4-Alkyl Radical Extrusion in the Cytochrome P-450-Catalyzed Oxidation of 4-Alkyl-1,4-dihydropyridines†

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ABSTRACT: Rat liver microsomal cytochrome P-450 oxidizes the 4-methyl, 4-ethyl (DDEP), and 4-isopropyl derivatives of 3,5-bis(carbethoxy)-2,6-dimethyl-1,4-dihydropyridine to mixtures of the corresponding 4-alkyl and 4-dealkyl pyridines. A fraction of the total microsomal enzyme is destroyed in the process. The 4-dealkyl to 4-alkyl pyridine metabolite ratio, the extent of cytochrome P-450 destruction, and the rate of spin-trapped radical accumulation are correlated in a linear inverse manner with the homolytic or heterolytic bond energies of the 4-alkyl groups of the 4-alkyl-1,4-dihydropyridines. No isotope effects are observed on the pyridine metabolite ratio, the destruction of cytochrome P-450, or the formation of ethyl radicals when [4-^{2}H]DDEP is used instead of DDEP. *N*-Methyl- and *N*-ethyl-DDEP undergo N-dealkylation rather than aromatization but *N*-phenyl-DDEP is oxidized to a mixture of the 4-ethyl and 4-deethyl *N*-phenylpyridinium metabolites. In contrast to the absence of an isotope effect in the oxidation of DDEP, the 4-deethyl to 4-ethyl *N*-phenylpyridinium metabolite ratio increases 6-fold when *N*-phenyl[4-^{2}H]DDEP is used. The results support the hypothesis that cytochrome P-450 catalyzes the oxidation of dihydropyridines to radical cations and show that the radical cations decay to nonradical products by multiple, substituent-dependent, mechanisms.

The oxidation of 3,5-bis(carbethoxy)-4-alkyl-2,6-dimethyl-1,4-dihydropyridines by cytochrome P-450 results in inactivation of the monooxygenase and, in most instances, in transfer of the 4-alkyl moiety to a pyrrole nitrogen of the prosthetic heme group (Figure 1) (Augusto et al., 1982). *N*-Alkylheme[1] adducts are formed with the 4-methyl, 4-ethyl, 4-propyl, and 4-isobutyl analogues but are not detectably formed with the 4-isopropyl and 4-benzyl analogues even though all the analogues destroy cytochrome P-450 (Augusto et al., 1982). Incubation of DDEP, the 4-ethyl analogue, with a spin trap and rat liver microsomes gives rise to an NADPH-dependent and cytochrome P-450 dependent EPR spectrum. The spin adduct responsible for the EPR spectrum has been isolated, and the trapped species has been unambiguously identified as the ethyl radical (Augusto et al., 1982). These observations led us to propose that oxidation of the dihydropyridine to a radical cation is followed by extrusion of the 4-alkyl group and aromatization of the 1,4-dihydropyridine ring (Figure 1).

N-alkylation of the prosthetic heme group of cytochrome P-450 isozymes that turn over DDEP and other 4-alkyl-1,4-dihydropyridines is of practical and theoretical interest. The reaction with cytochrome P-450 not only inactivates the monooxygenase but also generates *N*-alkylprotoporphyrin IX adducts that are potent ferrochelatase inhibitors (Tephly et al., 1979; De Matteis et al., 1980). Ferrochelatase catalyzes the insertion of iron into protoporphyrin IX in the final step of heme biosynthesis. The N-alkylated porphyrins generated by the reaction of cytochrome P-450 with the 4-alkyl-1,4-dihydropyridines are responsible, in fact, for the long-known porphyrinogenic activities of these substances (Marks, 1985). Extrusion of the 4-ethyl group of DDEP as a spin-trappable free radical provides, furthermore, one of the strongest pieces of evidence for one-electron oxidation of heteroatomic substrates by cytochrome P-450 (Augusto et al., 1982; Ortiz de Montellano, 1986). The 4-alkyl-1,4-dihydropyridines are thus highly informative probes of the catalytic mechanism of cytochrome P-450. We describe here further studies of the interactions of cytochrome P-450 with 4-alkyl-1,4-dihydropyridines that strengthen our understanding of the mechanisms by which nitrogen-containing substrates are oxidized.

Experimental Procedures

Materials. DDC, POBN, and NaB^2H_4 were purchased from Aldrich, and NADPH was from Sigma. DDEP, DDIP, and *N*-ethyl-DDEP were synthesized as reported previously (Augusto et al., 1982; Ortiz de Montellano et al., 1981a) by the procedure of Loev et al. (1974). Aromatization of the 4-alkyl-1,4-dihydropyridines was accomplished by oxidation with nitric acid as reported by Loev and Snader (1965). The structures of the compounds employed in this study are given in Figure 2. Cytochrome P-450b and cytochrome P-450 reductase were purified as previously described (Komives & Ortiz de Montellano, 1987) by the procedures, respectively, of Waxman and Walsh (1982) and Shephard et al. (1983). Reconstitution of cytochrome P-450b and incubations with the reconstituted enzyme system were carried out as described earlier (Komives & Ortiz de Montellano, 1987). Liver microsomes were prepared from Sprague-Dawley male rats pretreated with sodium phenobarbital (80 mg Kg^{-1} day^{-1} for 4 days) as previously reported (Ortiz de Montellano et al., 1981b).

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[1] Abbreviations: heme, iron protoporphyrin IX regardless of the oxidation and ligation state; POBN, 4-[(*tert*-butylimino)methyl]pyridine *N*,*N*′-dioxide; DDC, 3,5-bis(carbethoxy)-2,4,6-trimethyl-1,4-dihydropyridine; DDEP, 3,5-bis(carbethoxy)-2,6-dimethyl-4-ethyl-1,4-dihydropyridine; DDIP, 3,5-bis(carbethoxy)-2,6-dimethyl-4-isopropyl-1,4-dihydropyridine; HPLC, high-pressure liquid chromatography; EPR, electron paramagnetic resonance.

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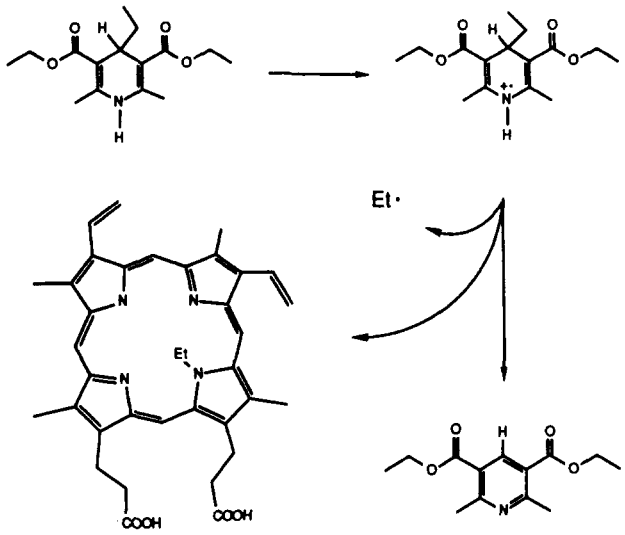
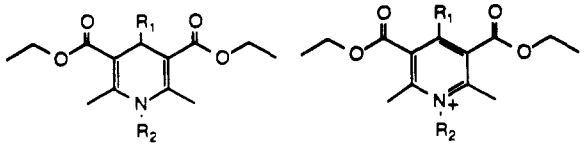


FIGURE 1: General mechanism proposed for the cytochrome P-450 catalyzed oxidation of 4-alkyl-1,4-dihydropyridines that results in transfer of the 4-alkyl group from the substrate to a nitrogen of the prosthetic heme group.



Dihydropyridine	R_1	R_2
DDC	CH_3	H
DDEP	CH_2CH_3	H
DDIP	$CH(CH_3)2$	H
N-Methyl-DDEP	CH_2CH_3	CH_3
N-Ethyl-DDEP	CH_2CH_3	CH_2CH_3
N-Phenyl-DDEP	CH_2CH_3	C_6H_6

Pyridinium	R_1	R_2
	H	H
	CH_3	H
	CH_2CH_3	H
	H	CH_3
	CH_2CH_3	CH_3
	H	CH_2CH_3
	CH_2CH_3	CH_2CH_3
	H	C_6H_6
	CH_2CH_3	C_6H_6

FIGURE 2: Structures of the dihydropyridines, pyridines, and pyridinium salts discussed in this paper.

Spectroscopic Studies. Electronic absorption spectra were recorded on a Hewlett-Packard 8450A UV/vis instrument. An Aminco DW2a spectrophotometer was used to quantitate cytochrome P-450. EPR spectra were obtained on a Varian Model 104 spectrometer custom interfaced with an IBM XT computer. NMR spectra were recorded in deuteriated chloroform at 80 MHz on a Varian FT-80 or (where indicated) on a custom-built 240-MHz instrument. Chemical shift values, reported in parts per million relative to tetramethylsilane, were measured relative to the residual chloroform peak. Electron impact mass spectra (70 eV) were obtained on a Kratos MS-25 mass spectrometer. LSIMS mass spectra were obtained with glycerol as the matrix on a Kratos MS-50 instrument. Gas–liquid chromatography was carried out on a Varian 2100 system equipped with flame ionization detectors and 6-ft glass columns. HPLC was performed on a gradient system consisting of two Altex Model 110A pumps, an Altex solvent programmer, and a Hewlett-Packard Model 1040A diode array UV/vis detector. Melting points are uncorrected. Analytical data were obtained by the Microanalytical Laboratory of the University of California, Berkeley.

[2-^2H]-2-Ethyl-4,4,6-trimethyltetrahydro-1,3-oxazine. A solution of 4.66 g (30 mmol) of 2-ethyl-4,4,6-trimethyldihydro-1,3-oxazine in 25 mL of dry tetrahydrofuran and 25 mL of [^{2}H]ethanol (>99 atom % deuterium) was mechanically stirred in an open flask immersed in a −40 °C dry ice–acetone bath while 9 N DCl/D_2O was added dropwise (200 drops total) until the pH was 7. A solution of sodium borodeuteride was prepared by adding 7 drops of 30% $NaOD/D_2O$ and 4.78 mL of D_2O to 1.25 g (30 mmol) of $NaBD_4$ (>98 atom % deuterium). This solution was added to the stirred dihydrooxazine solution in 20-drop portions, following each with 14 drops of 9 N DCl/D_2O and maintaining the cooling bath at −35 to −40 °C. Periodic checks ensured that a pH of 7 was maintained. When the addition was complete, the reaction mixture was stirred for 1 h at −30 to −40 °C and was then allowed to warm to 10 °C and combined with 150 mL of ether and 100 mL of water. After the aqueous layer was adjusted to pH 11 with 15% NaOH, the mixture was shaken, and the organic layer was separated, washed with saturated NaCl solution, dried over K_2CO_3, and concentrated under reduced pressure to give 4.70 g of a colorless oil: GLC retention time (10% carbowax, 77.5 °C) 6.89 min; 240-MHz ^{1}H NMR ($CDCl_3$) 0.96 (t, 3 H, J = 7.5 Hz), 1.11 (s, 3 H), 1.16 (d, 3 H, J = 6.1 Hz), 1.16 (s, 3 H), 1.43 (B of ABX, 1 H, J_{AB} = 13.1 Hz, J_{BX} = 2.2 Hz), 1.54 (q, 2 H, J = 7.5 Hz), and 3.75 ppm (ddq, 1 H, J = 11.4, 2.3, and 6.1 Hz). A signal at 4.15 (t, 1 H, J = 5.3 Hz) present in the nondeuteriated compound was absent in the deuteriated sample.

[4-^2H]-3,5-Bis(carbethoxy)-4-ethyl-2,6-dimethyl-1,4-dihydropyridine. A mixture of 4.29 g (27.1 mmol) of [2-^{2}H]-2-ethyl-4,4,6-trimethyltetrahydro-1,3-oxazine, 100 mL of water, and 4.55 mL (54.6 mmol) of concentrated HCl was heated at 95 °C for 1.5 h. The mixture was then cooled to 21 °C, diluted with 25 mL of water, and neutralized to pH 8 with 10.6 g of 15% NaOH. Ethyl acetoacetate (7.06 g, 54.4 mmol) and pulverized ammonium carbonate (13.0 g, 136 mmol) were added, and the mixture was capped and stirred for 4 days at 21 °C. The yellow solid was collected, washed with water, and dissolved in 100 mL of hot absolute ethanol. While heating, 70 mL of water was added, the solution was allowed to cool slowly, and after 2 days at 5 °C, the crystals that had formed were collected, washed with water, and dried under vacuum at 70 °C to give 4.00 g (52% yield) of fine yellow crystals: mp 106–107 °C (lit. mp for unlabeled compound 110 °C) (Engelmann, 1885); UV (ethanol) λ_{max} 232, 255 (shoulder), and 350 nm, $A_{232/250}$ = 2.18; 240-MHz ^{1}H NMR ($CDCl_3$) 0.75 (t, 3 H, J = 7.5 Hz), 1.29 (t, 6 H, J = 7.1 Hz), 1.36 (q, 2 H, J = 7.5 Hz), 2.29 (s, 6 H), 3.91 (t, J = 5.6 Hz, residual 4-^{1}H), 4.16 and 4.21 (AB of ABX_3, 4 H, J_{AB} = 10.8 Hz, J_{AX} = J_{BX} = 7.1 Hz), and 5.52 ppm (br s, 1 H); EIMS m/z 282 (M^+), 281, 280, 279, 253 (base peak, M − C_2H_5), 225, 197, 180, and 151. This compound only differs from the 4-^{1}H compound in the signals in the NMR 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 mass spectrum by a difference of 1 in the molecular ion and most of the fragment ions. A C-4 deuterium incorporation of 98.3% is indicated by the integral of the signal at 3.91 relative to the integrals for the signals at 4.16 and 4.21.

[4-^2H]-3,5-Bis(carbethoxy)-2,4,6-trimethyl-1,4-dihydropyridine. Ethyl acetoacetate (5.91 g, 45.4 mmol), [1-^{2}H]-acetaldehyde (Aldrich, >98 atom % deuterium, 1.02 g, 22.7 mmol), and ammonium carbonate solution [10.7 g of (N-

$H_4)_2CO_3$ in 100 mL of H_2O] were mixed and stirred at 21 °C for 1.5 h. The mixture was cooled to 4 °C and was swirled once a day for 6 days. The yellow solid that collected was washed with water and dissolved in 35 mL of hot absolute ethanol. After 1 mL of H_2O was added, the solution was gravity filtered and was diluted, while heating, with 34 mL of H_2O. After 1 day at 21 °C and 1 day at 4 °C, crystals were collected, washed with water, and dried under vacuum. Recrystallization from 27 mL of absolute ethanol and 27 mL of H_2O afforded, aftery drying overnight at 75 °C under high vacuum, 21.3 g (35% yield) of fine white crystals: 240-MHz ^1H NMR ($CDCl_3$) 0.96 (s, 3 H), 1.30 (t, 6 H, J = 7.1 Hz), 2.27 (s, 6 H), 4.17 and 4.21 (AB of ABX_3, 4 H, J_{AB} = 10.8, $J_{AX} = J_{BX}$ = 7.1 Hz), and 5.86 ppm (br s, 1 H); EIMS m/z 268 (M^+), 253 (base, M − CH_3), 225 (M − CH_3 − C_2H_4), 223 (M − OC_2H_5), and 197. Integration of the residual 4-^1H signal (3.83 ppm, q, J = 6.5 Hz) indicated that the product was 99.3% deuterium labeled.

N-Phenyl-3,5-bis(carbethoxy)-2,6-dimethyl-4-ethyl-1,4-dihydropyridine. A solution of ethyl acetoacetate (65.1 g, 0.5 mol), propionaldehyde (14.5 g, 0.25 mol), and aniline (23.3 g, 0.25 mol) in 50 mL of ethanol was refluxed overnight. The mixture was then cooled to room temperature, poured into 500 mL of ice/water, and extracted with diethyl ether (3 × 250 mL). The organic layer was dried over anhydrous sodium sulfate before the solvent was removed with a rotary evaporator. The crude product was subjected to flash column chromatography on silica gel with 8% (v/v) ethyl acetate in hexane as the solvent. The column fractions were monitored by thin-layer chromatography with 1:1 ethyl acetate/hexane as the solvent. The solvent was removed with a rotary evaporator from the appropriate fractions, and the residue was recrystallized twice from ethanol/water. The *N*-phenyl derivative was thus obtained in very low (1.2%) yield: mp 84–85 °C; UV (MeOH) λ_{max} 236 (ϵ = 11 800 M^{-1} cm^{-1}) and 345 nm (ϵ = 4200 M^{-1} cm^{-1}); ^1H NMR ($CDCl_3$) 0.90 (t, 3 H, J = 7.1 Hz, 4-CH_2CH_3), 1.25 (t, 6 H, J = 6.9 Hz, ethoxy methyl), 2.01 (s, 6 H, 2,6-dimethyl), 3.94 (t, 1 H, J = 6.3 Hz, 4-H), 4.20 (q, 4 H, J = 7.2 Hz, ethoxy methylene), and 7.09–7.19 and 7.38–7.47 ppm (m, 5 H, aromatic); EIMS (assignment, relative abundance) m/z 357 (M^+, 2), 328 (M^+ − 29, 100), 312 (M^+ − 45, 40), 300 (M^+ − 57, 50), and 272 (M^+ − 85, 55). Anal. Calcd for $C_{20}H_{27}NO_4$: C, 70.56; H, 7.61; N, 3.92. Found: C, 70.58; H, 7.70; N, 3.98.

N-Phenyl[4-^2H]-3,5-bis(carbethoxy)-2,6-dimethyl-4-ethyl-1,4-dihydropyridine. The synthesis of this material required the synthesis of [1-^2H]propionaldehyde. A solution of 2-ethyl-4,4,6-trimethyl-1,3-dihydrooxazine (0.137 mol) in 260 mL of 1:1 ethanol/tetrahydrofuran cooled to −30 °C was brought to pH 7 with 9 N HCl. A mixture of sodium borodeuteride (0.137 mol), 15% NaOH (2.5 mL), and water (21.5 mL) was added dropwise, alternating with addition of 9 N HCl to keep the pH constant. The mixture was then stirred for 1 h at −30 °C before it was allowed to warm to room temperature, and 10 mL of 15% NaOH and 400 mL of water were added. The mixture was extracted with 600 mL of diethyl ether, and the ether layer was washed with 500 mL of saturated NaCl solution and dried over K_2CO_3. Removal of the ether on a rotary evaporator under reduced pressure yielded 0.11 mol (80%) of [2-^2H]-2-ethyl-4,4,6-trimethyl-1,3-tetrahydrooxazine. To a solution of this material (0.161 mol) in 250 mL of water was added dropwise 31.5 mL of concentrated HCl. The resulting mixture was heated at 90 °C for 1 h before it was cooled to room temperature, and sufficient 15% NaOH was added to bring it to pH 7. [1-^2H]Propionaldehyde was distilled out of the reaction mixture in 68% yield (8 mL, 0.11 mol).

A mixture of aniline (0.11 mol), ethyl acetate (0.22 mol), [1-^2H]propionaldehyde (0.11 mol), and ethanol (20 mL) was heated at 100 °C for 24 h. The mixture was then cooled, poured into 200 mL of ice-cold water, and extracted three times with 200-mL portions of diethyl ether. The combined ether extracts were dried over anhydrous K_2CO_3, the solvent was removed under reduced pressure, and the product was purified from the resulting oil by flash chromatography on silica gel mesh 230–400 with 8% (v/v) ethyl acetate in hexane as the solvent. The chromatographic fractions were analyzed by thin-layer chromatography (1:1 ethyl acetate/hexane), and the desired fractions were pooled and concentrated under reduced pressure. The residual oil was crystallized from ethanol/water: EIMS m/z 358 (M^+, 3), 329 (M^+ − 29, 100), 313 (M^+ − 45, 32), 301 (M^+ − 57, 28), and 273 (M^+ − 85, 45); ^1H NMR ($CDCl_3$) 0.89 (t, 3 H, J = 7.2 Hz), 1.30 (t, 6 H, J = 7.2 Hz), 2.00 (s, 6 H), 4.20 (q, 4 H, J = 7.1 Hz), and 7.09–7.18 and 7.38–7.46 ppm (m, 5 H). The 4-H signal at 3.94 ppm is missing.

N-Phenyl-3,5-bis(carbethoxy)-2,6-dimethyl-4-isopropyl-1,4-dihydropyridine. This compound was synthesized by the procedure described for synthesis of the 4-ethyl analogue except that the purified oil did not crystallize from ethanol/water and was characterized as such: UV (MeOH) λ_{max} 242 (ϵ = 13 500 M^{-1} cm^{-1}) and 330 nm (ϵ = 5400 M^{-1} cm^{-1}); ^1H NMR ($CDCl_3$) 0.85 (d, 6 H, J = 7.3 Hz, isopropyl methyls), 1.27 (t, 6 H, J = 7.1 Hz, ethoxy methyls), 2.02 (s, 6 H, 2,6-dimethyls), 3.92 (d, 1 H, J = 6.0 Hz, 4-H), 4.15 (q, 4 H, J = 7.3 Hz, ethoxy methylenes), and 7.02–7.12 and 7.30–7.40 ppm (m, 5 H, aromatic); EIMS (assignment, relative abundance) m/z 371 (M^+, not detected), 328 (M^+ − 43, 100), 300 (M^+ − 71, 10), 272 (M^+ − 99, 15). Anal. Calcd for $C_{21}H_{29}NO_4$: C, 71.13; H, 7.87; N, 3.77. Found: C, 71.06; H, 7.84; N, 3.93.

N-Ethyl-3,5-bis(carbethoxy)-2,6-dimethyl-4-isopropyl-1,4-dihydropyridine. This compound was synthesized by the procedure previously used to prepare the 4-ethyl analogue (Augusto et al., 1982). A solution of ethyl acetoacetate (32.6 g, 0.25 mol), isobutyraldehyde (9.0 g, 0.125 mol), and ethylamine hydrochloride (14.3 g, 0.175 mol) in 24.3 mL of pyridine was heated at 100 °C for 1 h. After the mixture was cooled to room temperature, it was poured into 200 mL of ice/water, and the oil that separated was extracted with diethyl ether (3 × 200 mL). The combined extracts were dried over anhydrous K_2CO_3, and the solvent was removed with a rotary evaporator. The residual oil was crystallized from ethanol at −78 °C. The crystals were recrystallized twice from ethanol/water at room temperature, yielding the desired 4-isopropyl compound in 29% yield: mp 52–54 °C; UV (MeOH) λ_{max} 241 (ϵ = 13 300 M^{-1} cm^{-1}) and 338 nm (ϵ = 7700 M^{-1} cm^{-1}); ^1H NMR 0.74 (d, 6 H, J = 6.7 Hz, isopropyl methyls), 1.15 (t, 3 H, J = 7.0 Hz, NCH_2CH_3), 1.28 (t, 6 H, ethoxy methyls), 2.40 (s, 6 H, 2,6-dimethyls), 3.69 (q, 2 H, J = 7.3 Hz, NCH_2CH_3), and 4.17 ppm (q, 6 H, J = 6.6 Hz, ethoxy methylenes); EIMS (assignment, relative abundance) m/z 323 (M^+, 1), 280 (M^+ − 43, 100), 253 (M^+ − 70, 30), and 224 (M^+ − 99, 15). Anal. Calcd for $C_{18}H_{29}NO_4$: C, 66.85; H, 9.04; N, 4.33. Found: C, 66.72; H, 8.98; N, 4.28.

N-Methyl-3,5-bis(carbethoxy)-2,6-dimethyl-4-ethyl-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 reaction time was increased from 1 to 2 h: mp 86–88 °C; UV (MeOH) λ_{max} 234 (ϵ = 12 600 M^{-1} cm^{-1}) and 345 nm (ϵ = 6900 M^{-1} cm^{-1});

^{1}H NMR ($CDCl_3$) 0.66 (t, 3 H, J = 7.2 Hz, 4-ethyl CH_3), 1.23 (t, 6 H, J = 6.7 Hz, ethoxy methyls), 2.35 (s, 6 H, 2,6-dimethyls), 3.09 (s, 3 H, NCH_3), 3.83 (t, 1 H, J = 6.6 Hz, 4-H), and 4.12 ppm (q, 4 H, J = 6.9 Hz, ethoxy CH_2); EIMS (assignment, relative abundance) m/z 295 (M^+, 8), 266 ($M^+ - 29$, 100), 250 ($M^+ - 45$, 70), 238, ($M^+ - 57$, 80), and 210 ppm ($M^+ - 85$, 80). Anal. Calcd for $C_{16}H_{25}NO_4$: C, 65.06; H, 8.53; N, 4.74. Found: C, 65.23, H, 8.39; N, 4.69.

N-Methyl-3,5-bis(carbethoxy)-2,6-dimethyl-4-isopropyl-1,4-dihydropyridine. This compound was synthesized in the same manner as the *N*-methyl derivative of DDEP in 55% yield: mp 61–63 °C; UV (MeOH) λ_{max} 236 (ϵ = 11 500 M^{-1} cm^{-1}) and 340 nm (ϵ = 6900 M^{-1} cm^{-1}); ^{1}H NMR ($CDCl_3$) 0.72 (t, 6 H, J = 6.6 Hz, isopropyl methyls), 1.29 (t, 6 H, J = 7.3 Hz, ethoxy methyls), 2.40 (s, 6 H, 2,6-dimethyls), 3.13 (s, 3 H, *N*-Me), 3.87 (d, 1 H, J = 7.1 Hz, 4-H), and 4.18 ppm (q, 4 H, J = 7.3 Hz, ethoxy methylenes); EIMS (assignment, relative abundance) m/z 309 (M^+, 2), 266 ($M^+ - 43$, 100), 238 ($M^+ - 71$, 70), and 210 ($M^+ - 99$, 72). Anal. Calcd for $C_{17}H_{27}NO_4$: C, 65.99; H, 8.80; N, 4.53. Found: C, 66.15; H, 8.63; N, 4.44.

N-Ethyl-3,5-bis(carbethoxy)-2,6-dimethyl-4-ethylpyridinium Nitrate. The title compound was obtained by a modification of the procedure of Sugiyama et al. (1964). Concentrated nitric acid (2 mL) was added dropwise to a stirred solution of *N*-ethyl-DDEP (1 g) in 50 mL of ethanol, and the resulting solution was stirred 48 h at room temperature. After a second aliquot of nitric acid was added and stirred a further 48 h, the ethanol was removed on a rotary evaporator, and the residue was partitioned between 1:1 water/hexane (200 mL). Lyophilization of the water layer yielded the desired pyridinium salt in 25% yield: UV (MeOH) λ_{max} 281 nm (ϵ = 4700 M^{-1} cm^{-1}); ^{1}H NMR ([$^2H_6$]dimethyl sulfoxide) 1.10–1.53 (m, 12 H, methyls); 2.63 (partially obscured q, 2 H, J = 7.0 Hz, 4-ethyl CH_2), 2.79 (s, 6 H, 2,6-dimethyls), and 4.35–4.72 ppm (m, 6 H, *N*- and *O*-ethyl CH_2); LSIMS (glycerol) m/z 308 (M^+).

N-Ethyl-3,5-bis(carbethoxy)-2,6-dimethylpyridinium Nitrate. This compound was obtained in 38% yield by oxidation of *N*-ethyl-3,5-bis(carbethoxy)-2,6-dimethyl-4-isopropyl-1,4-dihydropyridine (1 g) with concentrated nitric acid (4 mL) by the procedure described for preparation of the *N*-ethyl-3,5-bis(carbethoxy)-2,4-dimethyl-4-ethylpyridinium analogue: UV (MeOH) λ_{max} 281 nm (ϵ = 1600 M^{-1} cm^{-1}); ^{1}H NMR ([$^2H_6$]dimethyl sulfoxide) 1.26–1.55 (m, 9 H, ethyl methyls), 3.04 (s, 6 H, 2,6-dimethyls), 4.25–4.82 (m, 6 H, ethyl CH_2), and 8.88 ppm (s, 1 H, 4-H); LSIMS (glycerol) m/z 280 (M^+).

N-Methyl-3,5-bis(carbethoxy)-2,6-dimethyl-4-ethylpyridinium Nitrate. This compound was derived from *N*-methyl-3,5-bis(carbethoxy)-2,6-dimethyl-4-ethyl-1,4-dihydropyridine in 42% yield as described for preparation of the *N*-ethyl-4-ethyl analogue: UV (MeOH) λ_{max} 281 nm (ϵ = 2800 M^{-1} cm^{-1}); ^{1}H NMR ([$^2H_6$]dimethyl sulfoxide) 1.08–1.42 (m, 9 H, ethyl methyls), 2.36 (q, 2 H, J = 6.2 Hz, 4-ethyl CH_2), 2.72 (s, 6 H, 2,6-dimethyls), 4.03 (s, 3 H, N-Me), and 4.51 ppm (q, 4 H, J = 7.3 Hz, ethoxy CH_2); LSIMS (glycerol) m/z 294 (M^+).

N-Methyl-3,5-bis(carbethoxy)-2,6-dimethylpyridinium Nitrate. Oxidation of *N*-methyl-3,5-bis(carbethoxy)-2,6-dimethyl-4-isopropyl-1,4-dihydropyridine as described for preparation of the *N*-methyl-4-ethyl analogue gave the named compound in 62% yield: UV (MeOH) λ_{max} 281 nm (ϵ = 5400 M^{-1} cm^{-1}); ^{1}H NMR ([$^2H_6$]dimethyl sulfoxide) 1.36 (t, 6 H, J = 7.1 Hz, ethoxy methyls), 2.99 (s, 6 H, 2,6-dimethyls), 4.16 (s, 3 H, *N*-Me), 4.43 (q, 4 H, J = 7.1 Hz, ethoxy CH_2), and 8.94 ppm (s, 1 H, 4-H); LSIMS (glycerol) m/z 266 (M^+).

N-Phenyl-3,5-bis(carbethoxy)-2,6-dimethyl-4-ethylpyridinium Nitrate. This compound was obtained by oxidation of *N*-phenyl-3,5-bis(carbethoxy)-2,6-dimethyl-4-ethyl-1,4-dihydropyridine as described for preparation of the *N*-ethyl analogue, except that the product partitions into the ethyl acetate rather than water layer. The residue from the ethyl acetate layer was partitioned between 1:1 methanol/hexane (200 mL total). Concentration of the methanol layer provided the desired compound in 60% yield: UV (MeOH) λ_{max} 221 (ϵ = 12 400 M^{-1} cm^{-1}) and 282 nm (ϵ = 7400 M^{-1} cm^{-1}); ^{1}H NMR ([$^2H_6$]dimethyl sulfoxide) 1.26–2.10 (m, 9 H, ethyl methyls), 2.44 (s, 6 H, 2,6-dimethyls), 2.78 (q, 2 H, J = 7.6 Hz, 4-ethyl CH_2), 4.49 (q, 4 H, J = 7.0 Hz, ethoxy methylenes), and 7.35–7.75 ppm (m, 5 H, aromatic); LSIMS (glycerol) m/z 356 (M^+).

N-Phenyl-3,5-bis(carbethoxy)-2,6-dimethylpyridinium Nitrate. This compound was obtained by oxidation of *N*-phenyl-3,5-bis(carbethoxy)-2,6-dimethyl-4-isopropyl-1,4-dihydropyridine as reported for the *N*-phenyl-4-ethyl analogue except that the product was in the aqueous rather than ethyl acetate layer and was therefore isolated in 57% yield by lyophilization of the aqueous layer: UV (MeOH) λ_{max} 219 (ϵ = 9400 M^{-1} cm^{-1}) and 283 nm (ϵ = 4100 M^{-1} cm^{-1}); ^{1}H NMR ([$^2H_6$]dimethyl sulfoxide) 1.37 (t, 6 H, J = 7.1 Hz, ethoxy methyls), 2.54 (s, 6 H, 2,6-dimethyls), 4.46 (q, 4 H, J = 7.0 Hz, ethoxy methylenes), 7.55–7.78 (m, 5 H, *N*-phenyl), and 9.19 ppm (s, 1 H, 4-H); LSIMS (glycerol) m/z 356 (M^+).

N-Phenyl-3,5-bis(carbethoxy)-2,6-dimethyl-4-ethyl-4-hydroxy-1,4-dihydropyridine. Approximately 1 mg of *N*-phenyl-3,5-bis(carbethoxy)-2,6-dimethyl-4-ethylpyridinium nitrate was dissolved in 5 mL of water, and the pH was adjusted to 10 (pH paper) with 15% NaOH. The absorption spectrum shifted instantly upon and addition of the NaOH from λ_{max} = 280 nm to λ_{max} = 252 and 315 nm. The latter dihydropyridine spectrum was stable even if the solution was acidified to pH 3. The basic solution was extracted with 10 mL of CH_2Cl_2. The CH_2Cl_2 was removed to give the desired 4-hydroxylated dihydropyridine: λ_{max} = 255 and 315 nm; ^{1}H NMR (in $CDCl_3$) 1.22–1.47 (m, ethyl methyls), 1.53 (s, 2,6-dimethyls), 2.57 (q, J = 7.5 Hz, 4-CH_2CH$_3$), 4.37 (q, J = 7.1 Hz, OCH_2CH$_3$), and 7.10–7.68 ppm (m, aryl protons); EIMS m/z (relative abundance) 327 (76, $M^+ - 46$), 312 (86, $M^+ - 61$), 298 (100, $M^+ - 75$), and 282 (34, $M^+ - 91$).

Destruction of the Cytochrome P-450 Chromophore. Incubation mixtures contained rat liver microsomes (4.0 nmol/mL), the dihydropyridine substrate (10 mM), and either NADPH (1.0 mM) or an NADPH regenerating system consisting of $NADP^+$ (1.0 mM), $MgCl_2$ (2.0 mM), glucose 6-phosphate (3.0 mM), and glucose-6-phosphate dehydrogenase (4 units/mL), all in 100 mM (pH 7.4) phosphate buffer containing KCl (150 mM) and diethylenetriaminepentaacetic acid (DETAPAC) (1.5 mM). The NADPH (or $NADP^+$) was added last to initiate the incubations, which were carried out at 25 °C. NADPH (or $NADP^+$) or the substrate was omitted from parallel control incubations. Aliquots were withdrawn as desired and the cytochrome P-450 chromophore was quantitated from the CO vs CO – reduced difference spectrum using a molar absorbance value of 91 000 (Estabrook et al., 1972).

Metabolite Identification and Quantitation. The incubation procedure used to examine metabolite formation was identical with that used for studies of cytochrome P-450 destruction except that the incubation volume was 2–3 mL and the $NADP^+$ regenerating system was invariably used. $NADP^+$

was omitted from control incubations. The mixtures were incubated at 37 °C for 10 min and were then added to tubes containing 2 mL of CH_2Cl_2, 0.4 mL of water containing 2 N NaCl and 1 M Na_2CO_3, and 10 μL of a 0.5 mg/mL solution of 3,5-bis(carbethoxy)-2,6-dimethyl-4-phenyl-1,4-dihydropyridine, the internal standard. The resulting biphasic mixture was vigorously agitated on a vortex mixer and was then centrifuged on a table-top centrifuge to separate the layers. For the analysis of pyridine and *N*-phenylpyridine metabolites, a 1.5-mL aliquot of the organic layer was transferred to a clean vial, and the solvent was removed under a stream of nitrogen. For the analysis of *N*-alkylpyridinium metabolites, the aqueous phase was lyophilized to dryness, the residue taken up in methanol, and the methanol removed. In both instances, the final residue was taken up in 100 μL of the solvent used for the chromatographic analysis, and a 10-μL aliquot was analyzed by HPLC. The dihydropyridine and pyridine metabolites were analyzed on a 240 × 4.6 mm Whatman Partisil silica column eluted with 25:75 tetrahydrofuran/hexane at a rate of 1.0 mL/min. The column effluent was monitored at 234 nm. The analysis of pyridinium metabolites was done by reverse-phase HPLC on a 250 × 4.6 mm Whatman Partisil ODS-3 column. The eluting solvents for the *N*-alkyl- and *N*-phenylpyridinium metabolites were respectively 1:1 methanol/water and 3:2 methanol/water, both containing 0.01 M methanesulfonic acid. The solvent flow rate was 1.0 mL/min, and the detector was set at 280 nm. Quantitative estimates of DDEP metabolite formation were obtained by adding 3,5-bis(carbethoxy)-2,6-dimethyl-4-phenyl-1,4-dihydropyridine as an internal standard. The standard was added before the incubation was worked up. The concentration of the metabolites was determined by comparison to a standard curve. The *N*-phenylpyridinium metabolites were similarly quantitated with *N*-methyl-3,5-bis(carbethoxy)-2,6-dimethylpyridinium nitrate as the standard except that the standard was added after the extraction step.

Spin Trapping of Carbon Radicals. Incubations were carried out with the standard microsomal system using the NADPH regenerating system, a 10 mM substrate concentration, and 20 mM POBN as the spin trap. Either the NADPH regenerating system or the substrate was omitted from control incubations. The mixtures were incubated at 25 °C and 50-μL aliquots were removed for EPR analysis. The following EPR parameters were typically employed: field set, 3400 G; scan range, 100 G; time constant, 0.25 s; scan time, 4 min; modulation amplitude, 1 G; modulation fequency, 100 Hz; receiver gain, (2.5–5.0) × 10^4; microwave power, 20 mW, microwave frequency, 9.517 GHz.

Results

Oxidation of 3,5-Bis(carbethoxy)-3,5-dimethyl-4-alkyl-1,4-dihydropyridines. Liver microsomes from phenobarbital-pretreated rats were incubated with DDC, DDEP, and DDIP, and the metabolic products (Figure 2, Table I) were quantitated by HPLC. Of these three substrates, DDC is the most rapidly oxidized and DDEP the least rapidly oxidized (Table I). DDC is oxidized almost exclusively to the corresponding 4-methylpyridine whereas DDIP is oxidized almost quantitatively to the 4-dealkyl pyridine. DDEP, in contrast, gives comparable amounts of the 4-ethyl and 4-deethyl pyridine metabolites (Figure 3). The HPLC retention times of the metabolites and their absorption and mass spectra were identical with those of authentic samples (not shown). The structure of the 4-isopropylpyridine, however, is based simply on its spectroscopic properties because it was not possible to prepare an authentic sample. No aromatization of DDC or

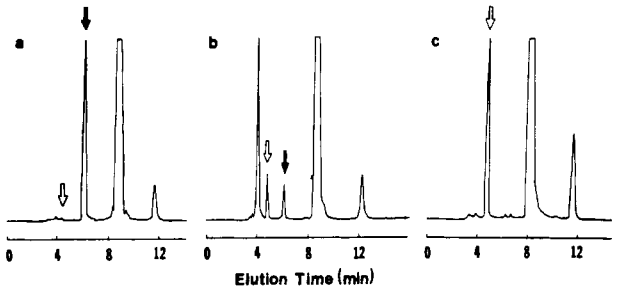


FIGURE 3: HPLC of the products isolated from the microsomal oxidation of (a) DDC, (b) DDEP, and (c) DDIP. The 4-unsubstituted (open arrow) and 4-substituted (closed arrow) pyridine metabolite is indicated in each chromatogram. The 4-substituted metabolite in panel c is assumed to be one of the trace peaks at 6–7 min, but it has not been unambiguously identified and is therefore not marked. The large unmarked peak is the substrate. The chromatographic conditions are given under Experimental Procedures.

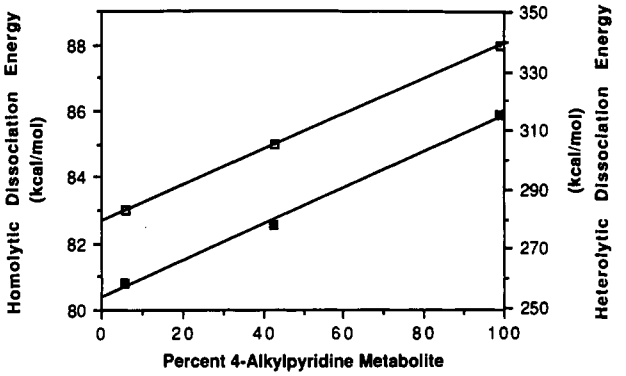


FIGURE 4: Correlation of the percent of 4-alkylpyridine metabolite versus the (a) 4-alkyl homolytic bond dissociation energies (■) and (b) 4-alkyl heterolytic bond dissocation energies (□). The homolytic dissociation energies are from Kerr (1966) and the heterolytic energies from Eggar and Cocks (1973).

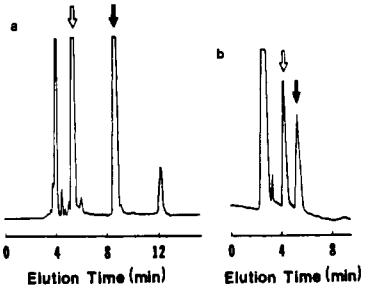


FIGURE 5: HPLC of the products formed in incubations of (a) *N*-ethyl-DDEP and (b) *N*-phenyl-DDEP with microsomal cytochrome P-450. In (a), *N*-ethyl-DDEP, the substrate, is indicated by the open arrow and DDEP, the primary product, by the closed arrow. No pyridinium products analogous to those in (b) were detected even though the authentic 4-unsubstituted *N*-ethylpyridinium nitrate was readily detected (not shown). In (b), the 4-unsubstituted (open arrow) and 4-ethyl-substituted (closed arrow) *N*-phenylpyridinium metabolites are indicated. The chromatographic conditions are described under Experimental Procedures.

DDEP is observed if NADPH is omitted from the incubations, but DDIP is oxidized to aromatic products at a reduced rate in the absence of NADPH. The proportion of the 4-alkylpyridine metabolite in the product mixture can be correlated linearly with both the homolytic and heterolytic bond energies of the 4-alkyl groups (Figure 4).

Oxidative Metabolism of N-Alkyl- and N-Phenyl-DDEP. Oxidation of *N*-ethyl- and *N*-methyl-DDEP by rat liver microsomes produces DDEP and a trace of the 4-ethyl and 4-deethyl pyridines expected from further metabolism of DDEP (Figure 5a). No metabolism is observed in the absence of

Table I: Pyridine Metabolite Formation in the Oxidation of Undeuteriated and 4-Deuteriated 4-Alkyl-1,4-dihydropyridines

	pyridine metabolites[b]			
	4-alkyl		4-dealkyl	
dihydropyridine[a]	nmol (nmol of P-450)$^{-1}$ min^{-1}	%	nmol (nmol of P-450)$^{-1}$ min^{-1}	%
4-methyl				
4-^{1}H	5.25 ± 0.55	99		1
4-^{2}H	4.25 ± 0.28	99		1
4-ethyl				
4-^{1}H	0.36 ± 0.01	43	0.48 ± 0.01	57
4-^{2}H	0.50 ± 0.01	41	0.35 ± 0.01	59
4-isopropyl		6	1.25 ± 0.31[c]	94
4-ethyl-*N*-phenyl				
4-^{1}H	0.081 ± 0.005	51	0.078 ± 0.005	49
4-^{2}H	0.012 ± 0.001	14	0.073 ± 0.004	86

[a] 3,5-Bis(carbethoxy)-2,6-dimethyl-1,4-dihydropyridines substituted at the 4-position with the indicated alkyl group and, if indicated, with a phenyl group on the dihydropyridine ring nitrogen. [b] 3,5-Bis(carbethoxy)-2,6-dimethylpyridines retaining at position 4 either the alkyl group or the hydrogen. The *N*-phenyl group is retained in the metabolites of *N*-phenyl-DDEP. The sum of the pyridine metabolites was taken as 100% in calculating the percent represented by each of the two in the mixture. In those instances where the minor component is present in too low a yield to accurately quantitate, only the percent determined from the relative peak heights of the products is given for the minor component. [c] This is the corrected value obtained by subtracting the rate of aromatic product formation observed in the absence of NADPH from that observed in its presence.

NADPH. No trace is detected of the *N*-alkylpyridinium metabolites expected from direct aromatization of *N*-methyl- or *N*-ethyl-DDEP even though the authentic pyridinium compounds are stable enough to be detected in our assay system (not shown). The *N*-alkyl derivatives of DDEP thus undergo cytochrome P-450 catalyzed oxidative dealkylation rather than aromatization.

The oxidation of *N*-phenyl-DDEP, which is not subject to N-dealkylation, by liver microsomes yields the 4-ethyl and 4-deethyl *N*-phenyl*pyridinium* metabolites in approximately a 1:1 ratio (Figure 5b, Table I). The pyridinium metabolites are formed at about one-fifth the rate of formation of the pyridine metabolites from DDEP itself (Table I). A third metabolite with a 1,4-dihydropyridine rather than pyridine or pyridinium chromophore is also detected. The structure of this metabolite has not been established because of its instability, but direct comparison by HPLC with an authentic sample (Partisil silica column eluted with 20% tetrahydrofuran in hexane) shows it is not the dihydropyridine metabolite with a hydroxyl at position 4. The instability of the metabolite and its dihydropyridine chromophore suggest it may be the *N*-(*p*-hydroxyphenyl) derivative of DDEP.

Deuterium Isotope Effects on Product Ratios. [4-^{2}H]DDC, [4-^{2}H]DDEP, and *N*-phenyl[4-^{2}H]DDEP were synthesized in the same manner as the unlabeled compounds with [1-^{2}H]-acetaldehyde or [1-^{2}H]propionaldehyde. The oxidation of [4-^{2}H]DDC and [4-^{2}H]DDEP by rat liver microsomes shows that deuterium substitution does not detectably alter the 4-dealkyl to 4-alkyl pyridine metabolite ratio (Table I). In contrast, the metabolite ratio is markedly shifted in favor of the 4-dealkyl metabolite in the case of *N*-phenyl[4-^{2}H]DDEP (Table I). The isotope effect for the reaction estimated from the change in product ratio is approximately $k_H/k_D = 6$. These results are consistent with competitive elimination of the hydrogen and 4-ethyl group in the aromatization of *N*-phenyl-DDEP but not DDC or DDEP.

Destruction of the Cytochrome P-450 Chromophore. Incubation of DDC with hepatic microsomes does not detectably decrease the enzyme chromophore, but analogous incubations

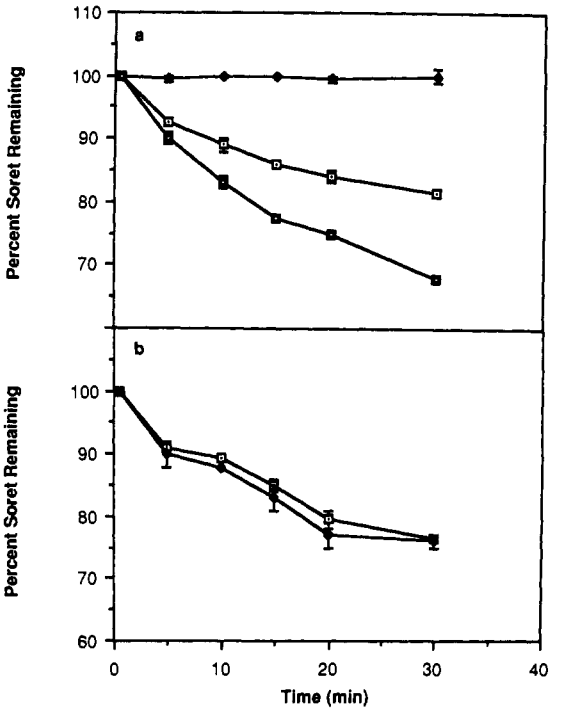


FIGURE 6: Destruction of cytochrome P-450 as a function of time by (a) DDC (◆), DDEP (□), and DDIP (■) and (b) DDEP (□) and [4-^{2}H]DDEP (◆). *N*-Phenyl-DDEP gives a plot similar to that for DDC. Error bars indicate maximum and minimum values. If no error bars are indicated, all the values fall within the symbol. The actual loss of cytochrome P-450 caused by DDEP and DDIP was respectively 2.6 and 4.1 nmol/10 min.

with DDEP and DDIP result in time-dependent loss of the Soret band (Figure 6a). DDIP destroys the enzyme chromophore more rapidly and more extensively than DDEP, but DDEP destroys the enzyme chromophore more efficiently than *N*-methyl- or *N*-ethyl-DDEP (not shown). *N*-Phenyl-DDEP does not detectably destroy the cytochrome P-450 chromophore (not shown). Direct comparison of the rate of chromophore destruction by DDEP and [4-^{2}H]DDEP shows that it is not significantly increased by deuterium substitution (Figure 6b). Neither [4-^{2}H]DDC nor *N*-phenyl[4-^{2}H]DDEP causes detectable chromophore loss (not shown). A comparison of DDEP metabolite formation (Table I) and cytochrome P-450 chromophore destruction indicates that approximately 26 molecules of DDEP are oxidized, 15 of them with loss of the 4-ethyl group, per prosthetic heme group that is destroyed.

Spin Trapping of Free Radicals. The 4-ethyl group is extruded as a spin-trappable ethyl radical in the cytochrome P-450 catalyzed aromatization of DDEP (Augusto et al., 1982). Extension of these spin-trapping studies to DDC and [4-^{2}H]DDC shows that neither of these substrates gives detectable EPR signals when incubated with microsomal cytochrome P-450 and POBN. Analogous oxidation of DDEP produces the expected six-line EPR signal of the POBN–ethyl radical adduct, but the rate and extent of accumulation of the EPR signal remain unchanged when [4-^{2}H]DDEP is used instead of DDEP (Figure 7b). A similar EPR signal is observed with DDIP, but the EPR signal of the adduct increases more rapidly (Figure 7a). Radical signal accumulation is much slower with *N*-methyl- and *N*-ethyl-DDEP, and no signal is observed with *N*-phenyl-DDEP or *N*-phenyl[4-^{2}H]DDEP (not shown).

Incubation of DDEP with Purified, Reconstituted Cytochrome P-450. Phenobarbital pretreatment increases the ability of DDEP to destroy the cytochrome P-450 chromophore

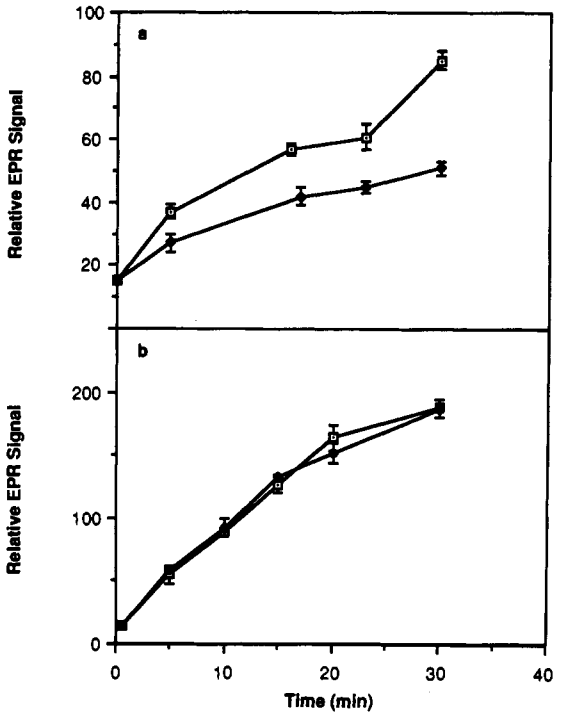


FIGURE 7: Time dependence of EPR signal intensity in incubations of the 4-alkyl-1,4-dihydropyridines with POBN and microsomal cytochrome P-450: (a) DDEP (◆) and DDIP (□) and (b) DDEP (◆) and [4-^{2}H]DDEP (□). Error bars indicate maximum and minimum values. If no error bars are indicated, all the values fall within the symbol.

and to cause ethyl radical accumulation. However, no loss of chromophore, no EPR-detectable radicals, and no metabolite formation are observed in incubations of DDEP with a 2:1 reconstituted mixture of purified cytochrome P-450 reductase and cytochrome P-450b, the principal isozyme induced by phenobarbital (not shown). DDEP therefore interacts with phenobarbital-inducible isozymes other than cytochrome P-450b. Recent work suggests that these isozymes include cytochromes P-450p and P-450h (Tephly et al., 1986; Guengerich et al., 1986; Correia et al., 1987).

Discussion

Oxidation of DDEP by microsomal cytochrome P-450 produces a flux of ethyl radicals that N-ethylate the prosthetic heme group and inactivate the enzyme (Augusto et al., 1982). These results led us to propose that the 4-ethyl group is eliminated as a free radical from the catalytically generated radical cation of DDEP (Figure 1). This mechanistic proposal is supported by the present demonstration that the 4-dealkylated pyridine metabolites are formed and that the importance of these metabolites increases in proportion to both the radical and cation stabilities of the 4-alkyl groups. A linear plot is thus obtained if the percent of the 4-alkylpyridine metabolite is plotted versus the homolytic or heterolytic bond energies for the methyl, ethyl, and isopropyl groups (Figure 4). A similar quantitative relationship is seen between the homolytic bond energies for the 4-alkyl groups and the initial rates of accumulation of the spin-trapped radicals (not shown). DDC thus yields no detectable radicals whereas DDIP yields a larger radical flux than DDEP (Figure 7a). Finally, the same quantitative relationship holds for the ability of the 4-methyl, 4-ethyl, and 4-isopropyl analogues to destroy the cytochrome P-450 chromophore (Figure 6a). The excellent correlation of the free radical stabilities of the 4-alkyl groups with the 4-dealkyl to 4-alkyl pyridine metabolite ratio, *carbon radical flux*, and enzyme chromophore destruction provides strong support for the proposed 4-alkyl radical extrusion mechanism.

If the dihydropyridine radical cation generated by cytochrome P-450 aromatizes by competitive elimination of either the 4-alkyl or 4-hydrogen, deuterium substitution at C-4 should result in increased elimination of the 4-alkyl group. Deuterium substitution, however, does not significantly alter the 4-dealkyl to 4-alkyl pyridine metabolite ratios obtained with either DDC or DDEP (Table I). The absence of an isotope effect on the DDEP product ratio is consistent with the finding that deuterium substitution does not detectably enhance either free radical release (Figure 7b) or cytochrome P-450 destruction (Figure 6b). These results preclude direct, competitive elimination of the 4-alkyl or 4-hydrogen from a common intermediate. The hydrogen and the alkyl group therefore must be eliminated from distinct species in the aromatization of DDEP.

If the ethyl radical is eliminated from the radical cation, the C-4 hydrogen must be eliminated from a different intermediate. In addition, mechanisms that eliminate the C-4 hydrogen as a proton rather than as a hydrogen radical are favored. These two requirements can be satisfied by deprotonating the *nitrogen* of the radical cation, transferring an electron from the resulting dihydropyridine radical to the enzyme to give the dihydropyridine cation, and deprotonating C-4 to give the 4-alkylpyridine metabolites (Figure 8). No isotope effect is expected by this mechanism if the alkyl group is extruded exclusively from the dihydropyridine radical cation and the C-4 proton from the final dihydropyridine cation. To test this hypothetical mechanism, we have investigated the

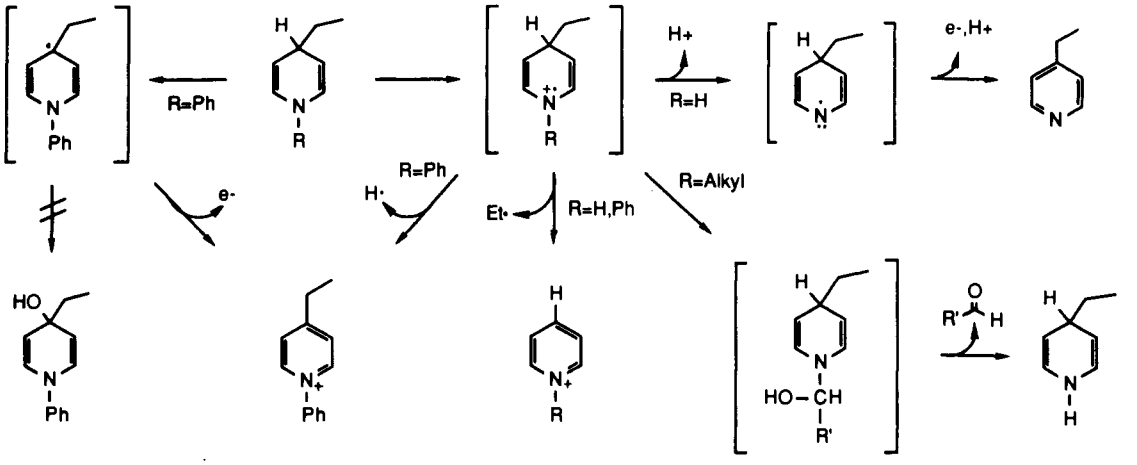


FIGURE 8: Mechanistic alternatives for the oxidation of N-substituted and N-unsubstituted 4-ethyl-1,4-dihydropyridines.

oxidation of N-substituted DDEP analogues that cannot lose a proton from the nitrogen. The oxidation of *N*-ethyl- and *N*-methyl-DDEP results in destruction of the cytochrome P-450 chromophore and accumulation of EPR-detectable POBN–radical adducts but at a slower rate than is observed with DDEP itself. Product analysis shows, furthermore, that the only detectable metabolites of the *N*-alkyl derivatives are DDEP and the pyridines expected from secondary oxidation of DDEP (Figure 5). No trace is found of the *N*-alkylpyridinium metabolites expected from direct aromatization of *N*-ethyl- or N-methyl-DDEP even though authentic standards show that the pyridinium metabolites are detectable. *N*-Methyl- and *N*-ethyl-DDEP are thus exclusively oxidized to DDEP rather than to the *N*-alkylpyridinium metabolites.

N-Dealkylation reactions are currently thought to involve oxidation of the nitrogen to the radical cation, deprotonation of the carbon vicinal to the nitrogen, and combination of the deprotonated carbon with the activated oxygen (Figure 8). The same nitrogen radical cation is therefore implicated in N-dealkylation and aromatization of the *N*-alkyl-DDEP analogues. The exclusive observation of N-dealkylation products therefore suggests that deprotonation of the *N*-alkyl moiety in the radical cation is much faster than the step which controls the aromatization reaction. Synchronous electron abstraction and 4-alkyl group elimination are specifically excluded by this result.

To avoid interference by the N-dealkylation reaction, we have investigated the oxidation *N*-phenyl-DDEP. The *N*-phenyl rather than *N-tert*-butyl derivative was chosen because the latter could not be synthesized. The cytochrome P-450 catalyzed oxidation of *N*-phenyl-DDEP yields the 4-ethyl and 4-deethyl *N*-phenylpyridinium metabolites in approximately a 1:1 ratio (Figure 5b, Table I). The only other metabolite detected in the incubation mixture retains the dihydropyridine chromophore. The structure of this metabolite is not known, but direct comparison with authentic material demonstrates it is not 4-hydroxylated *N*-phenyl-DDEP.

Formation of both the 4-deethyl *and* 4-ethyl *N*-phenylpyridinium metabolites shows that nitrogen deprotonation is not a mandatory prerequisite to elimination of the C-4 hydrogen (Figure 8). Phenyl substitution, however, alters the rate and may alter the mechanism of the oxidation reaction. *N*-Phenyl-DDEP does not detectably destroy the cytochrome P-450 chromophore or produce a detectable flux of ethyl radicals. This is due, in part, to the fact that *N*-phenyl-DDEP is only oxidized at about one-sixth the rate of DDEP itself. In addition, it is possible that reorientation of the substrate in the active site by *N*-phenyl substitution favors interception of the ethyl radical by the protein rather than the heme. A clear difference in the oxidation of *N*-phenyl-DDEP and DDEP is indicated by the fact that the aromatization of *N*-phenyl-DDEP is subject to a large product ratio isotope effect ($k_H/k_D = 6$) when deuterium is placed at C-4. This product ratio isotope effect suggests that removal of the C-4 hydrogen, possibility by the ferryl oxygen, competes with ethyl radical extrusion. This competition may result from competing routes for decomposition of the radical cation intermediate or may result from competition between electron abstraction to give the radical cation and direct abstraction of hydrogen at C-4. Removal of the hydrogen, however, cannot be followed by rebound addition of the activated oxygen because the 4-hydroxylated metabolite is not detectably formed even though it is sufficiently stable to be detected (Figure 8).

N-Phenyl substitution changes the mechanism of the oxidative aromatization from one in which eliminations of the C-4 hydrogen and 4-ethyl group do not complete to one in which they do. In practical terms, this limits the mechanistic alternatives for aromatization of *N*-phenyl-DDEP to those in which the nitrogen radical cation undergoes competitive elimination of the 4-ethyl or 4-hydrogen. The mechanisms for the aromatization of DDEP itself are not as narrowly limited by the present results. The nitrogen radical cation is still required to explain elimination of the ethyl radical from DDEP, but the details of the mechanism that results in elimination of the C-4 hydrogen are not yet clear. The absence of a kinetic isotope effect on the product ratio precludes competitive elimination of the ethyl and hydrogen from the radical cation, in agreement with the possibility that nitrogen deprotonation precedes aromatization, but the perturbations caused by nitrogen substitution make it difficult to obtain unambiguous evidence for such a deprotonation step.

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