Cytochrome P-450 Inactivation by 3-Alkylsydnones. Mechanistic Implications of *N*-Alkyl and *N*-Alkenyl Heme Adduct Formation†

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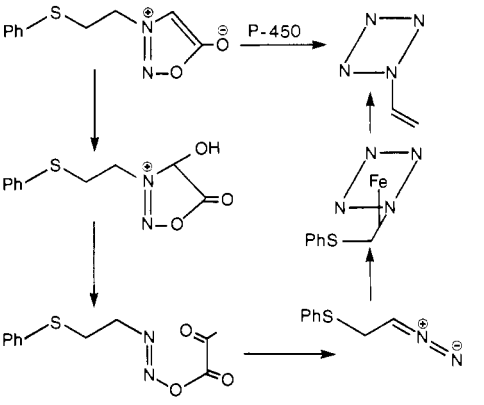
ABSTRACT: Incubation of 3-(2-phenylethyl)-4-methylsydnone (PMS) with liver microsomes from phenobarbital-pretreated rats or with reconstituted cytochrome P-450b results in loss of the enzyme chromophore. Chromophore loss is NADPH-dependent even though the sydnone decomposes by an oxygen- but not enzyme-dependent process to give pyruvic acid and, presumably, the (2-phenylethyl)diazonium cation. *N*-(2-Phenylethyl)protoporphyrin IX and *N*-(2-phenylethenyl)protoporphyrin IX have been isolated from the livers of rats treated with PMS. Both deuteriums are retained in the *N*-(2-phenylethyl) adduct derived from 3-(2-phenyl[1,1-^{2}H]ethyl)-4-methylsydnone, but one deuterium is lost in the *N*-(2-phenylethenyl) adduct. The *N*-(2-phenylethyl) to *N*-(2-phenylethenyl) adduct ratio is increased by deuterium substitution. No spectroscopically detectable intermediates precede chromophore loss in incubations of reconstituted cytochrome P-450b with PMS. Electron paramagnetic resonance (EPR)–spin trapping studies show that carbon radicals are formed in incubations of the sydnones with liver microsomes but by a process that is independent of chromophore destruction. It is proposed that the 2-phenylethyl radical formed by electron transfer to the sydnone-derived (2-phenylethyl)diazonium cation adds to the prosthetic heme group to give the *N*-(2-phenylethyl) adduct. This alkylation reaction is similar to that observed with (2-phenylethyl)hydrazine. Autoxidation of the Fe–CH(CH_2Ph)–N bridged species expected from insertion of 2-phenyldiazoethane into one of the heme Fe–N bonds is proposed to explain the unprecedented introduction of a double bond into the *N*-(2-phenylethenyl) adduct.

The metabolism of 3-[2-[(2,4,6-trimethylphenyl)thio]-ethyl]-4-methylsydnone (TTMS),[1] a representative of the pharmacologically interesting class of sydnones (Kier & Roche, 1967; Steward, 1964), has recently been shown to be a mechanism-based inactivator of rat liver microsomal cytochrome P-450 (Ortiz de Montellano & Grab, 1986). Destruction of the enzyme in vivo results in the accumulation of a hepatic pigment that has been isolated and identified as *N*-vinylprotoporphyrin IX. This *N*-alkylporphyrin is a potent inhibitor of ferrochelatase (Sutherland et al., 1986), the mitochondrial enzyme that inserts iron into protoporphyrin IX, and thus appears to be responsible for the potent porphyrinogenic activity of the parent sydnone (Stejskal et al., 1975).

The catalytic role of the enzyme in its own inactivation, the structure of the resulting heme adduct, the observation that pyruvic acid is concomitantly released, and the results of earlier model studies on the oxidation of sydnones (White & Egger, 1984) led us to formulate the mechanism in Scheme I for destruction of cytochrome P-450 by TTMS. Thus, cytochrome P-450 catalyzed hydroxylation of the sydnone ring triggers elimination of pyruvic acid and formation of the corresponding alkyldiazonium cation. Isomerization of the diazonium cation to the diazoalkane and reaction of the diazoalkane with the heme are suggested to result, in turn, in insertion of the diazoalkane carbon into one of the Fe–N bonds of the heme group

Scheme I: Mechanism Proposed for Inactivation of Cytochrome P-450 by TTMS[a]



[a] The prosthetic heme group of cytochrome P-450 is abbreviated as an iron atom in a square of four nitrogens.

to give a carbon-bridged intermediate. Finally, elimination of the thiomesityl moiety by the negative charge on the putative iron-coordinated carbon produces the *N*-vinyl adduct. In order to further define the mechanism of this reaction, we have examined the reaction of cytochrome P-450 with 3-(2-phenylethyl)-4-methylsydnone and 3-(2-phenyl[1,1-^{2}H]-ethyl)-4-methylsydnone, substrates that do not have a leaving group in the moiety expected to add to the nitrogen of the

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[1] Abbreviations: PMS, 3-(2-phenylethyl)-4-methylsydnone; PMS-d_2, 3-(2-phenyl[1,1-^{2}H]ethyl)-4-methylsydnone; TTMS, 3-[2-[(2,4,6-trimethylphenyl)thio]ethyl]-4-methylsydnone; POBN, α-(1-oxo-4-pyridyl)-*N*-*tert*-butylnitrone; SKF 252A, 2-(diethylamino)ethyl 2,2-diphenylpentanoate; heme, iron protoporphyrin IX regardless of the iron oxidation and coordination state; HPLC, high-pressure liquid chromatography; EPR, electron paramagnetic resonance; EIMS, electron impact mass spectrometry.

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prosthetic heme group. The results of these studies are reported here.

Experimental Procedures

Materials. NADPH, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, ethylenediaminetetraacetic acid (EDTA), and glutathione were purchased from Sigma; POBN and deuteriochloroform (100 atom % ^2H) were from Aldrich; dilauroylphoshatidylcholine was from Serdary Research Laboratories, Inc. (Port Huron, MI); and Chelex resin and protein assay kits were from Bio-Rad. Microanalyses were performed by the Microanalytical Laboratory of the University of California, Berkeley. The water used in all the biological work was deionized and double glass distilled. Buffers were passed through a Chelex column prior to use.

Microsomes and Purified Enzymes. Liver microsomes were prepared from phenobarbital-pretreated Sprague–Dawley male rats as previously described (Komives & Ortiz de Montellano, 1987; Ortiz de Montellano et al., 1981). Cytochrome P-450b was purified from the liver microsomes by the procedure of Waxman and Walsh (1982). Cytochrome P-450 reductase was purified by a minor modification (Komives & Ortiz de Montellano, 1987) of the procedure of Sheppard et al. (1983). Protein purity was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and by measurements of the specific cytochrome P-450 content (Komives & Ortiz de Montellano, 1987). Protein concentrations were measured with Bio-Rad kits.

Spectroscopic and Chromatographic Methods. Routine absorption spectroscopy and purified P-450 determinations were done on a Hewlett-Packard 8450A diode array spectrophotometer. Microsomal cytochrome P-450 determinations were carried out on an Aminco DW2a instrument. Routine ^1H NMR spectra were obtained at 80 MHz in $CDCl_3$ on a Varian FT 80 instrument. The ^1H NMR spectra of modified porphyrins were recorded in $CDCl_3$ at 500 MHz on a General Electric GN 500 instrument as reported earlier (Ortiz de Montellano & Grab, 1987). Chemical shift values are reported in parts per million relative to tetramethylsilane. Routine mass spectra were obtained on a Kratos MS 25 instrument operating in the electron impact (70-eV) mode. Mass spectra of the N-alkylated porphyrins were obtained on a Kratos MS 50 instrument operating in the field desorption mode or in the LSIMS mode with sulfolane as the matrix. EPR spectra were obtained on a Varian Model 104 spectrometer custom interfaced with an IBM XT computer. Cyclic voltammetry was done with a BAS Model CV-27 voltammograph. High-pressure liquid chromatography was carried out in a system consisting of two Beckman Model 110A pumps, a Beckman Model 420 controller, and a Hewlett-Packard 1040A diode array detector. Gas–liquid chromatography was carried out on a Varian 2100 instrument equipped with flame-ionization detectors and a Hewlett-Packard 3390A integrator.

Synthesis of 2-Phenyl[1,1-^2H]ethylamine (Nystrom, 1953). A solution of aluminum chloride (3.33 g, 25.0 mmol) in 40 mL of anhydrous ether was added under nitrogen via a dropping funnel to a stirred solution of lithium aluminum deuteride (1.05 g, 25.0 mmol) in 25 mL of anhydrous ether, and the mixture was stirred 5 min after the addition was complete. A solution of benzyl cyanide (2.93 g, 25 mmol) in 50 mL of anhydrous ether was then added dropwise with vigorous stirring. One hour later, water was added cautiously to quench the excess deuteride, and this was followed by the addition of 40 mL of 6 N HCl. The organic layer was separated, and the aqueous layer was washed with three 25-mL portions of ether. The aqueous layer was then brought to pH 11 by addition of KOH pellets before 100 mL of water was added. The resulting mixture was extracted with diethyl ether (3 × 100 mL), and the combined extracts were dried over anhydrous $MgSO_4$. Distillation through a short-path distillation column yielded 2.32 g (77% yield) of the desired product: 80-MHz ^1H NMR ($CDCl_3$) 7.21 (s, 5 H, phenyl), 1.72 (s, 2 H, methylene), 1.5 ppm (s, 3 H, NH); EIMS m/z 123 (M^+). The mass spectrometric analysis indicates 95% incorporation of deuterium.

Synthesis of N-(2-Phenylethyl)alanine and N-(2-Phenyl-[1,1-^2H]ethyl)alanine. To a solution of potassium *tert*-butoxide (4.15 g, 35 mmol) in 200 mL of freshly distilled *tert*-butyl alcohol was added 4.30 g (35 mmol) of 2-phenyl[1,1-^2H]ethylamine followed by 5.34 g (35 mmol) of 2-bromopropionic acid. The mixture was refluxed under nitrogen for 16 h before it was cooled and the solvent removed under reduced pressure. The residue was taken up in 100 mL of 2% NaOH, and the resulting aqueous solution was washed with 50 mL of diethyl ether before it was acidified to pH 5 with concentrated HCl. Filtration and washing with cold water afforded 3.41 g (50%) of the deuteriated amino acid: EIMS m/z 148 ($M^+ - CO_2$). The methyl ester was obtained by stirring a 0.05-g sample in 5 mL of 5% H_2SO_4 in methanol for 12 h: 80-MHz ^1H NMR of methyl ester ($CDCl_3$) 7.23 (s, 5 H, phenyl), 3.64 (s, 3 H, OCH_3), 3.38 [q, 1 H, J = 7.1 Hz, N-CH(Me)CO], 2.78 (s, 2 H, $PhCH_2$), 1.57 (s, 1 H, NH), 1.28 ppm (d, 3 H, J = 7.1 Hz, CH_3). The unlabeled compound, prepared by the same procedure, exhibited an additional NMR signal at 2.72 ppm (s, 2 H, CH_2N).

Synthesis of N-Nitroso-N-(2-phenylethyl)alanine and N-Nitroso-N-(2-phenyl[1,1-^2H]ethyl)alanine. A solution of *N*-(2-phenyl[1,1-^2H]ethyl)alanine (3.0 g, 15 mmol) and $NaNO_2$ (1.10 g, 16 mmol) in 100 mL of 1:1 CH_2Cl_2/H_2O was stirred at 0 °C while 1.5 mL of concentrated HCl was added dropwise over 10 min. The mixture was stirred for 2 h at 25 °C before the organic layer was separated, washed with saturated aqueous NaCl, dried over anhydrous Na_2SO_4, and concentrated on a rotary evaporator, providing 2.45 g (73%) of the desired nitrosamine (mixture of cis/trans isomers relative to the N=N—O function): 80-MHz ^1H NMR ($CDCl_3$) 8.33 (s, 1 H, CO_2H), 7.28 (m, 5 H, phenyl), 5.15 and 4.60 [2 q, 1 H, J = 7.1 Hz, *N*-CH(Me)CO], 3.17, 2.80, 2.82 (3 s, 2 H, $PhCH_2$), 1.63 and 1.40 ppm (2 d, 3 H, J = 7.4, CH_3). The unlabeled compound, prepared by the same procedure, exhibited a molecular ion in the mass spectrum at m/z 222, additional NMR signals at 3.74 and 4.37 ppm (2 m, 2 H, J = 7.4, 7.9 Hz, CH_2N), and splitting of the signals at 3.17, 2.80, and 2.82 ppm into triplets (J = 7.4, 7.9 Hz). Anal. Calcd for $C_{11}H_{14}N_2O_3$: C, 59.46; H, 6.35; N, 12.91. Found C, 59.32; H, 6.35; N, 12.51.

Synthesis of 3-(2-Phenylethyl)-4-methylsydnone. A solution of *N*-nitroso-*N*-(2-phenylethyl)alanine (8.30 g, 37.0 mmol) and 150 mL of freshly distilled acetic anhydride was allowed to stand at room temperature under nitrogen for 4 days. The reaction mixture was then poured into 300 mL of stirred ice water. The mixture was extracted with three 150-mL aliquots of CH_2Cl_2, and the combined extracts were washed with water, aqueous saturated $NaHCO_3$, and saturated aqueous NaCl before they were dried over anhydrous Na_2SO_4 and concentrated on a rotary evaporator. Recrystallization of the solid residue twice from ethyl acetate/hexanes afforded 4.31 g (58% yield) of the desired sydnone: ^1H NMR ($CDCl_3$) 7.26 (m, 5 H, phenyl), 4.42 (t, 2 H, J = 7.1 Hz, NCH_2), 3.22 (t, 2 H, J = 7.1 Hz, $PhCH_2$), 1.84 ppm (s, 3 H, methyl); IR ($CHCl_3$) 2925, 2256, 1729, 1455, 1377 cm^{-1}; EIMS m/z 204 (M^+).

Anal. Calcd for $C_{11}H_{12}N_2O_2$: C, 64.68; H, 5.92; N, 13.72. Found: C, 64.81; H, 5.95; N, 13.67.

3-(2-Phenyl[1,1-^{2}H]ethyl)-4-methylsydnone. To a solution of *N*-nitroso-*N*-(2-phenyl[1,1-^{2}H]ethyl)alanine (800 mg, 3.6 mmol) in 5 mL of dry diethyl ether was added 1 mL of trifluoroacetic anhydride, and the reaction mixture was stirred at 0 °C for 1 h. The solution was then concentrated in vacuo, and the residue, taken up in 25 mL of CH_2Cl_2, was washed with saturated $NaHCO_3$ and saturated aqueous NaCl before it was dried over anhydrous $MgSO_4$. Solvent removal and recrystallization from CH_2Cl_2/diethyl ether afforded 350 mg (48%) of the desired sydnone: mp 77–79 °C; 80-MHz ^{1}H NMR ($CDCl_3$) 7.24 (s, 5 H, phenyl), 3.24 (s, 2 H, $PhCH_2$), 1.83 ppm (s, 3 H, CH_3); EIMS *m/z* 206 (M^+). A comparison of the peaks at *m/z* 204 and 206 in the mass spectrum indicates the product is 95% deuteriated.

In Vitro Microsomal Metabolism of PMS. Standard 10-mL incubations contained microsomes from phenobarbital-pretreated rats (2.4 nmol of P-450/mL), KCl (150 mM), EDTA or diethylenetriaminepentaacetic acid (DETAPAC) (1.5 mM), NADPH (1 mM), and PMS (5 mM) in 0.1 M sodium/potassium phosphate buffer (pH 7.4). Control incubations were carried out simultaneously in the absence of either liver microsomes or NADPH. The reaction mixtures were incubated for periods of 15 or 60 min at 37 °C and then quenched by adding 5 mL of 10% (v/v) aqueous trichloroacetic acid. The resulting mixtures were centrifuged and extracted with cold (0 °C) diethyl ether (2 × 5 mL). The extracts were dried over anhydrous K_2CO_3 and analyzed by gas–liquid chromatography at 150 °C on a 6-ft glass column packed with 10% Carbowax on 120/140 mesh Chrom Q. Pyruvic acid formation was quantitated by the method of Friedemann (1957) as previously reported (Ortiz de Montellano & Grab, 1987).

Destruction of Microsomal Cytochrome P-450 by PMS. Incubation mixtures consisted of hepatic microsomes from phenobarbital-pretreated rats (3.0 nmol of P-450/mL), KCl (150 mM), EDTA or DETAPAC (1.5 mM), 100 μL of the regenerating system, and PMS (5 mM) in 0.1 M sodium/potassium phosphate buffer (pH 7.4). The regenerating system consisted of $MgCl_2$ (20.1 mg), glucose 6-phosphate (75 mg), $NADP^+$ (50 mg), NADPH (5 mg), and 120 units of glucose-6-phosphate dehydrogenase in 0.65 mL of 0.1 M sodium/potassium phosphate buffer (pH 7.4). The NADPH regenerating system or the sydnone was omitted from the control incubations. The mixtures were incubated at 4 or 37 °C, as stipulated in the text, for periods of 2–30 min. They were then placed on ice and their P-450 contents determined from their CO vs reduced CO difference spectrum by the procedure of Estabrook et al. (1972).

Aerobic Incubations with Purified Cytochrome P-450b. Cytochrome P-450b (2.5 nmol), cytochrome P-450 reductase (5 nmol), and 10 μL of dilauroylphosphatidylcholine were mixed and allowed to stand for 10 min at 25 °C before sufficient 0.1 M potassium phosphate buffer (pH 7.4) containing 20% glycerol and 25 μM DETAPAC was added to bring the volume to 0.5 mL. PMS or TTMS (approximately 1 mg) was then added, and the mixture was incubated for 5 min at 25 °C before NADPH (0.5 mM) was added to initiate the reaction. The absolute absorption spectrum was monitored as a function of time on a Hewlett-Packard 8450A diode array spectrophotometer. After 30 min, the incubation mixture was loaded onto a 14 × 1.7 cm column of Sephadex G-25 (fine) preequilibrated with 0.1 M potassium phosphate buffer (pH 7.4) containing 20% glycerol and 25 μM DETAPAC. The protein fractions eluted with the same buffer were collected and their absorption spectra recorded.

In one set of experiments, unreconstituted cytochrome P-450b (7 nmol) and PMS (1 mg) in 0.1 M potassium phosphate buffer (pH 7.4) containing 20% glycerol and 25 μM DETAPAC in a final volume of 0.4 mL were preincubated at 25 °C for either 2 or 15 min before sodium dithionite was added to reduce the cytochrome P-450. The absorption spectrum of the mixture was monitored during the incubation.

Anaerobic Incubations with Purified Cytochrome P-450b. The cytochrome P-450 system was reconstituted as described above, starting with 5.25 nmol of cytochrome P-450b, 10.5 nmol of cytochrome P-450 reductase, and 30 μL of dilauroylphosphatidylcholine in a final volume of 1.5 mL. The reconstituted enzyme was placed in an anaerobic cuvette system equipped with a plunger, a gas inlet/outlet, and a small magnetic stirring bar. The desired sydnone (1 mg) was then added to the incubation mixture, and NADPH (0.5 mM final concentration) and placed in the plunger. The system was flushed with argon for 30 min at 25 °C before the NADPH was transferred to the incubation mixture to start the reaction. The absorption spectrum of the mixture was monitored as a function of the incubation time.

EPR–Spin Trapping Studies. The reaction mixtures consisted of microsomal cytochrome P-450 (2.5 nmol/mL), POBN (20 mM), NADPH (1 mM), KCl (150 mM), EDTA (1.5 mM), and either PMS (5 mM) or TTMS (5 mM) in 0.1 M sodium/potassium phosphate buffer (pH 7.4). The mixtures were incubated at 37 °C. Aliquots were transferred at the desired times to 50-μL capillary tubes, and their EPR spectra were recorded.

Heme Adduct from Rats Treated with PMS. Each of 15 male Sprague–Dawley (250–260 g) rats injected intraperitoneally once a day for 5 days with an 80 mg/kg dose of phenobarbital was injected intraperitoneally on the sixth day with PMS (60–100 mg/250-g rat in 0.25 mL of dimethyl sulfoxide). The rats were decapitated 3 h later, and their livers were perfused with 1.15% KCl solution before they were excised and homogenized in 5% (v/v) H_2SO_4 in methanol with a Waring blender. The homogenate (100 mL/rat liver or 1.5 L) was allowed to stand for 16 h at 4 °C in the dark and was then filtered, diluted with 1 volume of distilled water, and extracted with CH_2Cl_2 (3 × 800 mL). The combined organic layers were washed with distilled water (2 × 1000 mL) and saturated aqueous NaCl solution (1 × 1000 mL) before they were dried over anhydrous Na_2SO_4, filtered, and concentrated on a rotary evaporator to a volume of approximately 100 mL. A solution of 15 mg of $Zn(OAc)_2$ in 10 mL of methanol was then added, and remaining solvent was removed on the rotary evaporator. The residue was chromatographed either on a column (20 × 2 cm) of Merck silica gel 60 (230–400 mesh) eluted sequentially with 1:1 (v/v) hexane/$CHCl_3$ (100 mL), $CHCl_3$ (100 mL), and 3:1 $CHCl_3$/acetone or on four preparative (2000-μm) silica gel G plates developed with 3:1 (v/v) chloroform/acetone. Electronic absorption and NMR spectra were obtained for the crude Zn^{2+}–porphyrin complex thus obtained. The zinc complex was then converted to its free base form by treatment with 5% (v/v) H_2SO_4 in methanol, extraction into CH_2Cl_2, and washing of the organic layer with saturated $NaHCO_3$ (Ortiz de Montellano et al., 1983). The free base was purified by high-pressure liquid chromatography on a 4.6 × 250 mm Partisil PAC 5 column eluted with 1% methanol in 1:1 (v/v) purified tetrahydrofuran/hexanes with the diode array detector set at 418 nm. The *N*-alkylporphyrin fractions were collected, concentrated, and spectroscopically

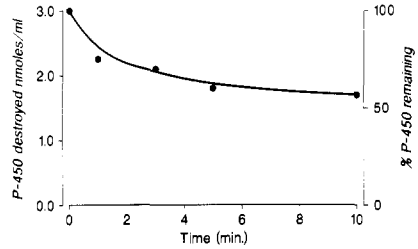


FIGURE 1: Loss of the cytochrome P-450 chromophore as a function of time in incubations of phenobarbital-induced microsomes with PMS at 4 °C. The same chromophore loss is achieved within 1–2 min at 37 °C, but no more enzyme is lost if the incubation is run for up to 30 min at 37 °C. The details of the incubation and assay are given under Experimental Procedures.

analyzed. The areas of the HPLC peaks were estimated both by triangulation and by cutting out and weighing the peaks.

RESULTS

Destruction of Microsomal Cytochrome P-450. Incubation of microsomes from phenobarbital-pretreated Sprague–Dawley male rats with PMS results in NADPH- and time-dependent destruction of the cytochrome P-450 chromophore (Figure 1). Control experiments show that the P-450 content is decreased by approximately 10% if the microsomes are incubated for 30 min with the sydnone but no NADPH or with NADPH but no sydnone. Subtraction of these control values from the 60% loss observed in the presence of both PMS and NADPH indicates that approximately 40% of the microsomal isozymes are susceptible to specific catalysis-dependent destruction. As shown in Figure 1, maximum chromophore loss is achieved within a few minutes even at 4 °C. No trace was detected in either aerobic or anaerobic incubations of species with Soret maxima above 450 nm. Addition of glutathione (10 mM) to the incubation mixtures provided no protection against inactivation by PMS (not shown).

Spectroscopic Changes Observed in Incubations of PMS and TTMS with Reconstituted Cytochrome P-450b. Incubation of reconstituted cytochrome P-450b with PMS or TTMS at 25 °C caused no time-dependent spectroscopic changes until NADPH was present. Within 30 s of the addition of NADPH, a marked decrease was observed in the intensity of the Soret band at 416 nm (Figures 2 and 3). The Soret band decreased to a minimum value within approximately 10 min of the addition of NADPH. Loss of the Soret band thus occurs more slowly in the reconstituted enzyme system than in the microsomal system. The reason for this is not known. A new absorption with a maximum at 444 nm is evident within 7–8 min and is a prominent feature of the final spectrum. The ratio of the absorbance at 416 nm to that at 444 nm after 30 min, which is essentially the same as that after 10 min, is 1.2:1.0 for both PMS and TTMS.

Passage of the mixtures through a Sephadex G-25 column 30 min after the incubations were started, at which time no further spectroscopic changes were discernible, caused a general broadening of the spectra (not shown). The same broadening was observed if the enzyme was passed through the G-25 column without first being incubated with PMS. The essential features of the spectrum, however, were not changed by passage through the Sephadex G-25 column. The Soret band at 416 nm remained highly attenuated, and the band at 444 nm reappeared if dithionite was added to reduce the cytochrome, although the latter band appeared as a shoulder rather than a well-resolved peak due to the spectral broadening.

Incubation of reconstituted cytochrome P-450b with PMS or TTMS under anaerobic conditions did not cause significant loss of the chromophore at 416 nm or detectable appearance

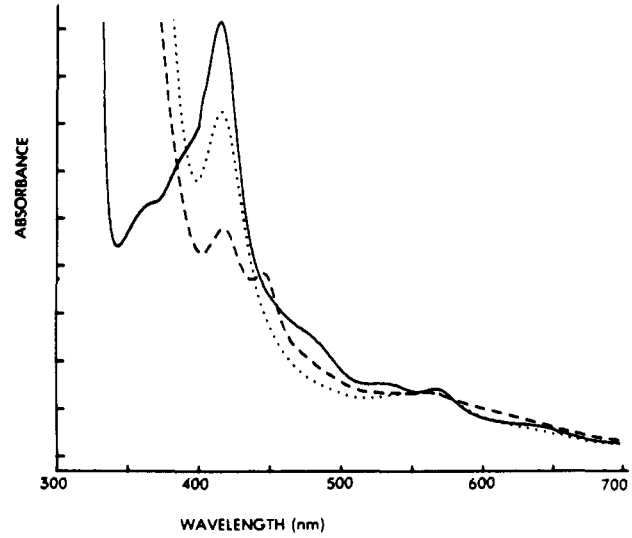


FIGURE 2: Alterations in the chromophore of purified, reconstituted cytochrome P-450b caused by incubation with PMS and NADPH: the enzyme and PMS before adding NADPH (—) and 10 min (···) and 30 min (- - -) after adding NADPH and incubating the mixture at 25 °C.

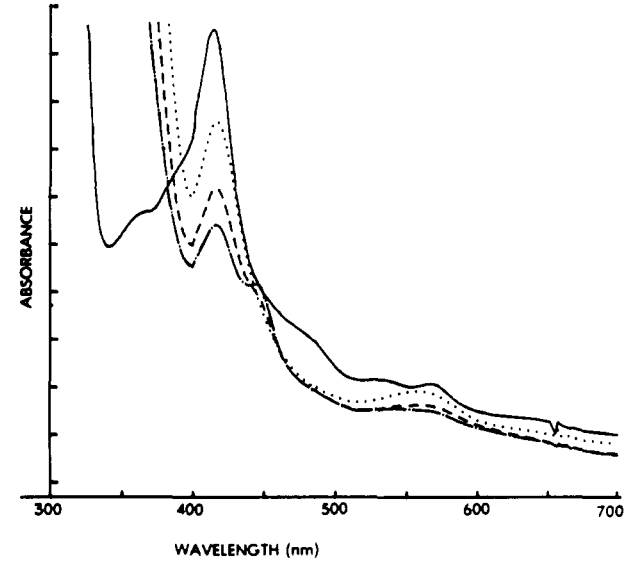


FIGURE 3: Alterations in the chromophore of purified, reconstituted cytochrome P-450b caused by incubation with TTMS and NADPH: the enzyme and TTMS before adding NADPH (—) and 30 s (···), 5 min (- - -), and 10 min (-·-) after adding NADPH and incubating the mixture at 25 °C.

of the peak at 444 nm (not shown). The spectra of the anaerobic incubations were not as clean as those of their aerobic counterparts due to strong end absorption in the 300–400-nm region by NADPH and reduced cytochrome P-450 reductase. Loss of the Soret band at 416 nm and appearance of the peak at 444 nm were also not observed when unreconstituted cytochrome P-450b was incubated with PMS in the presence of dithionite rather than cytochrome P-450 reductase and NADPH (Figure 4).

Products Formed from PMS. Incubation of hepatic microsomes with 5 mM PMS produces approximately 2000 nmol of pyruvic acid in a 10-mL incubation in 30 min whether NADPH is present or not. A comparable amount of pyruvic acid is obtained if PMS is simply incubated with chelex-treated pH 7.4 phosphate buffer. The release of pyruvic acid from PMS, in contrast to that from TTMS (Ortiz de Montellano & Grab, 1987), is mediated by an enzyme-independent mechanism. Parallel incubations of PMS and TTMS in

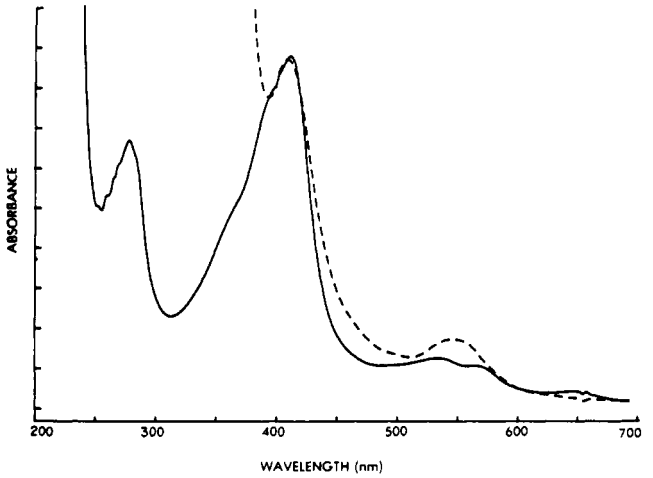


FIGURE 4: Effect of incubating purified, *un*reconstituted cytochrome P-450b with PMS and dithionite: enzyme and PMS before adding dithionite (—) and after adding dithionite and incubating the mixture for 10 min at 25 °C (– – –).

phosphate buffer confirmed that only PMS yields pyruvic acid in the absence of the microsomal enzyme system.

Three additional products were detected by gas–liquid chromatography in diethyl ether extracts of incubations of hepatic microsomes with PMS. Two of these products, as shown by comparison of their gas–liquid chromatographic retention times (3.92 and 11.95 min, respectively) and mass spectra with those of authentic samples, are phenylacetaldehyde and 2-phenylethanol. The third product, with a retention time of 7.43 min, has not been identified. Styrene was not detected even though 2-phenyldiazoethane reportedly rearranges slowly to styrene (Moritani et al., 1968). As observed with pyruvic acid, the formation of these products is not enzyme dependent. The same products in approximately the same ratios are obtained when PMS is simply incubated with phosphate buffer at pH 7.4 (not shown). Product formation is almost completely suppressed, however, if PMS is incubated *anaerobically* with phosphate buffer (not shown). The ratio of phenylacetaldehyde to 2-phenylethanol shifts slightly in favor of the alcohol when PMS-d_2 rather than PMS is incubated in phosphate buffer. Approximately 30% more 2-phenylethanol is formed from PMS-d_2. The data show that product release from PMS is mediated by an oxygen-dependent mechanism and that the distribution of products is somewhat sensitive to deuterium substitution.

EPR–Spin Trapping Studies. EPR-detectable free-radical signals are observed when PMS or TTMS is incubated with hepatic microsomes in the presence of the spin trap POBN (Figure 5). The EPR signals observed with both sydnones increase as a function of time for at least 1 h. No EPR signal is observed in microsomal incubations without NADPH or in incubations of the sydnones without microsomes or NADPH, but a low background signal is detected in microsomal incubations containing NADPH but no sydnone. The intensity of the EPR signal is diminished by the cytochrome P-450 inhibitor SKF 525A (Figure 5). The data show that carbon free radicals are formed by a process that depends on the presence of NADPH and liver microsomes. However, it is clear that the spin-trapped radicals are not generated by isozymes that are destroyed because the radical signal rises continually for at least 1 h whereas chromophore loss is complete within minutes.

Heme Adducts. Modified porphyrins are obtained when the livers of phenobarbital-induced rats treated with PMS are extracted with acidic methanol. Partial purification of the

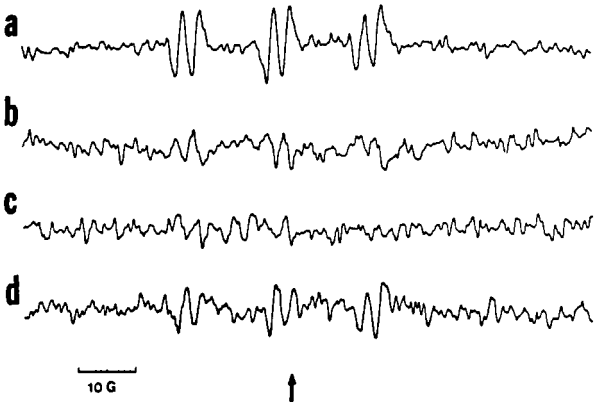


FIGURE 5: EPR of the spin adduct obtained in a 30-min incubation of hepatic microsomes from phenobarbital-pretreated rats with 1.0 mM NADPH, 25 mM POBN, and 5 mM PMS: (a) complete incubation system; (b) complete incubation system plus 1 mM SK-F525A; (c) incubation system minus NADPH; (d) incubation system minus the sydnone. The details of the incubations are given under Experimental Procedures. The arrow indicates the $g = 2.0000$ mark. Instrument settings: microwave power, 20 mW; modulation amplitude, 1 G; time constant, 25 s; scan range, 100 G; gain, 5×10^4; modulation frequency, 100 kHz. Similar data are obtained when 5 mM PMS is replaced in the incubations by 5 mM TTMS.

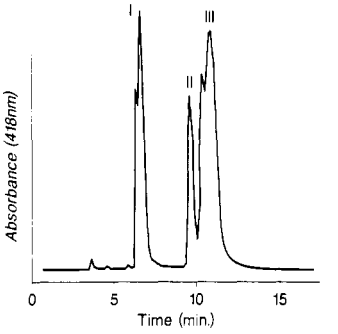


FIGURE 6: High-pressure liquid chromatograph of the pigment isolated from the livers of phenobarbital-induced rats treated with PMS. The porphyrin fractions are denoted as I, II, and III. The chromatographic conditions are given under Experimental Procedures.

porphyrins as the zinc complexes followed by demetalation yields a residue that is separated by HPLC into five or six porphyrins (Figure 6). The porphyrins were separated into three fractions. The first two overlapping peaks were collected as fraction I, the third peak as fraction II, and the overlapping fourth and fifth peaks as fraction III. In some chromatograms, the two peaks of fraction I were not clearly resolved but the leading peak of fraction III was resolved into two peaks. The electronic absorption spectra of the three fractions, which are nearly identical in both the free base and zinc-complexed forms (Table I), suggest that the compounds are *N*-alkylprotoporphyrin IX derivatives (Ortiz de Montellano & Mico, 1980).

The field desorption mass spectra of the isolated porphyrins exhibit molecular ions at m/z 693 (fractions I and II) or 695 (fraction III) (Table I). The molecular ion at m/z 695 is that expected for dimethyl-esterified *N*-(2-phenylethyl)protoporphyrin IX isomers (Ortiz de Montellano et al., 1983). In contrast, the porphyrins in fractions I and II, with a molecular ion at m/z 693, must bear a phenyl*ethenyl* rather than phenylethyl moiety or must have lost two hydrogens from the porphyrin framework. To differentiate between these alternatives, the modified porphyrins were isolated from rats treated with PMS-d_2. The molecular ions of the porphyrins in fraction III shifted by 2 mass units to m/z 697, as expected for isomers of *N*-(2-phenylethyl)protoporphyrin IX. In contrast, the molecular ions of fractions I and II shifted by only 1 mass unit

Table I: Electronic Absorption Maxima and Mass Spectrometric Molecular Ions of Porphyrin Fractions Isolated by High-Pressure Liquid Chromatography

fraction	absorption maxima[a] (nm) (relative intensity)	molecular ion,[b] (m/z) PMS	PMS-d_2
free base			
I	418 (100), 510 (11.0), 544 (5.1), 602 (2.2), 658 (1.1)	693	694
II	418 (100), 510 (10.0), 544 (4.9), 602 (1.4), 656 (0.4)	693	694
III	416 (100), 512 (11.0), 544 (6.5), 598 (3.5), 656 (2.8)	695	697
zinc complex			
I–III	432 (100), 544 (8.3), 594 (12.3), 634 (4.3)		

[a] Electronic absorption maxima of the dimethyl-esterified porphyrins. [b] Molecular ion of the dimethyl-esterified free base porphyrins. The molecular ions of the PMS and PMS-d_2 adducts are given.

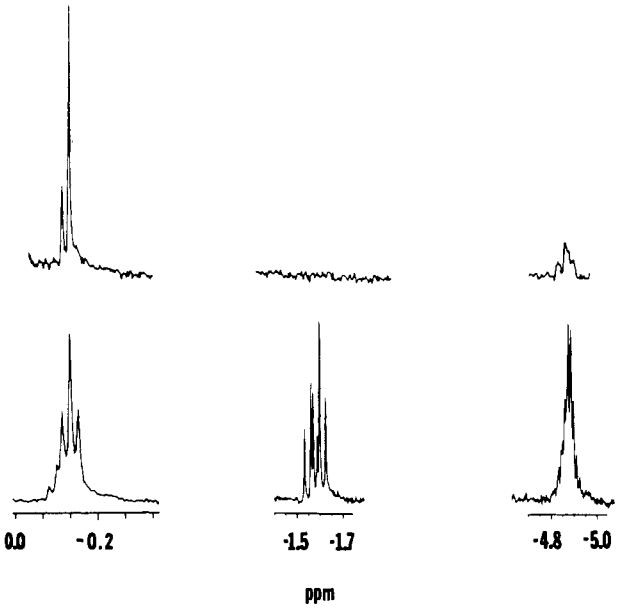


FIGURE 7: Upfield region of the 500-MHz ^{1}H NMR spectra of the dimethyl-esterified, zinc-complexed *N*-alkylporphyrin regioisomer mixtures obtained from phenobarbital-induced rats treated with PMS (lower spectrum) and PMS-d_2 (upper spectrum).

(from m/z 693 to m/z 694). One of the deuteriums of *PMS-d_2* is thus lost in the adduct. This is consistent with identification of the porphyrins in fractions I and II as dimethyl-esterified *N*-(2-phenylethenyl)protoporphyrin IX isomers.

The NMR spectra of all *N*-alkylprotoporphyrin IX derivatives consist of a relatively invariant set of signals due to the protoporphyrin IX skeleton and a variable set of signals due to the *N*-alkyl group. The NMR spectrum of the unseparated mixture of modified porphyrins was compared with that of authentic *N*-(2-phenylethyl)protoporphyrin IX (Ortiz de Montellano et al., 1983). The comparison shows that two isomers of the *N*-(2-phenylethyl) structure are present. This is clearly shown by the two triplets at −0.15 and −0.13 ppm due to the benzylic protons of the *N*-(2-phenylethyl) moieties in the two isomers, which collapse to a pair of singlets in the isomers obtained with PMS-d_2 (Figure 7). The adjacent methylene protons are found at −4.84 to −4.93 ppm as a multiplet approaching two partially resolved triplets that disappears in the isomers derived from PMS-d_2. The methylene protons at −4.84 to −4.93 ppm are thus coupled to those at −0.13 and −0.15 ppm. The corresponding protons of the

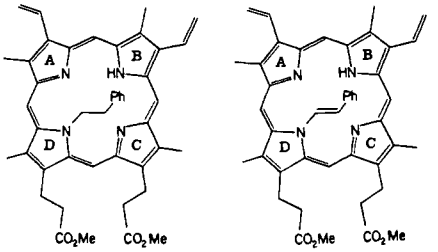


FIGURE 8: Structures of the two N-alkylated porphyrins isolated from rats treated with PMS. Only one of the four possible regioisomers is shown for each of the *N*-alkylporphyrins. The pyrrole rings of the porphyrin are identified by the letters.

single isomer obtained with (phenylethyl)hydrazine are found in the reference spectrum as triplets at −0.20 and−4.93 ppm (Ortiz de Montellano et al., 1983). The para phenyl protons of the *N*-(2-phenylethyl) moiety are found at 6.67 (t, 1 H, J = 7 Hz), the meta phenyl protons at 6.55 (t, 2 H, J = 7 Hz), and the ortho phenyl protons of the major isomer at 5.35 (d, J = 7 Hz) and of the minor isomer at approximately 5.37 ppm. Only the chemical shifts of the ortho phenyl protons appear to differ in the two isomers, presumably because they are more sensitive to differences in the porphyrin ring current. The ratios of the triplets at −0.15 and −0.13 ppm and of the ortho phenyl protons at 5.35 and 5.37 ppm suggest that the two isomers of *N*-(2-phenylethyl)protoporphyrin IX (Figure 8) are present in approximately a 3:1 ratio. Approximately the same ratio is obtained from the relative areas of the HPLC peaks (Figure 6).

In addition to the signals for the *N*-(2-phenylethyl) group, three doublets are found at −1.61, −1.58, and −1.56 ppm (J = 19 Hz) (Figure 7). The three doublets suggest that three additional regioisomers are present with the *N*-alkyl group on different pyrrole rings. The doublets appear at positions slightly downfield of that for the internal proton of the *N*-CH=CH_2 group in *N*-ethenylprotoporphyrin IX (−1.96 ppm) (Ortiz de Montellano & Grab, 1987). The finding that the three doublets disappear in the product mixture formed with PMS-d_2 shows that the proton in question is that of the original phenylethyl terminus. The results are consistent with identification of the porphyrins as regioisomers of *N*-(2-phenylethenyl)protoporphyrin IX. A small downfield shift is expected for the internal vinyl proton of the *N*-CH=CHPh moiety compared with that of the *N*-CH=CH_2 moiety because of the shielding effect of the phenyl group. The vinyl proton next to the nitrogen appears as a doublet because it is coupled to the second vinyl proton, but it has not been possible to specifically locate the second vinyl proton because it resonates in the 0–1 ppm region of the spectrum obscured by impurities. This is consistent with the earlier finding that the proton cis to the porphyrin in *N*-ethenylprotoporphyrin IX is at −0.13 ppm (J = 15.2 Hz) and that trans to the porphyrin is at 0.85 ppm (J = 8.5 Hz) (Ortiz de Montellano & Grab, 1987). The finding that the coupling constant in the present isomers is 19 Hz suggests, as would be expected, that the phenyl group is trans and the vinyl proton cis to the porphyrin. The phenyl ring protons of the three regioisomers are found as a complex pattern of overlapping signals in the region between 6.0 and 6.7 ppm (Table II). The NMR data thus fully support the proposal that the structures are regioisomers of *N*-(2-phenylethenyl)protoporphyrin IX (Figure 8). The ratio of the doublets in the −1.6 ppm region indicates that the three regioisomers are present in roughly a 1:2:1 ratio.

The protons of the protoporphyrin IX framework appear as complex multiplets in the NMR spectrum due to slight

Table II: NMR Data for the Mixture of Dimethyl-Esterified *N*-(2-Phenylethyl)- and *N*-(2-Phenylethenyl)protoporphyrin IX Regioisomers

protons	multiplicities	chemical shift (ppm)
	Protoporphyrin IX Skeleton	
meso	singlets[a]	10.14–10.54
internal vinyl	overlapping multiplets	8.03–8.28
external vinyl	overlapping multiplets	6.13–6.48
$CH_2CH_2CO_2H$	overlapping multiplets	4.10–4.40
methyls	singlets[b]	3.41–3.72
$CH_2CH_2CO_2H$	triplet	3.29 (J = 7 Hz)
	two multiplets	2.7–2.9
	N-Alkyl Group	
N-CH_2CH_2Ph	multiplets	−4.84 to −4.93
N-CH_2CH_2Ph	triplet	−0.15 (J = 7 Hz)
	triplet	−0.13 (J = 7 Hz)
C_6H_5	triplet (para)	6.67 (J = 7 Hz)
	triplet (meta)	6.55 (J = 7 Hz)
	doublet (ortho)[d]	5.35 (J = 7 Hz)
	doublet (minor)[d]	5.37
	multiplet[e]	5.99–6.02
N-CH=CHPh[c]	doublet	−1.56 (J = 18 Hz)
	doublet	−1.58 (J = 19 Hz)
	doublet	−1.61 (J = 19 Hz)

[a] Twenty singlets of different heights are found in the meso region of the spectrum, in agreement with the proposal that there are two *N*-phenylethyl and three *N*-phenylethenyl regioisomers in the mixture. [b] Fifteen singlets of different heights are found in the methyl region of the spectrum, in agreement with the proposal that there are two *N*-phenylethyl and three *N*-phenylethenyl regioisomers in the mixture. [c] Three sets of doublets, consistent with the presence of three *N*-phenylethenyl isomers. [d] Ortho protons of *N*-phenylethyl adducts. [e] Ortho protons of *N*-phenylethenyl adducts.

differences in the chemical shifts of the equivalent protons in the different regioisomers (Table II). The signal patterns show, however, that the major regioisomers bear the *N*-alkyl group on the nitrogens of pyrrole rings C and/or D. Thus, the signals for the internal protons of the 2- and 4-vinyl groups are not resolved into two distinct clusters, whereas the signals of the propionic acid methylene protons are clearly separated into two distinct sets of peaks. This pattern is diagnostic for *N*-alkyl substitution on pyrrole rings C and D rather than A and B (Kunze & Ortiz de Montellano, 1981). It is interesting in this context that only pyrrole ring D is detectably alkylated in the *N*-(2-phenylethyl) adduct obtained from the reaction of cytochrome P-450 with (2-phenylethyl)hydrazine (Ortiz de Montellano et al., 1983).

Effect of Deuterium Substitution on the Ratio of Saturated to Unsaturated N-Alkyl Products. The porphyrins in fractions I, II, and III from animals treated with unlabeled PMS, as shown by integration of the HPLC peak areas (Figure 6), represent 36, 15, and 49%, respectively, of the total porphyrin mixture. The same fractions from animals treated with PMS-d_2 represent 20, 10, and 70%, respectively, of the total mixture. Similar values were obtained in two independent experiments with PMS and PMS-d_2. Deuterium substitution thus favors the formation of products with a saturated *N*-alkyl moiety. An apparent isotope effect of about 2 is estimated from the fact that deuteriation changes the proportion of the porphyrins in fractions I + II to those in fraction III from 1.1:1 to 1:2.3.

Discussion

Incubation of PMS under catalytic turnover conditions with rat liver microsomal cytochrome P-450 (Figure 1) or reconstituted cytochrome P-450b (Figure 2) results in destruction of the monooxygenase. The chromophore changes caused by reaction with PMS are more clearly defined in the incubations with the purified, reconstituted enzyme. Time-dependent loss of the Soret band of the native enzyme at 412 nm is accompanied by the appearance of a new band with a maximum at 444 nm. These spectroscopic changes are only seen if NADPH, oxygen, and cytochrome P-450 reductase are present, as required if the destructive process depends on catalytic turnover of the enzyme. Direct evidence that the destructive process is irreversible is provided by the demonstration that the modified chromophore remains essentially unchanged when the reconstituted, PMS-treated enzyme is filtered through a Sephadex G-25 column to remove substances of low molecular weight and is rereduced. Nearly identical chromophore changes are seen when reconstituted cytochrome P-450b is incubated with TTMS (Figure 3). Cytochrome P-450b thus appears to be inactivated by PMS and TTMS by comparable mechanisms.

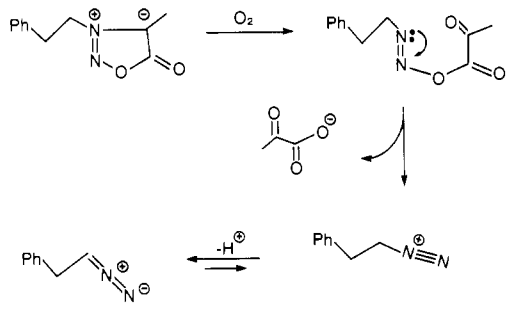
At no point in the incubations of sydnones with cytochrome P-450b were intermediates with absorption maxima above 450 nm detected. The chromophore changes caused by the sydnones thus resemble those caused by (phenylethyl)hydrazine except for the temporary appearance in the latter case of a peak with a maximum at 490 nm (B. A. Swanson and P. R. Ortiz de Montellano, unpublished data). The absence of intermediates with maxima above 450 nm in the reactions of PMS and TTMS is significant because all the complexes suggested to involve carbene–iron or alkyl–iron bonds exhibit such an absorbance. The phenyl–iron complex invoked in the reaction of phenylhydrazine with cytochrome P-450 thus has a maximum at 480 nm (Jonen et al., 1982; Delaforge et al., 1986; unpublished results), the σ-bonded Fe–$CHClCF_3$ adduct proposed in the halothane reaction a maximum at 470 nm (Ruf et al., 1984), and the carbene Fe=CCl_2 complex suggested in the reaction with CCl_4 a maximum at 460 nm (Wolf et al., 1977). The spectroscopic results indicate that carbene–iron or σ-bonded carbon–iron complexes do not accumulate in the reactions with sydnones but do not preclude their formation as unstable, transient intermediates or as intermediates in minor pathways.

Incubation of PMS with liver microsomes results in the formation of pyruvic acid, phenylacetaldehyde, and phenylethanol. However, in contrast to the formation of pyruvic acid from TTMS (Ortiz de Montellano & Grab, 1987), the same products are formed from PMS at roughly the same rates in the absence as in the presence of liver microsomes. The first step in the clearly enzyme-independent decomposition of PMS requires oxygen because PMS does not decompose under anaerobic conditions. The different chemical stabilities of TTMS and PMS are surprising in view of their nearly identical sydnone rings and oxidation potentials.[2] The reason for the different stabilities of PMS and TTMS is not known, but it is possible that chemical decomposition involves an autoxidative chain reaction that is efficiently quenched by the sulfur in TTMS.[3] In any case, it is likely that 2-phenylethanol is obtained by hydrolysis of the (2-phenylethyl)diazonium cation

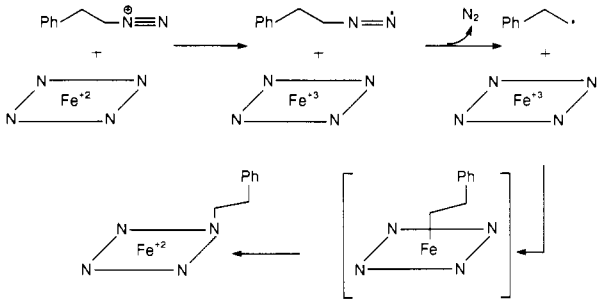
[2] The oxidation potentials of the sydnones were compared by cyclic voltammetry. The experiments were carried out with 5 mM solutions of the compounds in acetonitrile containing 0.1 M tetrabutylammonium perchlorate as supporting electrolyte. Cyclic voltammetry was carried out with a Pt electrode versus a standard calomel electrode over the range of 0–2 V at a scan rate of 0.25 V/s. Nearly identical voltammograms were obtained for PMS and TTMS with peak oxidation potentials (E_p) at 1.69 and 1.71 V, respectively.

[3] Comparison of the amounts of phenylacetaldehyde and 2-phenylethanol formed in incubations of PMS (5 mM) in 0.1 M phosphate buffer (pH 7.4) with and without added phenyl ethyl sulfide (5 mM) shows that the sulfide greatly decreases product formation.

Scheme II: Mechanism Proposed for Aerobic Decomposition of PMS to the (Phenylethyl)diazonium Cation and Isomerization of This Diazonium Cation to 2-Phenyl-2-oxodiazoethane



Scheme III: Mechanism Proposed for Formation of the *N*-(2-Phenylethyl) Heme Adduct in the Reaction of Cytochrome P-450 with PMS[a]



[a] The (2-phenylethyl)diazonium cation expected from oxidative metabolism of PMS (Scheme II) is shown as the starting substrate.

and phenylacetaldehyde by hydrolysis of the (2-phenylethyl)diazoethane rearrangement product. The finding that deuterium substitution increases the alcohol/aldehyde ratio is consistent with this rationale because deuterium substitution should slow down the isomerization reaction.

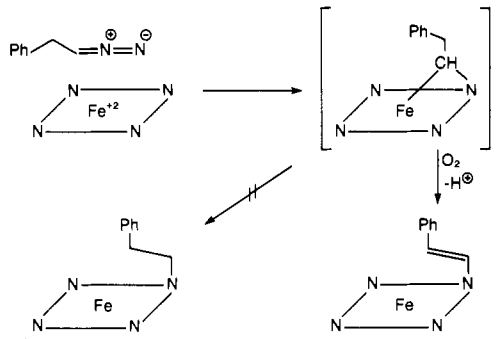
Inactivation does not simply reflect reaction of the enzyme with a degradation product of PMS because the degradation products are formed by an enzyme-independent mechanism whereas the enzyme is only destroyed under catalytic turnover conditions. In the case of TTMS, the finding that pyruvic acid formation lags behind enzyme destruction (Ortiz de Montellano & Grab, 1987) led us to postulate that the intermediate produced by enzymatic 4-hydroxylation of the sydnone ring partitions between rapid in situ decomposition and diffusion out of the active site followed by slow decomposition. Although both types of decomposition presumably yield pyruvic acid and the alkyldiazonium cation, the results indicate that the enzyme is primarily or exclusively inactivated by the products of in situ decomposition. The present findings are consistent with the earlier results in that catalytic turnover of PMS by the enzyme is required for inactivation even though PMS decomposes to apparently the same products by an enzyme-independent process. The thesis that the destructive species must be generated within the active site is supported by the finding that 10 mM glutathione does not protect the microsomal enzyme from inactivation.

The formation of two different types of N-alkylated porphyrins with PMS (Figure 8) suggests the existence of two different alkylation pathways. The HPLC and NMR data indicate that at least three of the four possible *N*-(2-phenylethenyl) regioisomers and two of the four possible *N*-(2-phenylethyl) regioisomers are formed, although it has not been possible to determine which pyrrole nitrogen is alkylated in each of the isomers. The *N*-(2-phenylethenyl) and *N*-(2-phenylethyl) adducts are isolated from PMS-treated rats in a 1.1:1 ratio, but the ratio changes to 1:2.3 when the adducts are isolated from PMS-d_2-treated rats. The pathway leading to saturated *N*-alkyl adducts is thus favored by deuterium substitution. This preference is readily rationalized if the saturated and unsaturated adducts are formed by reaction of the enzyme with respectively the (phenylethyl)diazonium cation and 2-phenyl-1-diazoethane expected from decomposition of PMS because deuterium should slow down diazonium cation–diazoalkane rearrangement (Scheme II). Indirect support for this view is provided by the finding that the 2-phenylethanol/phenylacetaldehyde ratio, which presumably depends on the rate of the alkyldiazonium cation to diazoalkane rearrangement, is increased by deuterium substitution.

The *N*-(2-phenylethyl) adduct obtained with PMS is identical, except for regioisomer distribution, with that obtained with (phenylethyl)hydrazine (Ortiz de Montellano et al., 1983). The earlier demonstration that phenylethyl radicals are formed in the reaction of (phenylethyl)hydrazine with liver microsomes led us to investigate whether carbon radicals are similarly formed with PMS and TTMS. In fact, EPR signals are observed when the sydnones are incubated with liver microsomes and POBN (Figure 5). However, even though radical formation requires NADPH *and* is suppressed by inhibition of the cytochrome P-450 system, it appears unlikely that chromophore destruction and EPR-detectable radical formation are closely linked because the EPR signal increases with time for at least 1 h whereas enzyme loss is complete at 37 °C within minutes. The 2-phenylethyl radical is, nevertheless, probably responsible for formation of the *N*-(2-phenylethyl) heme adduct because this adduct retains *both* of the deuterium atoms of PMS-d_2. Retention of both deuteriums specifically excludes the involvement of diazoalkane and carbene intermediates. The *N*-(2-phenylethyl) heme adduct thus appears to be formed by a mechanism analogous to that proposed earlier for inactivation of cytochrome P-450 by (phenylethyl)hydrazine, except that the phenylethyl radical would have to be formed from the sydnone by a reductive rather than oxidative step. This difference in the activation step is required by the fact that PMS yields an intermediate at the diazo oxidation state whereas the intermediate from (phenylethyl)hydrazine is at the diazene oxidation state. The data also suggest that the phenylethyl radical responsible for enzyme inactivation is produced and trapped within the active site of the target enzyme (Scheme III) (Ortiz de Montellano et al., 1983). The prolonged production of carbon radicals shows, however, that carbon radicals are also produced by resistant isozymes of cytochrome P-450 or by indirect, NADPH-dependent, processes.

The formation of *N*-(2-phenylethenyl)protoporphyrin IX is unprecedented. It is only superficially similar to the production of *N*-vinylprotoporphyrin IX from TTMS because the *N*-vinyl function is produced by elimination of a thiolate *anion* whereas formation of the *N*-(2-phenylethenyl) group requires elimination of a *proton* (Scheme I) (Ortiz de Montellano & Grab, 1987). We tentatively propose that the first step in both reactions is insertion of the diazoalkane into one of the iron–nitrogen bonds of heme to give a bridged species with the bridging carbon formally at the carbanion oxidation state. If a leaving group is present β to the iron-coordinated carbanion, as proposed for TTMS (Ortiz de Montellano & Grab, 1987), it is rapidly eliminated to give the *N*-vinyl function. In the absence of such a leaving group, however, the carbanion electron density may be removed by two one-electron oxidation steps with molecular oxygen as the probable terminal electron acceptor (Scheme IV). Elimination of a proton from the

Scheme IV: Mechanism Proposed for Formation of the *N*-(2-Phenylethenyl) Heme Adduct in the Reaction of Cytochrome P-450 with PMS[a]



[a] The 2-phenyl-2-oxodiazoethane metabolite expected from rearrangement of the (phenylethyl)diazonium cation (Scheme II) is shown as the starting substrate.

resulting cation then gives the observed *N*-(2-phenylethenyl) structure. In principle, the carbanion could pick up a proton to give the 2-(phenylethyl) moiety, but this would result, contrary to what is observed, in loss of one of the deuteriums in the *N*-(2-phenylethyl) adduct formed with PMS-d_2. The validity of the desaturation mechanism proposed above remains to be established, but it may be relevant that cytochrome P-450 has been found in two instances to desaturate an alkyl group (Rettie et al., 1987; Nagata et al., 1986).

The *N*-(2-phenylethyl) and *N*-(2-phenylethenyl) adducts may be formed by a single isozyme or by distinct isozymes of cytochrome P-450. As already described, formation of the saturated and unsaturated adducts is readily rationalized by differential reaction of cytochrome P-450 with alkyldiazonium cations and diazoalkanes, respectively. The shift from unsaturated to saturated adducts with PMS-d_2 supports this explanation but does not differentiate formation of both adducts by one isozyme from formation of the different adducts by different isozymes. This question can be resolved by analyzing the products formed by purified cytochrome P-450b, but it has not yet been possible to isolate the adducts from the purified enzyme in sufficient quantity for structural analysis.

Little is known about the reactions of diazo compounds with cytochrome P-450 beyond the fact that a transient complex with a Soret maximum at 468 nm is formed in the reaction of microsomal cytochrome P-450 with 1,1,1-trifluorodiazoethane (Mansuy et al., 1974) and that *N*-(carbethoxymethyl)protoporphyrin IX is formed in the reaction of ethyl diazoacetate with cytochrome P-450 (B. A. Swanson, E. A. Komives, and P. R. Ortiz de Montellano, unpublished results). Likewise, little is available in the literature on the chemical reactions of diazo compounds with iron porphyrins, although we have found that the reaction of diazoacetophenone with iron tetraphenylporphyrin yields the iron complex of *N*-(2-phenyl-2-oxoethyl)tetraphenylporphyrin (Komives et al., 1987). It is therefore necessary to look to the work on the reactions of diazocarbonyl compounds with cobalt porphyrins to find the chemical precedent for insertion of diazo compounds into metalloporphyrin metal–nitrogen bonds. Ethyl diazoacetate and diazoacetaldehyde thus react with cobalt porphyrins under reductive conditions to give adducts with the carbethoxymethylene or formylmethylene group, respectively, inserted into the cobalt–nitrogen bond (Johnson et al., 1975; Johnson & Ward, 1977; McLaughlin, 1974; Setsune & Dolphin, 1984, 1985). Oxidation of these cobalt–nitrogen bridged species converts them to the corresponding *N*-alkyl adducts. The available data suggest that bridged species are obtained by reductive insertion of diazoalkanes into porphyrin metal–nitrogen porphyrin bonds but that the iron bridged species may be less stable than the cobalt analogue. This may explain the absence of spectroscopically detectable intermediates in incubations of reconstituted cytochrome P-450b with PMS or TTMS. Good chemical models do exist for the formation of carbon radicals by electron transfer from ferrous heme to the diazonium function, as proposed for the reaction of cytochrome P-450 with sydnone-derived diazonium cations. Electron transfer from deoxyhemoglobin to substituted phenyldiazonium cations has been shown to result in the formation of aryl radicals and aryl–iron σ complexes (Doyle et al., 1985). The reactions proposed for the inactivation of cytochrome P-450 by PMS thus have at least partial precedent in reactions that have been observed in model systems.

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Use of Primary Deuterium and ^{15}N Isotope Effects To Deduce the Relative Rates of Steps in the Mechanisms of Alanine and Glutamate Dehydrogenases†

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ABSTRACT: We have used deuterium and ^{15}N isotope effects to study the relative rates of the steps in the mechanisms of alanine and glutamate dehydrogenases. The proposed chemical mechanisms for these enzymes involve carbinolamine formation, imine formation, and reduction of the imine to the amino acid [Grimshaw, C. E., Cook, P. F., & Cleland, W. W. (1981) *Biochemistry 20*, 5655; Rife, J. E., & Cleland, W. W. (1980) *Biochemistry 19*, 2328]. These steps are almost equally rate limiting for $V/K_{ammonia}$ with alanine dehydrogenase, while with glutamate dehydrogenase carbinolamine formation, imine formation, and release of glutamate after hydride transfer provide most of the rate limitation of $V/K_{ammonia}$. Release of oxidized nucleotide is largely rate limiting for V_{max} for both enzymes. When β-hydroxypyruvate replaces pyruvate, or 3-acetylpyridine NADH (Acpyr-NADH) or thio-NADH replaces NADH with alanine dehydrogenase, nucleotide release no longer limits V_{max}, and hydride transfer becomes more rate limiting. With glutamate dehydrogenase, replacement of α-ketoglutarate by α-ketovalerate makes hydride transfer more rate limiting. Use of Acpyr-NADPH has a minimal effect with α-ketoglutarate but causes an 8-fold decrease in V_{max} with α-ketovalerate, with hydride transfer the major rate-limiting step. In contrast, thio-NADPH with either α-keto acid causes carbinolamide formation to become almost completely rate limiting. These studies show the power of multiple isotope effects in deducing details of the chemistry and changes in rate-limiting step(s) in complicated reaction mechanisms such as those of alanine and glutamate dehydrogenases.

Alanine dehydrogenase from *Bacillus subtilis* catalyzes the reversible oxidative deamination of L-alanine:

$$NAD^+ + \text{L-alanine} \rightleftharpoons NH_4^+ + \text{pyruvate} + NADH + H^+ \quad (1)$$

Grimshaw and Cleland (1981) showed that the kinetic mechanism was ordered with NAD bound first and ammonia, pyruvate, and NADH released in that order. The isomerization of E·NAD is the slowest step for the oxidative deamination of alanine, but hydride transfer is also partially rate limiting.

Glutamate dehydrogenase from bovine liver catalyzes the reversible oxidative deamination of L-glutamate:

$$NAD(P)^+ + \text{L-glutamate} \rightleftharpoons NH_4^+ + \alpha\text{-ketoglutarate} + NAD(P)H + H^+ \quad (2)$$

The kinetic mechanism is random in the direction of oxidative deamination (Engel & Dalziel, 1969, 1970; Engel & Chen, 1975; Colen et al., 1972, 1977; Jallon et al., 1975; Silverstein & Sulebele, 1973; Rife & Cleland, 1980a). The reductive amination of α-ketoglutarate, however, involves ordered addition of NADPH, α-ketoglutarate, and NH_4^+ (Rife & Cleland, 1980a). Substrate inhibition by glutamate that is uncompetitive vs NADP and by α-ketoglutarate that is hyperbolic uncompetitive vs NH_4^+ suggests that release of both reduced and oxidized nucleotides is slow (Rife & Cleland, 1980a), while deuterium isotope effects of 1.1–1.2 at pH 7 (Rife & Cleland, 1980a; Cook, 1982) show that hydride transfer is only slightly rate determining. With the slow alternate substrate norvaline, hydride transfer is more rate limiting, and Srinivasan and Fisher (1985) have measured a primary deuterium isotope effect of about 4 (which is probably the intrinsic isotope effect on this step) on the oxidation of L-proline to Δ^1-pyrroline-carboxylic acid.

Although the degree of rate limitation by the hydride transfer step is known for both alanine and glutamate dehydrogenases, little is known of the contribution of other chemical steps within the interconversion of E·NAD(P)·amino acid and E·NAD(P)H·α-keto acid·ammonia central complexes. The present study presents evidence from deuterium and ^{15}N isotope effects that permits deduction of which of the several chemical steps in the reductive amination of the α-keto acids

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