 3.7.2). Formation of the 4-alkylpyridine is favored by increasing concentrations of ferricyanide and decreasing pH. The ferricyanide concentration dependence is consistent with the mechanism proposed for the oxidation of DDEP by cytochrome P-450 (Figure 3.2.15). The pathway for the 4-alkylpyridine requires the loss of a second electron; an increase in ferricyanide concentration should facilitate the loss of the second electron and thus increase the formation of the 4-alkylpyridine. The pH dependency, however, is opposite to that predicted by the mechanism in Figure 3.2.15. According to that mechanism, formation of the 4-alkylpyridines requires the loss of two protons and should therefore be facilitated by a basic environment, contrary to what is observed. The explanation for this pH dependence is not evident.

The cytochrome P-450-catalyzed oxidation of DDEP was shown to be insensitive to deuterium substitution at the 4-position. The ferricyanide oxidation of DDEP, however, exhibits a deuterium sensitivity (Figure 3.7.1). Product deuterium isotope effects of $k_H/k_D = 0.39 \pm 0.09$ and 1.69 ± 0.33 were observed for the yields of the 4-deethyl- and 4-ethylpyridine products, respectively. These translate to $k_H/k_D = 4.3$ for the 4-ethyl- to 4-deethylpyridine product ratio. Deuterium substitution increases formation of the 4-dealkylpyridine and decreases formation of 4-ethylpyridine, indicating a direct competition between loss of 4-alkyl and 4-

a)



b)

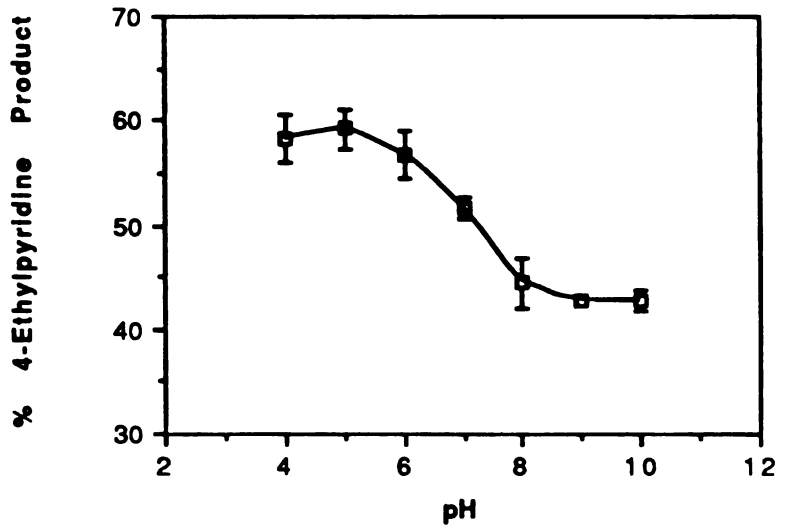


Figure 3.7.2 (a) Ferricyanide and (b) pH dependence of the production of the 4-ethylpyridine product as a % of the total pyridine products from DDEP oxidation by potassium ferricyanide. The error bars represent the standard deviations.

hydrogen moieties. This divergence in isotope effects between the enzymatic and chemical oxidations of DDEP clearly suggests that the two processes are different. In the enzymatic process, the residues surrounding the active site could influence the oxidation of DDEP to prevent the competition between the 4-ethyl and 4-hydrogen whereas ferricyanide oxidation occurs without the influence of such protein residues. A further difference between the two processes is that chemical reaction takes place in an aqueous environment whereas the enzymatic reaction takes place in a hydrophobic site. However, the stability of the 4-alkyl moiety as a radical still appears to determine whether the 4-alkyl or 4-hydrogen group is eliminated because the 4-alkyl- and 4-dealkylpyridine product ratios in the oxidation of DDC, DDEP, and DDIP by ferricyanide are consistent with those observed in the enzymatic reaction.

4.0 DIHYDROQUINOLINE

The cytochrome P-450 chromophore destruction, formation of *N*-alkylprotoporphyrin IX, and ferrochelatase inhibition properties of 4-alkyl-1,4-dihydropyridines have been exploited in studies of cytochrome P-450 and ferrochelatase. Oxidation of the dihydropyridine to its radical cation by electron abstraction by the iron-oxo species of cytochrome P-450 results in elimination of the 4-alkyl moiety as a free radical. Alkylation of a pyrrole nitrogen of the heme of cytochrome P-450 by the radical generates the inhibitor of ferrochelatase. 2,2-Dialkyl-1,2-dihydroquinolines have been reported to be oxidized to the corresponding quinolines with elimination of a 2-alkyl group (Engler and Riehm, 1885, Knovenagel, 1922, Reddelien and Thurm, 1932, and Craig, 1938) (Figure 4.1). In light of this chemical property of 2,2-dialkyl-1,2-dihydroquinolines, the oxidation of dihydroquinolines by cytochrome P-450 was investigated in order to determine if the oxidation of dihydroaromatic nitrogenous compounds to radical cations that can aromatize by elimination of an alkyl group is a general approach to

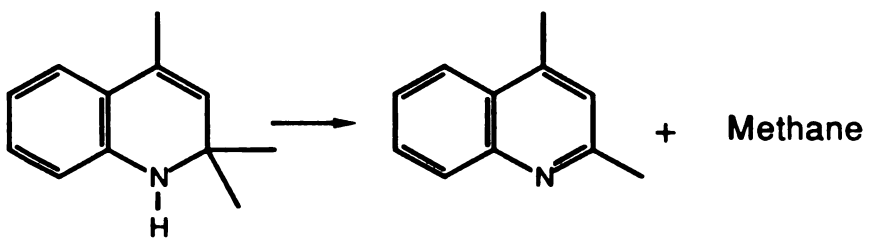


Figure 4.1 Oxidation of 2,2,4-trimethyl-1,2-dihydroquinoline to 2,4-dimethylquinoline.

the inactivation of cytochrome P-450 and the inhibition of ferrochelatase.

4.1 Synthesis of 2,2-Dialkyl-1,2-dihydroquinolines

Two dihydroquinolines, 2,4-diethyl-1,2-dihydro-2-methylquinoline (DDMQ) and 1,2-dihydro-2,2,4-trimethylquinoline (DTMQ), were tested for inactivation of cytochrome P-450 and inhibition of ferrochelatase. DTMQ was purchased from Aldrich Chemical CO. and DDMQ was synthesized following the general procedure of Layer (Layer, 1981) (Figure 4.1.1). Acid catalyzed

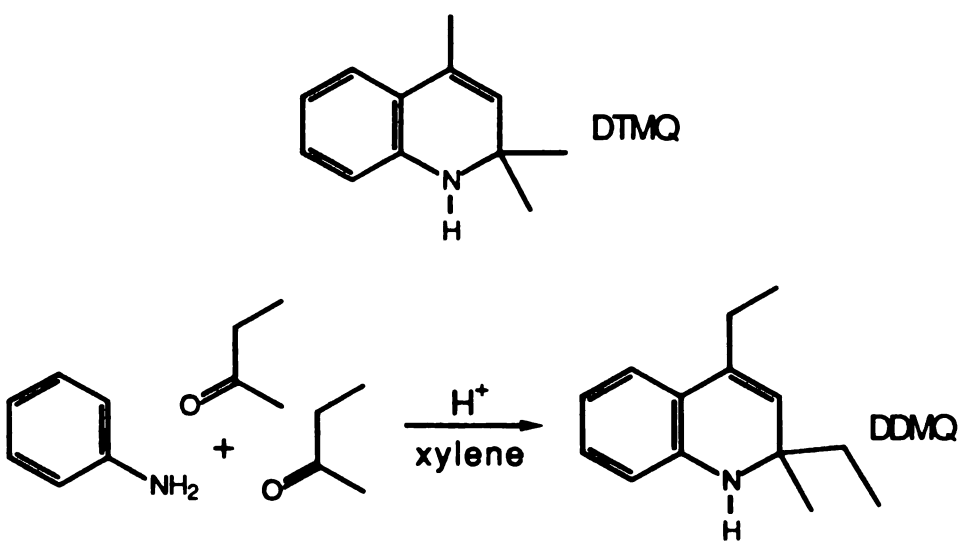


Figure 4.1.1 Structures and synthesis of the dihydroquinolines.

condensation of aniline with two molecules of butanone produced DDMQ. The H_2O generated in the condensation was removed from the reaction by the use of a Dean-Stark apparatus. DDMQ was purified

from the reaction mixture by acid-base extraction, preparative TLC, and distillation.

4.2 Destruction of Cytochrome P-450

Incubations of DDMQ with liver microsomes from rats pretreated with phenobarbital (PB) resulted in rapid but limited (8 % after 1 hour) loss of the cytochrome P-450 chromophore (Figure 4.2.1). Substitution of DDMQ with DTMQ in the incubations produced even less (<5 %) chromophore destruction. The percent loss of the cytochrome P-450 chromophore did not increase with microsomes from rats pretreated with 3-methylcholanthrene (3MC, a cytochrome

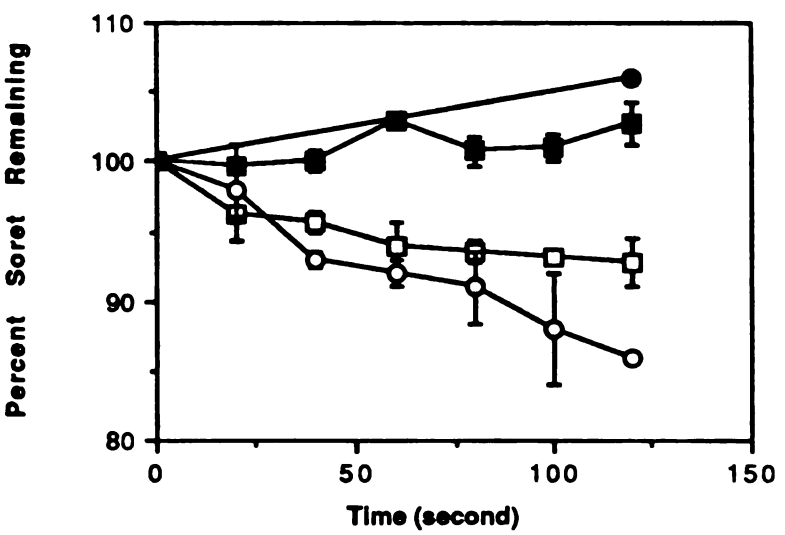


Figure 4.2.1 Destruction of the microsomal cytochrome P-450 chromophore by DDMQ. The open and closed markers represent incubations with and without NADPH, respectively. The square and circle markers indicate the loss of chromophore from microsomes made from rats pretreated with phenobarbital and dexamethasone, respectively.

P-450c inducer) or pregnenolone-16α-carbonitrile (PCN, a cytochrome P-450p inducer). However, a slightly greater loss of the chromophore was observed with microsomes made from rats pretreated with dexamethasone (DEX), which also induces cytochrome P-450p (Figure 4.2.1). The chromophore loss was turnover-dependent as indicated by the DDMQ, NADPH, and time dependence. The restricted destruction of the microsomal cytochrome P-450 levels from rats pretreated with PB, 3MC, PCN, and DEX indicates that cytochrome P-450b, c, and p are not the primary isozyme(s) involved in the oxidation of DDMQ. The uninvolvement of cytochrome P-450b and p was confirmed by assaying the 7-ethoxycoumarin *O*-deethylase and DDEP oxidase activities, respectively. Preincubations of microsomes from rats pretreated with PB with DDMQ did not alter the ability of the microsomal cytochrome P-450 to *O*-deethylate 7-ethoxycoumarin and oxidize DDEP in a time dependent manner (Table 4.2.1). The same results were obtained with purified, reconstituted cytochrome P-450b (Table 4.2.1), thus suggesting that cytochrome P-450b is not inactivated and does not oxidize DDMQ. As for DDEP, the discrepancy between the 7-ethoxycoumarin *O*-deethylase and DDEP oxidase activities of the +DDMQ and -DDMQ incubations suggests binding of DDMQ to the cytochrome P-450b and p active sites, respectively. Again, it is possible that the inhibitory property of DDMQ is a result of DDMQ binding to sites other than the active sites of the cytochromes P-450. Since DDMQ did not inactivate the DDEP oxidase activity of microsomes made from rats pretreated with PB, cytochrome P-450h, which oxidizes DDEP and is present in

Table 4.2.1 The yield of 7-ethoxycoumarin *O*-deethylation and DDEP oxidation after preincubation with and without DDMQ are presented here.

	+DDMQ		-DDMQ	
Activity	0 min[a]	5 min[b]	0 min[a]	5 min[b]
	(nmole/nmole cytochrome P-450/min)			
7-Ethoxycoumarin *O*-Deethylation				
cytochrome P-450b[c]	1.78	1.82	10.15	10.45
microsomes (PB)[d]	0.32	0.30	1.53	1.62
DDEP oxidation[e]				
microsomes (PB)[d]	0.25	0.29	0.25	0.28

[a] The length of preincubation was 0 minutes (control). [b] The length of preincubation was 5 minutes. [c] The enzyme is purifed, reconstituted cytochrome P-450b. [d] The source of the enzyme is hepatic microsomes prepared from rats pretreated with phenobarbital. [e] DDEP oxidation to 3,5-dicarbethoxy-2,6-dimethyl-4-ethylpyridine was measured.

microsomes made from PB treated rats, must also not be oxidizing DDMQ.

4.3 EPR Spin Trap Studies

EPR spin trapping studies with microsomes prepared from rats pretreated with phenobarbital were conducted by Dr. David Lukton. Incubations of DDMQ with hepatic microsomes made from rats treated with PB or DEX in the presence of POBN did not generate any

detectable EPR signals. However, a small EPR signal is detected if the incubation mixtures are extracted with CH_2Cl_2 and concentrated. Control incubations lacking DDMQ also generated small signals when extracted and concentrated thus indicating that the observed EPR signal is an artifact. Horseradish peroxidase, which is known to oxidize its substrates by one-electron abstraction, does catalyze the oxidation of DDMQ to a species that yields an EPR signal in the presence of POBN.

4.4 Formation of *N*-Alkylprotoporphyrin IX

This work was conducted by Dr. David Lukton (Lukton et al., 1988). Administration of DDMQ to rats pretreated with phenobarbital resulted in formation of *N*-methyl- and *N*-ethylprotoporphyrin IX. *N*-Ethylprotoporphyrin IX was formed in greater yield than *N*-methylprotoporphyrin IX, probably because of the greater stability of ethyl as opposed to methyl radicals. The proposed mechanism for these reactions is cytochrome P-450-catalyzed oxidation of DDQM to the radical cation which aromatizes to quinoline by elimination of either the 2-methyl or 2-ethyl group as a free radical (Figure 4.4.1). This mechanism is analogous to the mechanism proposed for DDEP oxidation. Administration of DTMQ to rats pretreated with phenobarbital generated only *N*-methylprotoporphyrin IX as expected from the DDMQ data. The *N*-alkylprotoporphyrins IX were identified by NMR, mass spectrometry, absorption spectroscopy, and co-elution with authentic samples.

4.5 Porphyrinogenic Activity of Dihydroquinolines

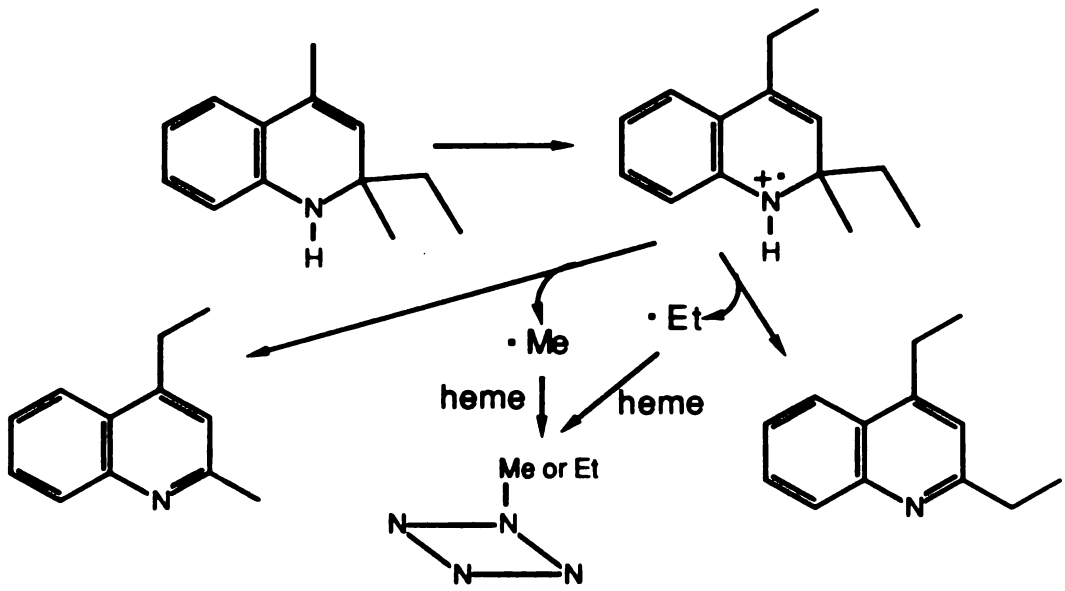


Figure 4.4.1 Oxidation of DDMQ by cytochrome P-450 to generate *N*-methyl and *N*-ethylprotoporphyrin IX.

In light of the fact that the oxidations of 2,2-dialkyl-1,2-dihydroquinolines by cytochrome P-450 causes the formation of *N*-alkylprotoporphyrins IX and that *N*-alkylprotoporphyrins IX inhibit ferrochelatase, the dihydroquinolines were tested for porphyrinogenic activity. The porphyrinogenic activity studies were conducted in the laboratory of Dr. Gerald S. Marks at Queen's University, Kingston, Ontario, Canada. Examination of cultured chick embryo hepatocytes after incubation with the dihydroquinolines indicated that dihydroquinolines induce porphyrin biosynthesis (Figure 4.5.1). Protoporphyrin IX has been determined to be the major porphyrin accumulated, suggesting that dihydroquinolines behave like dihydropyridines in inhibiting ferrochelatase (Lukton et al., 1988). The 5-aminolevulinic acid synthase activity has also been shown to be induced by the dihydroquinolines (Figure 4.5.2).

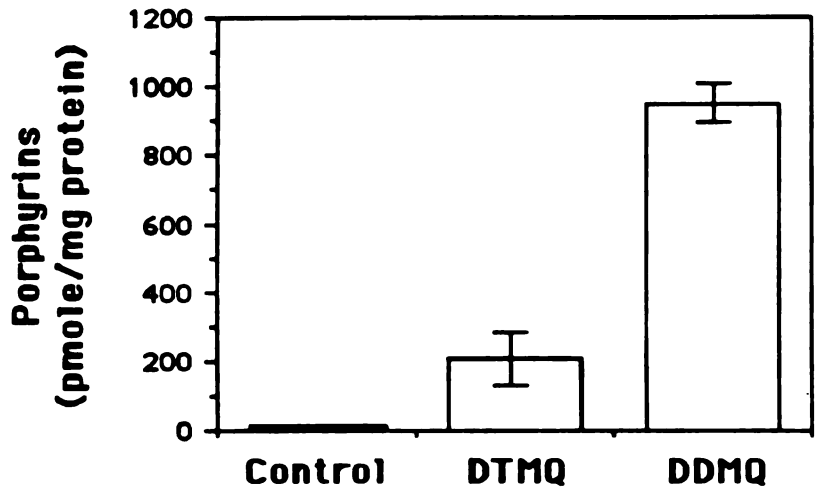


Figure 4.5.1 Accumulation of protoporphyrins 24h after administration of 95 % ethanol (control), DTMQ (100 μM), and DDMQ (100 μM) in 95 % ethanol to cultured chick embryo hepatocytes. The error bars represent the mean standard deviation.

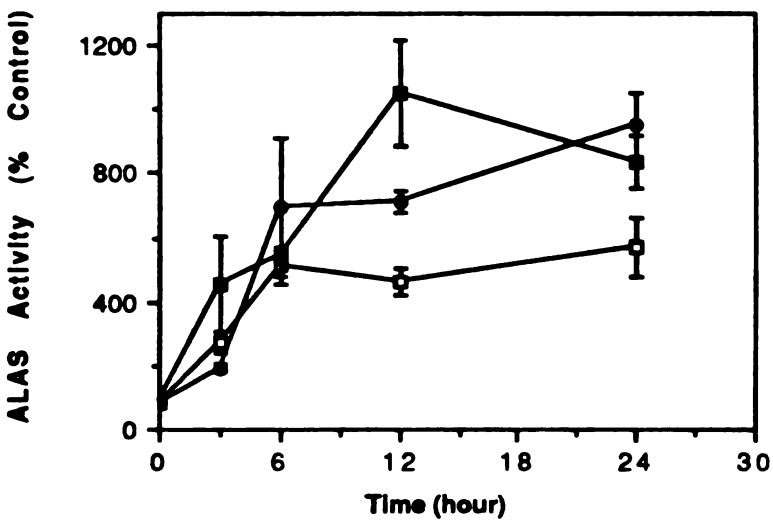


Figure 4.5.2 5-Aminolevulinic acid synthase activity at various times after exposure of chick embryo hepatocytes to (closed circles) DDEP (4 μM), (open squares) DTMQ (100 μM), and (closed squares) DDMQ (100 μM). The results are expressed as % of control. The error bars represent the standard deviations.

5-Aminolevulinic acid synthase is the first enzyme in the biosynthesis of heme and is under feedback control by the endogenous level of heme. The low level of heme that results from ferrochelatase inhibition and possibly also cytochrome P-450 induction must lead to induction of the activity of 5-aminolevulinic acid synthase. DDMQ was found to be more potent in inhibiting ferrochelatase and inducing 5-aminolevulinic acid synthase than DTMQ (Figures 4.5.1 and 4.5.2).

As expected, dihydroquinolines are oxidized to the corresponding radical cations which aromatize by extruding alkyl radicals. Alkylation of one of the pyrrole nitrogens by the free radical generates *N*-alkylprotoporphyrin IX which inhibits ferrochelatase. This similarity between the dihydroquinolines and dihydropyridines strongly suggests the generality of the radical cation-alkyl radical extrusion mechanism for cytochrome P-450-catalyzed oxidation of dihydroaromatic nitrogenous compounds.

5.0 CONCLUSIONS

Oxidations catalyzed by cytochrome P-450 have been extensively studied over the last 20 years. The mechanism of the reactions still remains unclear, although a non-concerted radical mechanism is now generally accepted for cytochrome P-450-catalyzed oxidations. The most direct evidence for a radical mechanism has been provided by the nitrogen oxidation of DDEP (Augusto et al., 1982). Incubation of DDEP with microsomes results in turnover-dependent inactivation of cytochrome P-450 and generation of spin trappable ethyl radicals. The inactivation correlates with the formation of *N*-ethylprotoporphyrin IX (Augusto et al., 1982) which inhibits ferrochelatase (Ortiz de Montellano et al., 1981). The source of the ethyl moiety has been shown to be the 4-ethyl group of DDEP (Ortiz de Montellano et al., 1981b and De Matteis et al., 1981). The ethyl radicals can be trapped by POBN, resulting in spin adducts that have been isolated and characterized (Augusto et al., 1982). The generation of spin trappable radicals is partially inhibited by the presence of cytochrome P-450 inhibitors (SKF525A and carbon monoxide). These results indicate that cytochrome P-450 is involved in the production of the spin trappable radicals. The catalytic extrusion of the ethyl radical is best explained by one-electron oxidation of DDEP to a radical cation that aromatizes by eliminating the 4-ethyl group (Figure 1.7.3.7).

It is possible that the proposed radical process is only a minor pathway of several dihydropyridine oxidation pathways catalyzed by cytochrome P-450. Since the concentration of the 4-dealkylpyridine

metabolite in the incubation of DDEP with cytochrome P-450 calculated from Table 3.2.3 is approximately 20 μM after 10 minutes of incubation, the concentration of the free 4-ethyl moiety should also be approximately 20 μM. A comparison of cytochrome P-450 chromophore destruction (Figure 3.2.1) and DDEP metabolite formation (Table 3.2.3) indicates that approximately 15 molecules of DDEP are oxidized with loss of the 4-ethyl group per prosthetic heme group that is destroyed. The ethyl groups that alkylate the heme only account for approximately 7 % of the released ethyl groups. The concentration of the spin adduct is very difficult to measure. However, since the receiver gain setting on the EPR was set to near maximum, it can be estimated to be approximately 1 μM, which is the limit of detection of EPR spectroscopy. This large discrepancy between the yield of the 4-deethylpyridine metabolite (or free 4-ethyl moiety) and concentration of the spin adduct from the cytochrome P-450-catalyzed oxidation of DDEP indicates either that the radical process is only a minor pathway of dihydropyridine oxidation or that the ethyl radicals are quenched by various microsomal components before they can be spin trapped. Another possible explanation for the discrepancy is that the radicals resulting from the 4-alkyl group of the dihydropyridines are hydroxylated by radical recombination with the Fe^{IV}-O species of cytochrome P-450 before they can escape the active site.

The formation of *N*-alkylprotoporphyrin IX by alkyl radicals extruded by the cytochrome P-450-catalyzed oxidation of dihydroaromatic nitrogenous compounds can be generalized. The

dihydroquinolines (DDMQ and DTMQ) catalytically inactivate cytochrome P-450 with concomitant formation of *N*-alkylprotoporphyrins IX that inhibit ferrochelatase.

The dihydropyridines have been suggested to be oxidized by cytochrome P-450 isozymes c, h, and p (Tephly et al., 1986, Guengerich et al., 1986, Correia et al., 1987, and Riddick et al., 1988). Studies conducted with purified, reconstituted cytochromes P-450b and c have confirmed the involvement of cytochrome P-450c in the oxidation of dihydropyridines and have established that cytochrome P-450b does not oxidize the dihydropyridines.

The proposed radical mechanism (Figure 1.7.3.7) of dihydropyridine oxidation is supported by formation of the corresponding 4-deethylpyridine metabolite in the cytochrome P-450-catalyzed oxidation of DDEP. The degree to which the 4-alkyl moieties are lost in the cytochrome P-450-catalyzed oxidation of a series of 4-alkyl-1,4-dihydropyridines is proportional to both the homolytic and heterolytic dissociation bond energies of the 4-alkyl groups (the easier the cleavage of the 4-alkyl group from the dihydropyridine, the greater the formation of the 4-dealkylpyridine metabolite). The abilities of the dihydropyridines to destroy the cytochrome P-450 chromophore and to generate spin trapped radicals also exhibit similar correlations with the degree of 4-alkyl group elimination. The correlation that exists between the stabilities of the 4-alkyl groups as free radicals and the degree of the loss of the 4-alkyl groups, destruction of the cytochrome P-450 chromophore, and generation of spin trapped radicals strongly

supports the proposed 4-alkyl radical extrusion mechanism (Figure 1.7.3.7).

The oxidation of the dihydropyridines results in production of 4-alkyl- and 4-dealkylpyridines. The mechanism for the formation of 4-dealkylpyridine is clear (Figure 5.1, path A) but the mechanism for formation of the 4-alkylpyridine is less well defined. The mechanism of 4-alkylpyridine formation was studied by deuterium substitution at the 4-position of the dihydropyridines. If loss of the 4-alkyl group competes with loss of the 4-hydrogen, then isotope effects should be observed for the pyridine metabolite product ratio, destruction of cytochrome P-450 chromophore, and generation of spin trapped radicals. Deuterium substitution has no effect on any of these parameters, however, indicating that loss of the 4-alkyl group does not compete directly with loss of the 4-hydrogen. A mechanism in which the 4-hydrogen is lost as a proton from the nitrogen cation (Figure 5.1, path B) rather than nitrogen radical cation is consistent with the absence of such isotope effects.

Recently, elimination of the 4-hydrogen as a proton from the nitrogen radical cation has been proposed (Guengerich and Bocker, 1988) following observation of a kinetic deuterium isotope effect at the 4-position on oxidation of the 4-phenyldihydropyridine analogue. The isotope effect was only observed when the oxidation was catalyzed by purified cytochrome P-450h rather than by microsomal cytochrome P-450. Since the 4-phenyldihydropyridine analogues are only oxidized to the corresponding 4-phenylpyridines, the observed

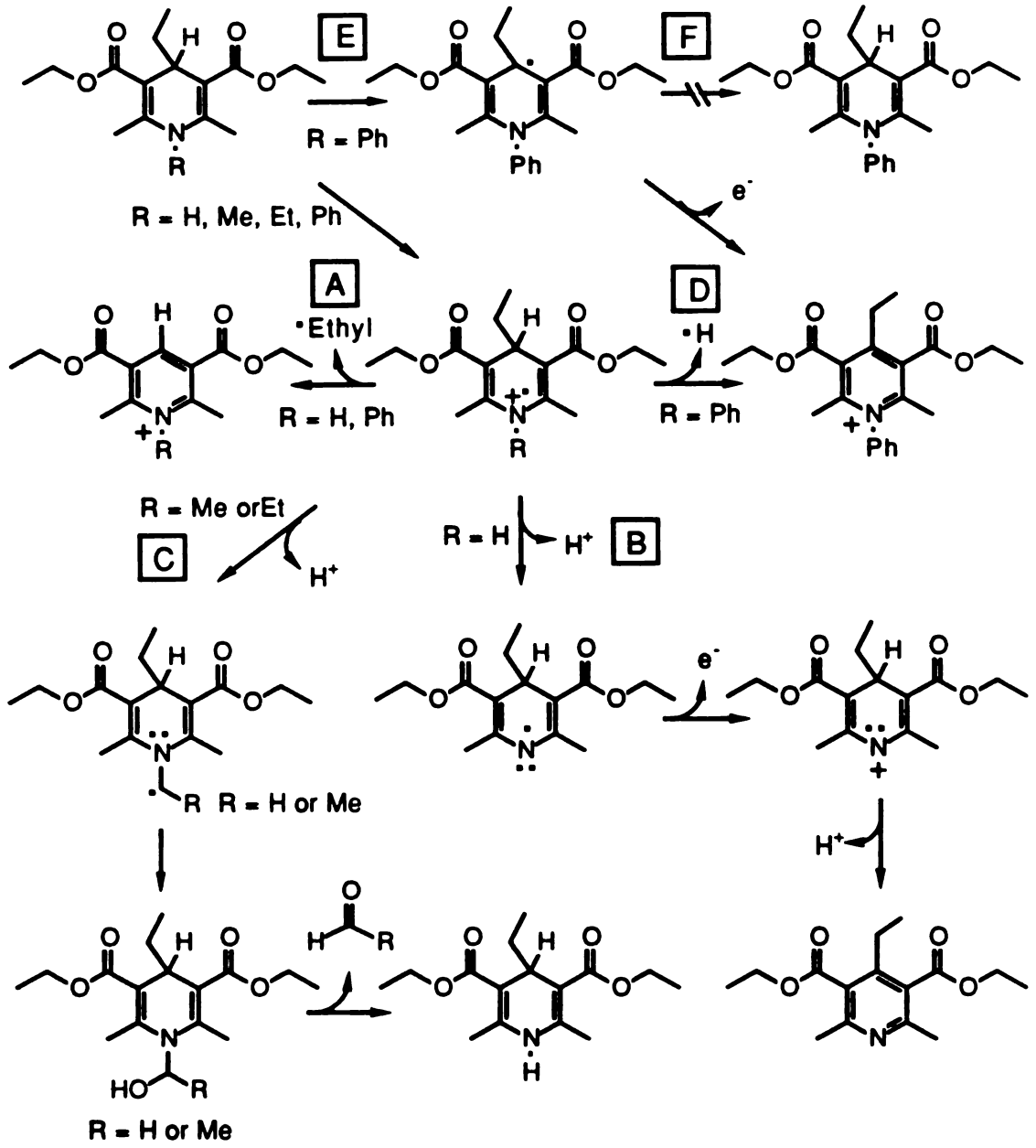


Figure 5.1 Proposed mechanisms for the oxidation of dihydropyridines by cytochrome P-450.

effect is a kinetic isotope effect on the oxidation 4-phenyldihydropyridine and not a product isotope effect resulting from competition between loss of the 4-substituent and 4-hydrogen,

as it is in the oxidation of DDEP. The difference between the two isotope effects explains the observed discrepancy between the presence (kinetic) and absence (product) of isotope effects.

Attempts to verify the mechanism of the 4-hydrogen loss by methyl, ethyl, and phenyl substitution on the nitrogen have failed. Substitution on the nitrogen should prevent the nitrogen deprotonation necessary for production of the 4-alkylpyridine metabolite according to the mechanism in Figure 5.1 (path B). Only the 4-dealkylpyridinium metabolite should therefore be generated. Cytochrome P-450, however, exclusively oxidizes *N*-alkylDDEP to DDEP (*N*-dealkylation) (Figure 5.1, path C). Aromatized *N*-alkylpyridinium metabolites are not detected. The absence of the pyridinium metabolites is unexpected, especially that of the 4-deethylpyridinium, since formation of the aromatic ring should strongly stabilize the pyridinium products. This is interesting because the *N*-dealkylation is likely to proceed through the same radical cation that is proposed for the aromatization reaction. It can therefore be construed that deprotonation of the *N*-alkyl group must occur much faster than aromatization (elimination of 4-ethyl or 4-hydrogen moiety) of the radical cation intermediate. The reason for the dominance of the *N*-alkyl deprotonation over the aromatization reaction is not clear. It is possible that a basic moiety in the active site of cytochrome P-450 facilitates the deprotonation but, to this day, no evidence exist for the presence of such a basic moiety. A more likely explanation is that the hydrogen α to the nitrogen is more acidic than the 4-hydrogen due to inductive

effects thus resulting in *N*-dealkylation exclusively. Another possible explanation is that *N*-dealkylation does not proceed through the radical cation intermediate whereas aromatization does. Hanzlik (Hall and Hanzlik, 1987 and Hall et al., 1989) has shown that electron withdrawing groups next to nitrogen result in *N*-dealkylation with large kinetic isotope effects similar to those observed for *O*-dealkylation (Foster et al., 1974 and Miwa et al., 1984). This suggests that highly electron deficient nitrogens undergo direct α hydrogen abstraction leading to *N*-dealkylation, as proposed for *O*-dealkylation. The electron withdrawing nature of the two α,β unsaturated carbonyl groups (the double bonds and ester groups) of DDEP, although not fully conjugated with the nitrogen, could force abstraction of an α hydrogen rather than a nitrogen electron abstraction by the activated oxygen species of cytochrome P-450. Since α hydrogen abstraction leads only to *N*-dealkylation, this explanation would account for the absence of aromatized products.

In order to avoid the complication introduced by the *N*-dealkylation reaction, *N*-phenylDDEP was used to test the proposal that nitrogen deprotonation precedes loss of the 4-hydrogen. The oxidation of *N*-phenylDDEP produced both the 4-ethyl and 4-deethyl-*N*-phenylpyridinium metabolites. The presence of the 4-ethyl-*N*-phenylpyridinium metabolite indicates that nitrogen deprotonation is not necessary for loss of the 4-hydrogen. Further studies, however, indicate that *N*-phenyl substitution alters the mechanism of oxidation of the dihydropyridines. A large product isotope effect

(k_H/k_D = 6) is observed with deuterium substitution at the 4-position. Phenyl substitution on the nitrogen thus alters the mechanism of dihydropyridine oxidation from one that is insensitive to one that is quite sensitive to deuterium substitution at the 4-position. The isotope effect can be explained either by direct competition between loss of the 4-ethyl and 4-hydrogen as radicals from the radical cation intermediate (Figure 5.1, paths A and D) or by abstraction of an electron from the nitrogen leading to the 4-deethyl product and abstraction of the hydrogen radical from the 4-position leading to the 4-ethyl product (Figure 5.1, paths A and E). However, radical recombination between the C-4 radical and Fe^{IV}-OH (Figure 5.1, path F) does not occur since the 4-hydroxy-*N*-phenylDDEP (carbon hydroxylation product) is not detected.

Several possible explanations exist for the change in the mechanism of dihydropyridine oxidation imposed by *N*-phenyl substitution. The phenyl moiety could alter the binding of the substrate. For an example, the dihydropyridine ring could be partially displaced from the activated oxygen species preventing oxidation of the dihydropyridine ring and/or altering the site of oxidation. This idea is supported by the formation of a polar but non-ionic metabolite that retains the dihydropyridine ring system on oxidation of *N*-phenylDDEP. Altered binding of the dihydropyridine due to the phenyl substitution could also place the 4-H near the activated oxygen species leading to abstraction of 4-hydrogen (Figure 5.1, pathway E). Alternatively, the phenyl group could influence the electronic nature of the dihydropyridine ring, causing a

change in oxidation, even though the phenyl group is not fully conjugated to the nitrogen of the dihydropyridine ring because of the steric interference from 2,6-dimethyl groups.

The electron abstraction-electron transfer sequence (Figure 5.1, pathway E), which is essentially a dehydrogenation, leads to formation of the 4-ethylpyridinium product via two consecutive one-electron oxidations of the substrate. Although dehydrogenation is not a reaction normally catalyzed by cytochrome P-450, precedents exist in the literature for such reactions. Acetaminophen has been shown to be dehydrogenated by cytochrome P-450 to *N*-acetyl-*p*-benzoquinone imine (Dahlin et al., 1984) and testosterone (Nagata et al., 1986) and valproic acid (Rettie et al., 1987) have been shown to be desaturated by cytochrome P-450 to the corresponding olefins (17β-hydroxy-4,6-androstadiene-3-one and Δ^4-valproic acid, respectively). These dehydrogenated products were not formed as artifacts by dehydration of the corresponding hydroxylated metabolites. The mechanism proposed for formation of the olefins involves two consecutive hydrogen abstractions by the activated oxygen species of cytochrome P-450. It is possible that electron transfer-deprotonation as proposed here is a more accurate description for the second hydrogen abstraction step.

6.0 EXPERIMENTAL

Materials DDC, DTMQ, POBN, 7-ethoxycoumarin, 7-hydroxycoumarin, $MgCl_2$, [1-^{2}H]-acetaldehyde (>98 atom% deuterium) and NaB^2H_4 (98 atom% deuterium) were purchased from Aldrich Chemical Co. (Milwaukee, WI); DETAPAC, EDTA, dexamethasone, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, $NADP^+$, and NADPH were purchased from Sigma Chemical Co. (St. Louis, MO); resorufin (hydroxyphenoxazone) and 7-ethoxyresorufin were purchased from Pierce Chemical Co. (Rockford, IL); 99.8% ^{18}O-labeled molecular oxygen was obtained from MSD Isotopes (St. Louis, MO); sodium phenobarbital was obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ); pregnenolone-16α-carbonitrile was obtained from Searle and CO. (Skokie, IL) as a gift; dilauroylphosphatidylcholine was purchased from Seredary Research Labs (Port Huron, MI). DDC and DTMQ were recrystalized from ethanol/H_2O and distilled, respectively, prior to use. The buffers were prepared from doubly distilled and deionized H_2O. The trace metals were removed by chelexation with chelex 100. Liver microsomes were prepared from male Sprague-Dawley rats obtained from Bantin and Kingman (Fremont, CA).

Spectroscopic Studies The electronic absorption spectra were recorded on a Hewlett-Packard 8450A UV/vis spectrophotometer. Cytochrome P-450 was quantitated using an Aminco DW2a spectrophotometer. The fluorescence of 7-hydroxycoumarin and resorufin was measured on a Perkin-Elmer model 650-10S

fluorescence spectrophotometer equipped with a Perkin-Elmer xenon power supply model 150. The EPR spectra were obtained on a Varian Model 104 spectrometer custom interfaced with an IBM XT computer. The proton NMR spectra were recorded on a Varian FT 80 or a custom built 240 MHz instrument. Deuterated chloroform and deuterated DMSO were used as NMR solvents for the non-ionic and ionic compounds, respectively. The chemical shift values are reported in parts per million relative to tetramethylsilane or the residual chloroform peak. The electron impact probe mass spectra (70 eV) and gas chromatogram/mass spectra were recorded on a Kratos MS-25 mass spectrometer. The LSIMS were obtained with glycerol as a matrix on a Kratos MS-50 mass spectrometer. Gas liquid chromatography was conducted on a Hewlett-Packard model 5890 capillary gas chromatogram equipped with a DB-5 column, flame ionization detectors, and a Hewlett-Packard 3390A integrator. HPLC was performed on a system consisting of either two Altex Model 110A pumps, an Altex solvent programmer, a Knauer variable wavelength detector, and a Hewlett-Packard 3390A integrator or two Beckman 110A pumps, a Beckman 420 controller, a Hewlett-Packard 1040A diode array UV/vis detector, a Hewlett-Packard 85 computer, and a Hewlett-Packard 3390A integrator. Oxidation potentials were determined on a Bioanalytical Systems model CV-27 cyclic voltammogram equipped with a Soltec VP-6414S X-Y recorder. Rat liver microsomes were prepared using a Sorvall Superspeed RC2-B centrifuge (Sorvall SC34 rotor) and a Beckman L-2 Ultracentrifuge (Type SW 28 rotor). Elemental analyses were

determined by the Microanalytical Laboratory of the University of California, Berkeley.

Synthesis of 3,5-Dicarbethoxy-2,6-dimethyl-4-ethyl-1,4-dihydropyridine DDEP was synthesized as reported (Augusto et al.,1982 and Ortiz de Montellano et al., 1981) by the procedure of Loev and colleagues (Loev et al., 1974). A mixture of ethyl acetoacetate (65.1 g, 0.5 mole), propionaldehyde (14.5 g, 0.25 mole), ammonium hydroxide (21.2 mL of 58% solution, 0.35 mole) and ethanol (150 mL) was heated at reflux for 24 hours and cooled to room temperature. The solution was poured into ice cold H_2O (500 mL) and the resulting yellow solid was filtered and washed with cold H_2O. Recrystallization (3 times) of the yellow solid from the ethanol/H_2O system produced pure DDEP in 55% yield. The solid was dissolved in a minimum volume of boiling 100% ethanol and H_2O was added dropwise until the solution became slightly cloudy. The ethanol/H_2O solution was placed in the refrigerator overnight to generate the crystal. The characterizations of the compound was identical to those reported in the literature.

Synthesis of 3,5-Dicarbethoxy-2,6-dimethyl-4-isopropyl-1,4-dihydropyridine DDIP was synthesized by the same procedure as DDEP, except isobutyraldehyde (18.0 g, 0.25 mole) was used instead of propionaldehyde. The characterizations of the compound was identical to those reported in the literature.

Synthesis of [4-^{2}H]-3,5-Dicarbethoxy-2,4,6-trimethyl-1,4-dihydropyridine [4-^{2}H]-DDC was synthesized by Dr. Neil E. Jacobsen.

A mixture of ethyl acetoacetate (5.91 g, 45.4 mmole), [1-^{2}H]-acetaldehyde (1.02 g, 22.7 mmole), and ammonium carbonate solution (10.7 g in 100 mL of H_2O) was stirred at 21 °C for 1.5 hours. The mixture was cooled to 4 °C and was swirled once a day for 6 days. The yellow solid was filtered and washed with H_2O. The solid was recrystallized from the ethanol/H_2O system as mentioned previously to produce fine white crystals in 35% yield: ^{1}H NMR ($CDCl_3$) 0.96 (s, 3 H, 4-methyl), 1.30 (t, 6 H, J = 7.1 Hz, O-$CH_2C\underline{H}_3$), 2.27 (s, 6 H, 2,6-dimethyls), 4.19 (q, 4 H, J = 7.1 Hz, O-$C\underline{H}_2CH_3$), and 5.86 ppm (broad s, 1 H, N-H); EIMS *m/z* 268 (M^+), 253 (base, M^+ - 15), 225 (M^+ - 43), 223 (M^+ - 45), and 197 (M^+ - 71). The integration of the residual 4-^{1}H signal (3.83, q, J = 6.5 Hz) indicated that the product was 99.3% deuterium labeled.

Synthesis of 2-Ethyl-4,4,6-trimethyldihydro-1,3-oxazine 2-Ethyl-4,4,6-trimethyldihydro-1,3-oxazine was synthesized following the procedure of Tillmanns and Ritter (Tillmanns and Ritter, 1957). To a flask containing propionitrile (27.5 g, 0.35 mole), H_2SO_4 (167 g, 91 mL of 98% solution) was added dropwise over 2 hours and then followed by a dropwise addition of 2,4-dihydroxy-2-methylpentane (41.3 g, 0.35 mole) (2 - 3 drops min^{-1}). The flask was kept cold in an ice bath for the duration of the additions. The reaction mixture was poured into stirring ice cold H_2O (350 mL). The solution was neutralized to pH 6 with NaOH solution (160 g in 250 mL of H_2O) and extracted with an equal volume of $CHCl_3$. To the aqueous phase the rest of the NaOH solution was added and then extracted with diethyl ether (500 mL, 3 times).

The combined ether layer was concentrated on a rotary evaporator and dried with K_2CO_3. The solvent was removed with the rotary evaporator and the resulting oil was distilled under vacuum (67 °C at 24 mm Hg) to yield 15% of the product: ^1H NMR ($CDCl_3$) 0.98 - 1.29 (m, 12 H, 2-$CH_2C\underline{H}_3$ and 4,4,6-trimethyls), 1.61 (d, 2 H, J = 3.2 Hz, 5-CH_2), 2.11 (q, 2 H, J = 7.6 Hz, 2-$C\underline{H}_2CH_3$), and 4.05 ppm (m, 1 H, 6-CH); EIMS *m/z* 155 (M^+), 114 (M^+ - 41), 102 (M^+ - 53), 84 (M^+ - 71), 74 (M^+ - 81), and 58 (base, M^+ - 97).

Synthesis of [2-^{2}H]-2-Ethyl-4,4,6-trimethyltetrahydro-1,3-oxazine [2-^{2}H]-Ethyl-4,4,6-trimethyltetrahydro-1,3-oxazine was synthesized following a procedure from Meyers and colleagues (Meyers et al., 1967). A solution of 2-ethyl-4,4,6-trimethyldihydro-1,3-oxazine (21.3 g, 0.137 mole) in 260 mL of a dry 50/50 mixture of ethanol/THF was placed in a dry ice-acetone bath (-40 °C). The pH was brought to 7 with a dropwise addition of HCl (9N). A mixture of NaB^2H_4 (5.85 g, 0.137 mole), NaOH (2.5 mL of 15% solution), and H_2O (21.5 mL) was added dropwise to the stirring dihydrooxazine solution, alternating with HCl (9N) to maintain the pH at 7, while the temperature of the reaction was maintained at -35 to -40 °C. Following the completion of the additions, the reaction mixture was stirred for one hour at -35 to -40 °C and then allowed to warm up to 10 °C. The reaction mixture was poured into H_2O (400 mL) containing NaOH (10 mL of 15% solution) and extracted with diethyl ether (600 mL). The ether layer was concentrated on a rotary evaporator and dried with K_2CO_3. The solvent was removed on a rotory evaporator to give the [2-^{2}H]-2-ethyl-4,4,6-

trimethyltetrahydro-1,3-oxazine in 80% yield: ^{1}H NMR ($CDCl_3$) 0.96 (t, 3 H, J = 7.5 Hz, 2-$CH_2C\underline{H}_3$), 1.11 (s, 3 H, 4-methyl), 1.16 (d, 3 H, J = 6.1 Hz, 6-methyl), 1.16 (s, 3 H, 4-methyl), 1.43 (B of ABX, 1 H, J_{AB} = 13.1 Hz, J_{BX} = 2.2 Hz, 5-CH), 1.54 (q, 2 H, J = 7.5 Hz, 2-$C\underline{H}_2CH_3$), and 3.75 ppm (ddq, 1 H, J = 11.4, 2.3, 6.1 Hz, 6-CH). A signal at 4.15 (t, 1 H, J = 5.3 Hz) present in the nondeuterated compound was absent in the deuterated sample.

Synthesis of [4-^{2}H]-3,5-Dicarbethoxy-2,6-dimethyl-4-ethyl-1,4-dihydropyridine [4-^{2}H]-DDEP was synthesized by Dr. Neil E. Jacobsen. A mixture of [2-^{2}H]-4-ethyl-4,4,6-trimethyltetrahydro-1,3-oxazine (4.3 g, 27.1 mmole), H_2O (100 mL), and concentrated HCl (4.55 mL, 54.6 mmole) was heated at 95 °C for one hour. The mixture was then cooled to room temperature, diluted with H_2O (25 mL), and neutralized to pH 8 with NaOH (10.6 mL of 15% solution). Ethyl acetoacetate (7.06 g, 54.4 mmole) and pulverized ammonium carbonate (13.0 g, 136 mmole) were added and the mixture was capped and stirred for 4 days at room temperature. The yellow solid that formed was filtered, washed with H_2O, and recrystalized from the ethanol/H_2O system to give [4-^{2}H]-DDEP in 52% yield: mp 106-107 °C (lit. mp of the unlabeled compound 110 °C) (Engelmann, F., 1885); UV (ethanol) λ_{max} 232 (255 shoulder) and 350 nm; ^{1}H NMR ($CDCl_3$) 0.75 (t, 3 H, J = 7.5 Hz, 4-$CH_2C\underline{H}_3$), 1.29 (t, 6 H, J = 7.1 Hz, O-$CH_2C\underline{H}_3$), 1.36 (q, 2 H, J = 7.5 Hz, 4-$C\underline{H}_2CH_3$), 2.29 (s, 6 H, 2,6-dimethyls), 3.91 (t, residual 4-^{1}H, J = 5.6 Hz), 4.16 and 4.21 (AB of ABX_3, 4 H, J_{AB} = 10.8 Hz, J_{AX} = J_{BX} = 7.1 Hz, O-$C\underline{H}_2CH_3$), and 5.52 ppm (broad s, 1 H, N-H); EIMS m/z 282 (M^+), 253 (base peak, M^+ -

29), 191 (M^+ - 91), 180 (M^+ - 102), and 151 (M^+ - 131). This compound differs from the unlabelled compound in the NMR signals at 1.36 (dq, 2 H, J = 5.7 and 7.5 Hz) and 3.91 ppm (t, 1 H, J = 5.6 Hz) and in the molecular ion by one in the mass spectrum. The integration of the residual 4-^{1}H signal (3.91 ppm) indicated that the product was 98.3% deuterium labeled.

Synthesis of 3,5-Dicarbethoxy-2,6-dimethyl-4-ethylpyridine 3,5-Dicarbethoxy-2,6-dimethyl-4-ethylpyridine was synthesized by the procedure of Loev and Snader (Loev and Snader, 1965). To a solution of DDEP (5.0 g, 18 mmole) in glacial acetic acid (15 mL), $NaNO_2$ (2.7 g, 39 mmole) was added over several hours at room temperature. The solution was then poured into ice cold H_2O (200 mL) and extracted with diethyl ether (2 x 200 mL). The ether layer was extracted with HCl (3N) (3 x 100 mL) and the aqueous layer was neutralized with $NaHCO_3$. The product was extracted with CH_2Cl_2 and the solvent was dried with Na_2SO_4. The solvent was removed with the rotary evaporator and the resulting oil was distilled under vacuum (175-180 °C at 25 mmHg) to produce 3,5-dicarbethoxy-2,6-dimethyl-4-ethylpyridine in 60% yield: UV (methanol) λ_{max} 270 nm; ^{1}H NMR ($CDCl_3$) 1.30 (two overlapping triplets, 9 H, J = 7.5 Hz, 4-$CH_2C\underline{H}_3$ and O-$CH_2C\underline{H}_3$), 2.40 (s, 6 H, 2,6-dimethyls), 2.65 (q (partially buried under the singlet at 2.40 ppm), 2 H, J = 7.7 Hz, 4-$C\underline{H}_2CH_3$), and 4.30 ppm (q, 4 H, J = 6.7 Hz, O-$C\underline{H}_2CH_3$).

Synthesis of 3,5-Dicarbethoxy-2,4,6-trimethylpyridine 3,5-Dicarbethoxy-2,4,6-trimethylpyridine was synthesized using DDC as the starting material by following the same procedure as the 4-

ethylpyridine derivative. The 4-methylpyridine analogue was not distilled: ^{1}H NMR ($CDCl_3$) 1.38 (t, 6 H, J = 7.1 Hz, O-$CH_2C\underline{H}_3$), 2.27 (s, 3 H, 4-CH_3), 2.51 (s, 6 H, 2,6-dimethyls), and 4.41 ppm (q, 4 H, J = 7.1 Hz, O-$C\underline{H}_2CH_3$).

Synthesis of 3,5-Dicarbethoxy-2,6-dimethylpyridine 3,5-Dicarbethoxy-2,6-dimethylpyridine was synthesized using DDIP as used for the starting material and the same procedure as the 4-ethylpyridine analogue. The product precipitated when the aqueous layer was neutralized and thus organic extraction was not necessary. The precipitate was filtered to give 75% yield. The solid was crystallized from hexane: mp 68-70 °C; UV (methanol) λ_{max} 234 and 272 (280 shoulder) nm; ^{1}H NMR ($CDCl_3$) 1.40 (t, 6 H, J = 7.5 Hz, O-$CH_2C\underline{H}_3$), 2.82 (s, 6 H, 2,6-dimethyls), 4.33 (q, 4 H, J = 7.5 Hz, O-$C\underline{H}_2CH_3$), and 8.60 ppm (s, 1 H, 4-CH).

Synthesis of 3,5-Dicarbethoxy-2,6-dimethyl-4-ethyl-*N*-phenyl-1,4-dihydropyridine A solution of ethyl acetoacetate (65.1 g, 0.5 mole), propionaldehyde (14.5 g, 0.25 mole), and aniline (23.3 g, 0.25 mole) in ethanol (50 mL) was heated at reflux overnight. The mixture was then cooled to room temperature, poured into ice cold H_2O (500 mL), and extracted with diethyl ether (3 x 250 mL). The combined ether layer was dried over Na_2SO_4 and the solvent was removed on a rotary evaporator. The resulting crude oil was subjected to silica gel (mesh 230-400) flash column chromatography using 8% (v/v) ethyl acetoacetate in hexane as the solvent. The column fractions were monitored by thin layer chromatography with 50% (v/v) ethyl acetate in hexane used as the

solvent. The desired fractions were pooled and the solvent was removed with a rotary evaporator. The resulting oil was crystalized and recrystalized twice from the ethanol/H_2O system to give the *N*-phenyl derivative in very low 1.2% yield: mp 84-85 °C; UV (methanol) λ_{max} 236 and 345 nm; ^1H NMR ($CDCl_3$) 0.90 (t, 3 H, J = 7.1 Hz, 4-$CH_2C\underline{H}_3$), 1.25 (t, 6 H, J = 6.9 Hz, O-$CH_2C\underline{H}_3$), 2.01 (s, 6 H, 2,6-dimethyls), 3.94 (t, 1 H, J = 6.3 Hz, 4-CH), 4.20 (q, 4 H, J = 7.2 Hz, O-$C\underline{H}_2CH_3$), and 7.09-7.19 and 7.38-7.47 ppm (m, 5 H, aromatic); EIMS *m/z* (assignment, relative abundance %) 357 (M^+, 2), 328 (M^+ - 29, 100), 312 (M^+ - 45, 40), 300 (M^+ - 57, 50), and 272 (M^+ - 85, 55); elemental analysis for $C_{20}H_{27}NO_4$: calculated C,70.56; H, 7.61; N,3.92, found C, 70.58; H, 7.70; N, 3.98.

Synthesis of 3,5-Dicarbethoxy-2,6-dimethyl-4-isopropyl-*N*-phenyl-1,4-dihydropyridine This compound was synthesized by the procedure described for the synthesis of the 4-ethyl analogue except that isobutyraldehyde was used instead of propionaldehyde as the starting material. UV (methanol) λ_{max} 242 and 330 nm; ^1H NMR ($CDCl_3$) 0.85 (d, 6 H, J = 7.3 Hz, 4-CH($C\underline{H}_3$)$_3$), 1.27 (t, 6 H, J = 7.1 Hz, O-$CH_2C\underline{H}_3$), 2.02 (s, 6 H, 2,6-dimethyls), 3.92 (d, 1 H, J = 6.0 Hz, 4-CH), 4.15 (q, 4 H, J = 7.3 Hz, O-$C\underline{H}_2CH_3$), and 7.02-7.12 and 7.30-7.40 ppm (m, 5 H, aromatic); EIMS *m/z* (assignment, relative abundance %) 371 (M^+, not detected), 328 (M^+ - 43, 100), 300 (M^+ - 71, 10), and 272 (M^+ - 99, 15); elemental analysis for $C_{21}H_{29}NO_4$, calculated C, 71.13; H, 7.87; N, 3.77, found C, 71.06; H,7.84; N, 3.93.

Synthesis of [1-^{2}H]-Propionaldehyde Concentrated HCl (31.5 mL) was added dropwise to a solution of [2-^{2}H]-2-ethyl-4,4,6-

trimethyltetrahydro-1,3-oxazine (24.4 g, 0.161 mole) in H_2O (250 mL). The resulting mixture was heated at 90 °C for an hour before cooling to room temperature. The pH of the solution was raised to 7 with NaOH (15% solution) and [1-^{1}H]-propionaldehyde was distilled out of the reaction mixture in 68% yield; ^{1}H NMR ($CDCl_3$) 1.16 (t, 3 H, J = 6.8 Hz, CH_3) and 2.50 ppm (q, 3 H, J = 7.0 Hz, CH_2). The singlet at 9.75 ppm (1-CH), which is present in the unlabelled compound, is absent.

Synthesis of [4-^{2}H]-3,5-Dicarbethoxy-2,6-dimethyl-4-ethyl-*N*-phenyl-1,4-dihydropyridine A mixture of aniline (10.2 g, 0.11 mole), ethyl acetoacetate (28.6 g, 0.22 mole), [1-^{2}H]-propionaldehyde (7.6 g, 0.11 mole), and ethanol (50 mL) was heated at 100 °C for 24 hours. The mixture was then cooled, poured into ice cold H_2O (200 mL), and extracted with diethyl ether (3 x 200 mL). The combined ether layer was dried with K_2CO_3 and the solvent was removed with a rotary evaporator. The resulting oil was purified by flash chromatography using silica gel mesh 230-400 with 8% (v/v) ethyl acetate in hexane as the solvent. The fractions were analyzed by thin layer chromatography using 50% (v/v) ethyl acetate in hexane as the solvent. The desired fractions were pooled and the solvent was removed with a rotary evaporator. The resulting oil was crystallized from the ethanol/H_2O system: ^{1}H NMR ($CDCl_3$) 0.89 (t, 3 H, J = 7.2 Hz, 4-$CH_2C\underline{H}_3$), 1.30 (t, 6 H, J = 7.2 Hz, O-$CH_2C\underline{H}_3$), 2.00 (s, 6 H, 2,6-dimethyls), 4.20 (q, 4 H, J = 7.1 Hz, O-$C\underline{H}_2CH_3$), and 7.09-7.18 and 7.38-7.46 ppm (m, 5 H, aromatic); EIMS *m/z* 358 (M^+, 3), 329 (M^+ - 29, 100), 313 (M^+ - 45, 32), 301 (M^+ - 57, 28), and 273

(M^+ - 85, 45). The signal corresponding to 4-H at 3.94 ppm is not present indicating quantitative incorporation of deuterium.

Synthesis of 3,5-Dicarbethoxy-2,6-dimethyl-4-ethyl-*N*-ethyl-1,4-dihydropyridine *N*-EthylDDEP was synthesized as previously reported (Augusto et al., 1982). A mixture consisting of ethyl acetoacetate (65 g, 0.5 mole), propionaldehyde (16.5 g, 0.25 mole), ethylamine hydrochloride (30.6 g, 0.375 mole), and pyridine (49.4 g, 0.625 mole) was heated at 100 °C for one hour and then cooled to room temperature. The reaction mixture was poured into ice cold H_2O (400 mL). The resulting oil was extracted into diethyl ether (3 x 250 mL). The ether layer was dried with K_2CO_3 and the solvent was removed. The oil was dissolved in a minimum volume of ethanol and placed in a dry ice/acetone bath to force the precipitation of the oil. The precipitates were allowed to settle and the ethanol solution was decanted and replaced with fresh ethanol, which has been pre-chilled in a dry ice/acetone bath. The precipitates were filtered and washed with cold H_2O. Yellow crystals were obtained in 50% yield after recrystallization (three times) from an ethanol/H_2O system: UV (ethanol) λ_{max} 238 (260 shoulder) and 345 nm; ^1H NMR ($CDCl_3$) 0.75 (t, 3 H, J = 7.2 Hz, 4-$CH_2C\underline{H}_3$), 1.15 (t, 3 H, J = 7.1 Hz, *N*-$CH_2C\underline{H}_3$), 1.29 (t, 6 H, J = 6.9 Hz, O-$CH_2C\underline{H}_3$), 2.39 (s, 6 H, 2,6-dimethyls), 3.73 (m (an overlap of q and d), 3 H, J = 7.4 Hz (*N*-$C\underline{H}_2CH_3$) and J = 6.0 Hz (4-CH)), and 4.18 ppm (q, 4 H, J = 7.0 Hz, O-$C\underline{H}_2CH_3$).

Synthesis of 3,5-Dicarbethoxy-2,6-dimethyl-4-isopropyl-*N*-ethyl-1,4-dihydropyridine This compound was synthesized by the

procedure used for synthesis of the 4-ethyl analogue (Augusto et al., 1982) except that isobutyraldehyde (18 g, 0.25 mole) was used as the starting material instead of propionaldehyde. The yield for the 4-isopropyl analogue was 29%: mp 52-54 °C; UV λ_{max} 241 and 338 nm; ^{1}H NMR ($CDCl_3$) 0.74 (d, 6 H, J = 6.7 Hz, 4-CH($C\underline{H}_3$)$_3$), 1.15 (t, 3 H, J = 7.0 Hz, N-$CH_2C\underline{H}_3$), 1.28 (t, 6 H, J = 6.8 Hz, O-$CH_2C\underline{H}_3$), 2.40 (s, 6 H, 2,6-dimethyls), 3.69 (q, 2 H, J = 7.3 Hz, N-$C\underline{H}_2CH_3$), and 4.17 ppm (q, 4 H, J = 6.6 Hz, O-$C\underline{H}_2CH_3$); EIMS *m/z* (assignment, relative abundance %) 323 (M^+, 1), 280 (M^+ - 43, 100), 253 (M^+ - 70, 30), and 224 (M^+ - 99, 15); elemental analysis for $C_{18}H_{29}NO_4$, calculated C, 66.85; H, 9.04; N, 4.33, found C, 66.72; H, 8.98; N, 4.28.

Synthesis of 3,5-Dicarbethoxy-2,6-dimethyl-4-ethyl-*N*-methyl-1,4-dihydropyridine This compound was synthesized in 53% yield by the procedure described for preparation of the *N*-ethyl analogue (Augusto et al., 1982) except that the length of the reaction was increased from one to two hours and that methylamine hydrochloride (33.8 g, 0.5 mole) was used as the starting material instead of ethylamine hydrochloride: mp 86-88 °C; UV λ_{max} 234 and 345 nm; ^{1}H NMR ($CDCl_3$) 0.66 (t, 3 H, J = 7.2 Hz, 4-$CH_2C\underline{H}_3$), 1.23 (t, 6 H, J = 6.7 Hz, O-$CH_2C\underline{H}_3$), 2.35 (s, 6 H, 2,6-dimethyls), 3.09 (s, 3 H, *N*-methyl), 3.83 (t, 1 H, J = 6.6 Hz, 4-CH), and 4.12 ppm (q, 4 H, J = 6.9 Hz, O-$C\underline{H}_2CH_3$); EIMS *m/z* (assignment, relative abundance %) 295 (M^+, 8), 266 (M^+ - 29, 100), 250 (M^+ - 45, 70), 238 (M^+ - 57, 80), and 210 (M^+ - 85, 80); elemental analysis on $C_{16}H_{25}NO_4$, calculated C, 65.06; H, 8.53; N, 4.74, found C, 65.23; H, 8.39; N, 4.69.

Synthesis of 3,5-Dicarbethoxy-2,6-dimethyl-4-isopropyl-*N*-methyl-1,4-dihydropyridine This compound was synthesized in the same manner as *N*-methylDDEP in 55% yield except that isobutyraldehyde (18 g, 0.25 mole) was used as the starting material instead of propionaldehyde: mp 61-63 °C; UV λ_{max} 236 and 340 nm; ^{1}H NMR ($CDCl_3$) 0.72 (d, 6 H, J = 6.6, 4-CH($C\underline{H}_3$)$_3$), 1.29 (t, 6 H, J = 7.3, 4-$CH_2C\underline{H}_3$), 2.40 (s, 6 H, 2,6-dimethyls), 3.13 (s, 3 H, *N*-methyl), 3.87 (d, 1 H, J = 7.1 Hz, 4-CH), and 4.18 ppm (q, 4 H, J = 7.3 Hz, O-$C\underline{H}_2CH_3$); EIMS *m/z* (assignment, relative abundance %) 309 (M^+, 2), 266 (M^+ - 43, 100), 238 (M^+ - 71, 70), and 210 (M^+ - 99, 72); elemental analysis on $C_{17}H_{27}NO_4$, calculated C, 65.99; H, 8.80; N, 4.53, found C, 66.15; H, 8.63; N, 4.44.

Synthesis of 3,5-Dicarbethoxy-2,6-dimethyl-4-ethyl-*N*-ethylpyridinium Nitrate This compound was obtained through the modified procedure of Sugiyama and colleagues (Sugiyama et al., 1964). Concentrated nitric acid (2 mL) was added dropwise to a stirring solution of *N*-ethylDDEP (1 g, 3.6 mmole) in ethanol (50 mL), and the resulting solution was stirred at room temperature for 48 hours. After 48 hours a second aliquot of concentrated nitric acid (2 mL) was added. The reaction mixture was stirred for another 48 hours. The ethanol was removed on a rotary evaporator, and the residue was partitioned between 1:1 H_2O/hexane (200 mL total). The H_2O layer was lyophilized to give the desired pyridinium salt in 25% yield: UV (methanol) λ_{max} 281 nm; ^{1}H NMR ([$^2H_6$]-DMSO) 1.10-1.53 (m, 12 H, 4-$CH_2C\underline{H}_3$, O-$CH_2C\underline{H}_3$, and *N*-$CH_2C\underline{H}_3$), 2.63 (partially obscured q, 2 H, J = 7.0 Hz, 4-$C\underline{H}_2CH_3$), 2.79 (s, 6 H, 2,6-dimethyls), and 4.35-

4.72 ppm (m, 6 H, O-C$\underline{H_2}CH_3$ and *N*-C$\underline{H_2}CH_3$); LSIMS (glycerol) *m/z* 308 ($M^+ - NO_3$).

Synthesis of 3,5-Dicarbethoxy-2,6-dimethyl-*N*-ethylpyridinium Nitrate This compound was obtained in 38% yield by oxidation of *N*-ethylDDIP (1 g, 3.1 mmole) with concentrated nitric acid (4 mL) by the procedure described for the preparation of the 3,5-dicarbethoxy-2,6-dimethyl-4-ethyl-*N*-ethylpyridinium nitrate analogue: UV (methanol) λ_{max} 281 nm; ^1H NMR ([$^2H_6$]-DMSO) 1.26-1.55 (m, 9 H, O-$CH_2C\underline{H_3}$ and *N*-$CH_2C\underline{H_3}$), 3.04 (s, 6 H, 2,6-dimethyls), 4.25-4.82 (m, 6 H, O-C$\underline{H_2}CH_3$ and *N*-C$\underline{H_2}CH_3$), and 8.88 ppm (s, 1 H, 4-CH); LSIMS (glycerol) *m/z* 280 ($M^+ - NO_3$).

Synthesis of 3,5-Dicarbethoxy-2,6-dimethyl-4-ethyl-*N*-methylpyridinium Nitrate This compound was obtained in 42 % yield from the oxidation of *N*-methylDDEP (1 g, 3.4 mmole) by concentrated nitric acid (4 mL) as described for the preparation of the 4-ethyl-*N*-ethylpyridinium nitrate analogue: UV (methanol) λ_{max} 281 nm; ^1H NMR ([$^2H_6$]-DMSO) 1.08-1.42 (m, 9 H, O-$CH_2C\underline{H_3}$ and 4-$CH_2C\underline{H_3}$), 2.36 (q, 2 H, J = 6.2 Hz, 4-C$\underline{H_2}CH_3$), 2.72 (s, 6 H, 2,6-dimethyls), 4.03 (s, 3 H, *N*-methyl), and 4.51 ppm (q, 4 H, J = 7.3 Hz, O-C$\underline{H_2}CH_3$); LSIMS (glycerol) *m/z* 294 ($M^+ - NO_3$).

Synthesis of 3,5-Dicarbethoxy-2,6-dimethyl-*N*-methylpyridinium Nitrate 3,5-Dicarbethoxy-2,6-dimethyl-*N*-methylpyridinium nitrate was obtained from oxidation of *N*-methylDDIP (1 g, 3.2 mmole) with concentrated nitric acid (4 mL) in the same manner as mentioned for the oxidation of *N*-methylDDEP in

62% yield: UV (methanol) λ_{max} 281 nm; ^1H NMR ([$^2H_6$]-DMSO) 1.36 (t, 6 H, J = 7.1 Hz, O-$CH_2\underline{CH_3}$), 2.99 (s, 6 H, 2,6-dimethyls), 4.16 (s, 3 H, *N*-methyl), 4.43 (q, 4 H, J = 7.1 Hz, O-$\underline{CH_2}CH_3$), and 8.94 ppm (s, 1 H, 4-CH); LSIMS (glycerol) *m/z* 266 ($M^+ - NO_3$).

Synthesis of 3,5-Dicarbethoxy-2,6-dimethyl-4-ethyl-*N*-phenylpyridinium Nitrate This compound was obtained by oxidation of *N*-phenylDDEP (1 g, 2.8 mmole) as described for the preparation of the *N*-ethylpyridinium nitrate analogue, except that the product partitions into the ethyl acetate layer instead of the H_2O layer. The ethyl acetate was removed with a rotary evaporator and the resulting residue was partitioned between 1:1 methanol/hexane (200 mL total). Removal of the methanol layer provided the desired compound in 60% yield: UV (methanol) λ_{max} 282 nm; ^1H NMR ([$^2H_6$]-DMSO) 1.26-2.10 (m, 9 H, O-$CH_2\underline{CH_3}$ and 4-$CH_2\underline{CH_3}$), 2.44 (s, 6 H, 2,6-dimethyls), 2.78 (q, 2 H, J = 7.6 Hz, 4-$\underline{CH_2}CH_3$), 4.49 (q, 4 H, J = 7.0 Hz, O-$\underline{CH_2}CH_3$), and 7.35-7.75 ppm (m, 5 H, aromatic); LSIMS (glycerol) *m/z* 356 ($M^+ - NO_3$).

Synthesis of 3,5-Dicarbethoxy-2,6-dimethyl-*N*-phenylpyridinium Nitrate This compound was obtained by oxidation of *N*-phenylDDIP (1 g, 2.7 mmole) as reported for 4-ethyl-*N*-phenylpyridinium nitrate analogue except that the product partitioned into the aqueous layer. Lyophilization of the aqueous layer resulted in the desired compound in 57% yield: UV (methanol) λ_{max} 283 nm; ^1H NMR ([$^2H_6$]-DMSO) 1.37 (t, 6 H, J = 7.1 Hz, O-$CH_2\underline{CH_3}$), 2.54 (s, 6 H, 2,6-dimethyls), 4.46 (q, 4 H, J = 7.0 Hz, O-$\underline{CH_2}CH_3$),

7.55-7.78 (m, 5 H, aromatic), and 9.19 ppm (s, 1 H, 4-CH); LSIMS (glycerol) *m/z* 328 ($M^+ - NO_3$).

Synthesis of 3,5-Dicarbethoxy-2,6-dimethyl-4-ethyl-4-hydroxy-*N*-phenyl-1,4-dihydropyridine 3,5-Dicarbethoxy-2,6-dimethyl-4-ethyl-*N*-phenylpyridinium nitrate (1 mg, 2.4 μmole) was dissolved in H_2O (5 mL) and the pH was adjusted to 10 using NaOH (15% solution). The absorption spectrum changed instantly from λ_{max} = 280 nm to λ_{max} = 252 and 315 nm. The latter dihydropyridine spectrum was stable even under acidic conditions (pH 3). The basic solution was extracted with CH_2Cl_2 (10 mL). The solvent was removed with a stream of N_2 to give the corresponding 4-hydroxylated 1,4-dihydropyridine: UV (methanol) λ_{max} 252 and 315 nm; ^1H NMR ($CDCl_3$) 1.22-1.47 (m, 9 H, 4-CH_2CH_3 and O-CH_2CH_3), 1.53 (s, 6 H, 2,6-dimethyls), 2.57 (q, 2 H, J = 7.5 Hz, 4-CH_2CH_3), 4.37 (q, 4 H, J = 7.1 Hz, O-CH_2CH_3), and 7.10-7.68 ppm (m, 5 H, aromatic); EIMS (assignment, relative abundance %) *m/z* 373 (M^+, 0), 327 (M^+ - 46, 76), 312 (M^+ - 61, 86), 298 (M^+ - 75, 100), and 282 (M^+ - 91, 34). The NMR signal corresponding to 4-H of *N*-phenylDDEP at 3.94 ppm is absent.

Synthesis of 2,4-Diethyl-1,2-dihydro-2-methylquinoline 2,4-Diethyl-1,2-dihydro-2-methylquinoline was synthesized based on the general approach of Layer (Layer, 1981). A mixture of aniline (14.0 g, 0.15 mole), butanone (32.4 g, 0.45 mole), *p*-toluenesulfonic acid (0.57 g, 3.0 mmole), and xylene (50 mL) was refluxed for 4 days in an apparatus fitted with a Dean-Stark trap to remove H_2O evolved in the reaction. The reaction mixture was cooled to room

temperature and extracted with HCl solution (0.5 N, 3 x 200 mL). The aqueous layer was neutralized with $NaHCO_3$ and back extracted with diethyl ether (3 x 200 mL). The combined ether layers were dried with K_2CO_3 and the solvent was removed with a rotary evaporator. The product was isolated on silica preparatory thin layer chromatography plates (2000μm) with 25 % methylene chloride in hexane as the solvent system. The fluorescent band was collected and the resulting oil was distilled under vacuum to yield the product which was stored in sealed ampules at -20 °C: ^1H NMR ($CDCl_3$) 0.90 (t, 3 H, J = 6.8 Hz, 2-$CH_2C\underline{H}_3$), 1.15 (t, 3 H, J = 7.5 Hz, 4-$CH_2C\underline{H}_3$), 1.21 (s, 3 H, 2-methyl), 1.45 (q, 2 H, J = 6.4 Hz, 2-$C\underline{H}_2CH_3$), 2.34 (q, 2 H, J = 7.3 Hz, 4-$C\underline{H}_2CH_3$), 3.46 (broad s, 1 H, NH), 5.18 (s, 1 H, 3-CH), and 6.36-7.13 ppm (m, 4 H, aromatic); EIMS *m/z* 201 (M^+) and 172 (M^+ - 29, base peak); elemental analysis on $C_{14}H_{19}N$, calculated C, 83.53; H, 9.51; N, 6.96, found C, 83.51; H, 9.55; N, 6.89.

Preparation of Rat Liver Microsomes Rat liver microsomes were prepared from rats weighing 250-260 g and injected intraperitoneally once a day for four days with sodium phenobarbital (80 mg Kg^{-1} rat), 3-methylcholanthrene (40 mg Kg^{-1}), pregnenolone-16α-carbonitrile (50 mg Kg^{-1}), or dexamethasone (100 mg Kg^{-1}, 2 days and 80 mg Kg^{-1}, 2 days). The sodium phenobarbital was injected with H_2O as the vehicle, and the 3-methylcholanthrene, pregnenolone-16α-carbonitrile, and dexamethasone were injected with corn oil as the vehicle (Ortiz de Montellano et al., 1981). On the fifth day the rats were sacrificed by decapitation and their livers were perfused through the inferior vena cava with an ice cold

solution of KCl (1.15 % w/v in distilled H_2O, approximately 100 mL). The livers were removed and homogenized (one at a time) with the ice cold KCl solution (1.15% w/v in H_2O, 20 mL). An electric drill fitted with a pestle was used to homogenize the livers (10 strokes). The homogenates were centrifuged at 10,000 x g at 4 °C for 30 minutes. The supernatants were transferred to ultracentrifuge tubes and the tubes were filled to their capacity with the KCl solution (1.15 % w/v in distilled H_2O). The tubes were centrifuged at 100,000 x g at 4 °C for 60 minutes. The pellets were then removed, leaving behind the clear glycogen layers which stick to the bottoms of the centrifuge tubes. The pellets were homogenized with KCl solution (1.15 %) and were recentrifuged at 100,000 g at 4 °C for 60 minutes. The pellets were either suspended in pH 7.4 phosphate (100 mM) buffer containing DETAPAC (1.5 mM) and KCl (150 mM) for use or they were frozen with liquid nitrogen and stored at -70 °C. The frozen microsomal pellets were thawed by alternating between an ice bath and 40 °C H_2O bath for one minute in each. The cytochrome P-450 content was determined from the CO vs. CO - reduced difference spectrum (Omura and Sato, 1964).

Destruction of the Cytochrome P-450 Chromophore by 4-Alkyl-1,4-dihydropyridine in Hepatic Microsomes Incubations consisted of rat liver microsomes (4.0 nmole cytochrome P-450/mL), dihydropyridine (10 mM), and either NADPH (1.0 mM) or an NADPH regenerating system which contained $NADP^+$ (1.0 mM), $MgCl_2$ (2.0 mM), glucose-6-phosphate (3.0 mM), and glucose-6-phosphate dehydrogenase (4 units/mL). The incubations were conducted in pH

7.4 phosphate buffer (100 mM) containing KCl (100 mL) and DETAPAC (1.5 mM). The NADPH or the NADPH regenerating system was added to initiate the incubations which were conducted at 37 °C. The control incubations lacked either the substrate or NADPH (or NADPH regenerating system). An aliquot (approximately 1 mL) was removed from the reaction mixture and the cytochrome P-450 chromophore was quantitated from the CO vs. CO - reduced difference spectrum (Omura and Sato, 1964).

Destruction of the Cytochrome P-450 Chromophore by 2,2-Dialkyl-1,2-dihydroquinoline in Hepatic Microsomes The substrate was dissolved in ether (0.2 mM) and 50 µL was placed in a vial and the ether was removed with a stream of N_2. Rat liver microsomes (4.0 nmole cytochrome P-450/mL) in phosphate buffer were added to the residue. The incubation was started by the addition of NADPH (1.0 mM) or NADPH regenerating system. The phosphate buffer and NADPH regenerating system were identical to those mentioned in the previous section. The control incubations lacked either the substrate or NADPH. The incubation was conducted at 25 °C. Aliquots (approximately 1 mL) were transferred every 20 seconds to test tubes which were placed in an ice bath. The samples were analyzed for cytochrome P-450 content using CO vs. CO - reduced difference spectroscopy (Estabrook, et al., 1972).

Destruction of the Cytochrome P-450 Chromophore in Reconstituted Pure Cytochrome P-450 Systems The cytochrome P-450 reconstituted system was prepared by mixing purified cytochrome P-450 (1 nmole mL^{-1}) and purified cytochrome P-450

reductase (1 nmole mL^{-1}). This solution was stirred for approximately one minute and dilauroylphosphatidylcholine (40 μg mL^{-1}) was added to generate the lipid environment. After a minute of stirring, the mixture was diluted to the desire concentration by addition of pH 7.4 phosphate buffer (100 mM) containing glycerol (20% v/v) and DETAPAC (1.5 mM). The mixture was stirred for 10 minutes before it was used to allow the necessary interactions to develop. To the reconstituted system, dihydropyridine (1.0 mM) was added and the mixture was heated to 37 °C. The incubation was then started by the addition of NADPH (1.0 mM). At the desired time points, aliquots were removed and the cytochrome P-450 content was measured using CO vs. CO - reduced difference spectroscopy (Estabrook, et al., 1972).

4-Alkyl-1,4-dihydropyridine Metabolite Studies The incubation conditions used to examine metabolite formation were identical to those used in the cytochrome P-450 chromophore destruction studies except that the substrate concentration was 1 mM and the incubation volume was 2 mL. The incubation was initiated by addition of the NADPH regenerating system. After 10 minutes at 37 °C, the incubation mixture was transferred to a tube containing CH_2Cl_2 (2 mL), 3,5-dicarbethoxy-2,6-dimethyl-4-phenyl-1,4-dihydropyridine (10 μL, 0.5 mg mL^{-1}) as internal standard, and a solution of NaCl (1 N) and Na_2CO_3 (2 M) (400 μL). The resulting biphasic mixture was vigorously agitated on a vortex mixer and a table top centrifuge was used to separate the layers. For the analysis of the pyridine and *N*-phenylpyridinium metabolites, the

organic layer (1.5 mL) was transferred to a new vial and the solvent was removed with a stream of nitrogen. For the analysis of the *N*-alkylpyridinium metabolites, the aqueous layer (2 mL) was transferred to a new vial and the H_2O was lyophilized. The dry residue was taken up in methanol (2 mL). The undissolved materials were filtered and the methanol was removed with a stream of nitrogen. In both cases, the final residue was taken up in the solvent (100 μL) used for the chromatographic analysis and a 10 μL aliquot was then analyzed by HPLC. The pyridine metabolites were separated on a Whatman Partisil silica gel column (250 x 4.6 mm) eluted with 25% (v/v) THF in hexane at a rate of 1 mL min^{-1}. The column effluent was monitored at 234 nm. The analysis of the pyridinium metabolites was carried out on a reverse phase Whatman Partisil ODS-3 column using an ion-pair technique. The mobile phases for the separations of the *N*-alkyl- and *N*-phenylpyridinium metabolites were 50% (v/v) methanol in H_2O and 60% (v/v) methanol in H_2O, respectively. Methanesulfonic acid (0.01 M) was used as the ion-pair agent for both analyses. The solvent flow rate was 1 mL min^{-1} and the effluent was monitored at 280 nm. The metabolites were quantitated by comparison to a standard curve. The *N*-phenylpyridinium metabolites were quantitated using 3,5-dicarbethoxy-2,6-dimethyl-*N*-methylpyridinium as the internal standard which was added after the extraction step.

The incubation conditions for the analysis of metabolites resulting from the 4-alkyl groups were same as those of pyridine metabolite studies except that the incubation volume was 20 mL

instead of 2 mL. The 4-phenylethyl-1,4-dihydropyridine analogue was use as the substrate. The incubations were initiated by the addition of NADPH regenerating system. After 10 minutes of incubation, 10 µL of hydroxycinnamaldehyde solution (10 µg in 10 mL of CH_2Cl_2) was added as internal standard and the incubations were terminated by freezing the incubation mixtures with a dry ice/acetone bath. The control incubations lack the NADPH regenerating system. The incubation mixtures were extracted with CH_2Cl_2 (50 mL) and the organic layers were splitted into two equal fractions. To one of the fraction, BSTFA (100 µL) was added in order to detect alcoholic metabolites. The volume of both of the fractions was reduced to approximately 1 mL and the resulting samples were analyzed on a capillary gas chromatogram equipped with DB-5 column. The gradient was programmed to maintain 35 °C for one min, rise from 35 to 50 °C at a rate of 50 °C/min, rise from 50 to 150 °C at a rate of 6 °C/min, and maintain 150 °C for 15 min.

The incubation conditions for the $^{18}O_2$ atmosphere study were identical to those mentioned in the previous paragraph. The complete incubation components except NADPH regenerating system were placed in a round bottom flask equipped with valves leading to a vacuum pump, a nitrogen inlet, a balloon, and a 250 mL break seal ampule containing 99.8 % [$^{18}O_2$]-molecular oxygen. The NADPH regenerating system was placed into a separate flask which was attached to the vacuum system. This system was evacuated with vacuum pump and purged with nitrogen to remove $^{16}O_2$ from the incubation environment. This cycle was repeated five times. The break seal of the $^{18}O_2$ ampule was then broken and the incubation

was initiated by addition of NADPH regenerating system. The incubation was worked up as mention in the previous paragraph. The sample was analyzed by gas chromatography/mass spectrometry.

Spin Trapping of Radical from 4-Alkyl-1,4-dihydropyridine

The incubation conditions were the same as those mentioned in the cytochrome P-450 chromophore destruction studies. POBN (20 mM) was used as the spin trap. The control incubations lacked either the substrate or the NADPH regenerating system. The incubations were conducted at 37 °C. At the desired time points, 50 μL aliquots were transferred to melting point capillary tubes which were placed into an EPR tube for the EPR analyses. The following EPR parameters were typically used: field set, 3400 G; scan range, 100 G; time constant, 0.25 second; scan time, 4 minutes; modulation amplitude, 1 G; modulation frequency, 100 Hz; receiver gain, 2.5-5.0 x 10^4; microwave power, 20 mW; and microwave frequency, 9.517 GHz.

The procedure for the generation of spin-adduct from horseradish peroxidase oxidation of DDEP was same as the oxidations supported by cytochrome P-450. The incubations consisted of DDEP (5 mM), horseradish peroxidase (0.5 μM), H_2O_2 (0.5 mM), and POBN (20 mM) in pH 7.4 phosphate buffer (100 mM). The H_2O_2 was added to start the incubations. The EPR parameters were identical to those of cytochrome P-450 incubations.

Spin Trapping of Radical from 2,2-Dialkyl-1,2-dihydroquinoline The procedures for the spin trap studies with 2,2-dialkyl-1,2-dihydroquinolines were identical as the procedures for

the 4-alkyl-1,4-dihydropyridines except that the total incubation volume was 5 mL instead of 1 mL. The incubation mixture was extracted and concentrated to a final volume of approximately 100 μL. The EPR spectrum of the concentrated sample was recorded. The control incubation, which lacked the substrate, was carried out in parallel.

The incubations for the horseradish peroxidase supported production of spin-adducts consisted of DDMQ (3 mM), horseradish peroxidase (1.3 μM), H_2O_2 (147 mM), and POBN (34 mM) in pH 7.4 phosphate buffer (100 mM). The H_2O_2 was added to initiate the incubations. The EPR parameters were identical to those used in oxidation of the dihydropyridines.

Assay for the Inactivation of Reconstituted Cytochrome P-450

The inactivation incubations consisted of the cytochromes P-450b or c reconstituted system as mentioned in the cytochrome P-450 chromophore destruction studies, inhibitor (1 mM), and NADPH (1 mM), and they were incubated at 37 °C for 20 minutes. At the desired time points, aliquots (20 or 50 μL, respectively) of the inactivation incubation mixture were transferred for the analysis of 7-ethoxycoumarin or 7-ethoxyresorufin *O*-deethylase activity, respectively. For the 7-ethoxycoumarin *O*-deethylase assay (Greenlee and Poland, 1978), the 20 μL aliquot was added to a mixture containing NADPH (0.5 mM) and 7-ethoxycoumarin (0.5 mM) in pH 7.4 phosphate buffer (100 mM) consisting of glycerol (20%, v/v) and DETAPAC (1.5 mM). The final concentrations of the incubation mixture were 20 pmole mL^{-1} cytochrome P-450b, 20

pmole mL^{-1} cytochrome P-450 reductase, and 0.08 μg mL^{-1} dilauroylphosphatidylcholine. The incubation was conducted at 37 °C for 10 minutes at which time the reaction was stopped with addition of HCl (100 μL, 2 N). The mixture was then added to a tube containing CH_2Cl_2 (2 mL). The tube was vigorously agitated on a vortex mixer and the biphasic mixture was separated on a table top centrifuge. The CH_2Cl_2 layer (1 mL) was transferred to a tube containing pH 9.2 borate buffer (30 mM, 2.5 mL) and back extracted into the aqueous layer by agitating the mixture vigorously and then centrifuging it. The aqueous layer was analyzed for fluorescence of 7-hydroxycoumarin at 456 nm when excited at 368 nm. For the 7-ethoxyresorufin *O*-deethylase assay (Burke and Mayer, 1974), the 50μL aliquots of the inactivation incubation were added to a mixture containing NADPH (1 mM) and 7-ethoxyresorufin (5 μM) in pH 7.4 phosphate buffer (100 mM) consisting of glycerol (20%, v/v) and DETAPAC (1.5 mM). The final concentrations of the incubation mixture were 50 pmole mL^{-1} cytochrome P-450c, 50 pmole mL^{-1} cytochrome P-450 reductase, and 0.20 μg mL^{-1} dilauroylphosphatidylcholine. The incubation was conducted at 37 °C for 2 minutes and the reaction was terminated by adding ice cold acetone (3 mL). This mixture was vigorously agitated on a vortex mixer and centrifuged on a table top centrifuge. The solution was analyzed for fluorescence of resorufin at 585 nm when excited at 535 nm.

DDEP Oxidase Inactivation by 2,2-Dialkyl-1,2-dihydroquinoline

DDMQ (1 mM) was incubated with microsomal cytochrome P-450 (10

nmole mL^{-1}) and NADPH regenerating system in phosphate buffer. The NADPH regenerating system and phosphate buffer were identical to those mentioned in the cytochrome P-450 chromophore destruction studies. The incubations were conducted at 25 °C. The control incubations, lacking DDMQ, were conducted in parallel. At the appropiate time points, aliquots (900 µL) were transferred to the DDEP assay mixture consisting of NADPH (1 mM) and DDEP (1 mM). The final concentrations of the incubation mixture were 3 nmole mL^{-1} cytochrome P-450 and 0.3 mM DDMQ in 3 mL total volume. The incubations were conducted at 37 °C for 10 minutes. The reaction workup and analysis were the same as these indicated in the metabolite studies section.

Measurement of 4-Alkyl-1,4-dihydropyridine Oxidation Potentials Cyclic voltammetry was performed on a 1.0 mM solution of the 4-alkyl-1,4-dihydropyridines in acetonitrile containing tetrabutylammonium perchlorate (0.1 M) as the supporting electrolyte. The electrical cell was equipped with a saturated calomel reference electrode, a freshly polished platinum working electrode, and a platinum auxiliary electrode. The measurements were determined at 25 °C after bubbling the solution with nitrogen. The potential was varied from -1.5 to 2.5 V at a sweep rate of 500 mV sec^{-1}.

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