

Carbon Radicals in the Metabolism of Alkyl Hydrazines*

(Received for publication, March 14, 1983)

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The metabolism of phenelzine (2-phenylethyldiazine) by rat liver microsomes yields phenylacetaldehyde, 2-phenylethanol, and ethylbenzene. A carbon radical is formed during the oxidative metabolism of phenelzine that reacts with the prosthetic heme of cytochrome P-450 and irreversibly inactivates the enzyme. The radical has been spin-trapped, isolated, and shown by mass spectrometry to be the 2-phenylethyl radical. The metal-free porphyrin derived from the prosthetic heme group has been isolated and identified as *N*-(2-phenylethyl)protoporphyrin IX. The metabolism of phenelzine, an alkyl hydrazine, thus yields a carbon radical that inactivates cytochrome P-450, is converted to a hydrocarbon by hydrogen atom abstraction, and reacts with spin traps or (presumably) alternative cellular targets.

and phenelzine (16) has shown that at least part of the inhibition of oxidative metabolism engendered by hydrazines reflects irreversible inactivation of microsomal cytochrome P-450. Inactivation requires catalytic turnover of the hydrazines by vulnerable cytochrome P-450 enzymes and is associated with the formation of unidentified hepatic pigments. In order to definitively identify the reactive species produced during the metabolism of alkyl hydrazines, and to explore the mechanism by which these substrates inactivate cytochrome P-450, we have undertaken to (a) identify the major microsomal metabolites of phenelzine, (b) determine if this agent is metabolically oxidized to a carbon radical, and (c) characterize the hepatic pigment formed during the accompanying destruction of cytochrome P-450. The results demonstrate that alkyl hydrazines are oxidized by hepatic microsomes to carbon radicals that react with the prosthetic heme of cytochrome P-450.

EXPERIMENTAL PROCEDURES

Materials—Benzyl hydrazine·HCl, phenylacetaldehyde, 2-phenylethanol, ethylbenzene, α -phenyl *N*-*tert*-butyl nitron, and α -(4-pyridyl)-1-oxide *N*-*tert*-butyl nitron were purchased from Aldrich Chemical Co. The phenylacetaldehyde was shown by gas chromatographic analysis to be free of 2-phenylethanol. Phenelzine·HCl (2-phenylethyldiazine·HCl) was obtained from ICN Pharmaceuticals, Inc. Bovine superoxide dismutase (type I), isocitrate dehydrogenase, isocitrate, glucose-6-phosphate dehydrogenase, glucose 6-phosphate, NADPH, NADH, bovine thymol-free catalase (nominally 10,000–25,000 units/mg), and human hemoglobin (type IV) were from Sigma. The catalase was dissolved in 0.1 M phosphate buffer (pH 7.4) at a concentration of 3 mg/ml and was extensively dialyzed against the same buffer before being used. Protein determinations were carried out with Bio-Rad protein assay kits. Deionized water was used throughout this investigation. The water was also glass-distilled before it was used for the EPR experiments.

Metabolite Identification and Cytochrome P-450 Inactivation—Hepatic microsomes were obtained as previously described (17) from 250–300-g male Sprague-Dawley rats after pretreatment with sodium phenobarbital (80 mg/kg/day) for 8 days. DETAPAC was substituted for EDTA in all of the procedures, however. Standard incubation mixtures contained the following: cytochrome P-450 (4.2–4.4 nmol/ml), DETAPAC (1.5 mM), NADP (0.78 mM), glucose 6-phosphate (7.8 mM), glucose-6-phosphate dehydrogenase (0.8 unit/ml), MgCl₂ (2.55 mM), and KCl (150 mM), all in 0.1 M phosphate buffer (pH 7.4). The NADPH-generating system was not included in control incubations. The concentration of phenelzine or benzyl hydrazine in the incubation, when present, was 5 mM, and the incubation volume, unless otherwise indicated, was 25 ml. The incubations were normally carried out at 37 °C in a reciprocating water bath for 60 min before they were terminated by the addition of trichloroacetic acid (5% w/v final concentration). The mixtures were centrifuged to remove the precipitated protein and the supernatants were extracted with UV grade (Burdick and Jackson, Inc.) hexane (1 ml/2.5 ml of incubation volume). A known concentration of propylbenzene was then added as an internal standard and the hexane extract was analyzed by gas-liquid chromatography on a 6-foot glass column packed with 10% Carbowax 20M on 120/140 mesh Chrom Q. The column temperature, initially at 50 °C, was programmed to rise to 200 °C at 15 °C/min upon injection of the sample. A Varian model 2100 instrument

Hydrazine derivatives are widely employed not only in a variety of industrial and technological applications but also in human medicine. Drugs that owe their pharmacological activity to the presence of a hydrazine function include the monoamine oxidase inhibitors isocarboxazid, nialamide, and phenelzine,¹ the antihypertensive agent hydralazine, the antitubercular isoniazid, and the anticancer drug procarbazine (1). The utility of hydrazine derivatives as drugs, however, is compromised by the fact that they elicit a variety of metabolism-dependent toxic effects. The metabolism of isoniazid is, in some instances, the overture to hepatic necrosis (2), that of hydralazine to both carcinogenesis (3) and lupus erythematosus (4), and that of phenelzine to carcinogenesis (5, 6). The metabolic activation of hydrazines is believed to involve the generation of cationic or radical alkylating agents (7, 8). We have recently reported preliminary evidence for the intervention of radicals (9).

Alkyl, aryl, and acyl hydrazines have been known for some time to impair the oxidative metabolism of co-administered substrates (10–14). Recent work with phenylhydrazine (15)

* This research was supported in part by a grant from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The trivial names and abbreviations used are: phenelzine, 2-phenylethyldiazine; heme, iron protoporphyrin IX regardless of the iron oxidation state; POBN, α -(4-pyridyl)-1-oxide *N*-*tert*-butyl nitron; PBN, α -phenyl *N*-*tert*-butyl nitron; DETAPAC, diethylenetriamine pentaacetic acid; SKF 525A, 2-diethylaminoethyl 2,2-diphenylpentanoate.

equipped with flame ionization detectors and a Hewlett-Packard model 3390A calculating integrator was used for these studies. The metabolites of phenelzine were located by comparing the gas chromatograms obtained from extracts of normal incubations with those of the incubation mixtures before addition of the NADPH-generating system. The structures of the metabolites were established by direct comparison of their gas chromatographic retention times and electron impact (70 eV) mass spectra with those of authentic samples. The mass spectrometric studies were carried out on a Kratos MS-25 instrument in-line with a Varian model 3700 gas chromatograph. A 24-m fused silica capillary column coated with OV-1 was used in these studies to separate the metabolites for mass spectrometric characterization. The column, initially at 40 °C, was programmed to rise 4 min after sample injection at a rate of 15 °C/min to 250 °C.

Aliquots were withdrawn from the incubations at various time points in the appropriate experiments and their cytochrome P-450 content was determined by difference spectroscopy on an Aminco DW-2A instrument (17). A molar absorbance of 100 mm⁻¹ cm⁻¹ was used for the absorbance difference between the peak at 450 nm and the asymptotic base-line at approximately 520 nm of dithionite-reduced CO-saturated versus unreduced CO-saturated microsomes to calculate the cytochrome P-450 concentrations.

Spin-trapping of Free Radicals—The incubation mixtures used for spin-trapping studies contained cytochrome P-450 (2.5 nmol/ml), phenelzine or benzyl hydrazine (5 mM), POBN (25 mM), NADPH (1 mM), KCl (150 mM), and DETAPAC (1.5 mM) in 0.1 M phosphate buffer (pH 7.4). The mixtures were normally incubated for 60 min at 37 °C. Additional components or components omitted in control incubations are indicated in the text. Aliquots, transferred from the incubation mixtures to capillary tubes (50- μ l capillary pipettes sealed at one end) at appropriate time points, were analyzed on a Varian E-3 or E-4 EPR spectrometer. Preliminary experiments established that the same EPR results were obtained with glass capillary tubes as with gas-permeable tubing (internal diameter 0.81 mm, wall thickness 0.05 mm, Zeus Industrial Products, Inc., Raritan, NJ). The permeable tubing was therefore only used for the characterization of radicals in benzene solution after they had been extracted and purified.

Isolation and Characterization of Phenelzine Spin-adducts—The radical trapped by POBN during the chemical oxidation of phenelzine was first isolated and characterized in order to (a) develop a protocol for purification of the biologically formed spin-adduct and (b) provide a reference to which the biological adduct could be spectroscopically compared. Phenelzine·HCl (52 mg) and POBN (39 mg) were stirred in 10 ml of 0.05 M carbonate buffer (pH 10) containing 0.1 mM CuCl₂. The reaction, monitored by the height of the EPR signal, was complete after approximately 90 min. The solution was then neutralized with HCl and was lyophilized. The residue was extracted with 10 ml of acetone. The acetone was removed at a rotary evaporator and the residue, taken up in chloroform, was chromatographed on a column of Silica Gel 60 (1 × 26 cm) eluted with 5% methanol in chloroform. The eluant fractions were checked by EPR for the presence of radicals and by UV spectroscopy for the presence of POBN. The EPR-active fractions not contaminated with POBN were combined, concentrated, and rechromatographed on a prepacked Merck Silica Gel 60 Lobar column (10 × 240 mm). The column was eluted with the same solvent as before under low pressure conditions. The column effluent was continuously monitored with a variable wavelength detector set at 280 nm (the UV maximum of the radical spin-adduct). The appropriate fractions (fractions of 0.5 ml volume were collected) were combined and the solvent was removed under vacuum. The purified spin-trapped radical thus obtained (approximately 300 μ g by weight on a Cahn balance) was characterized by electronic absorption spectroscopy and mass spectrometry.

The biological spin-adduct was obtained in a large scale incubation (125 ml) containing microsomal cytochrome P-450 (2.5 nmol/ml), phenelzine (5 mM), POBN (20 mM), NADPH (0.3 mM), isocitrate dehydrogenase (5 units/ml), isocitrate (5 mM), MgSO₄ (5 mM), KCl (150 mM), and DETAPAC (1.5 mM) in 0.1 M phosphate buffer (pH 7.4). The mixture, after incubation at 37 °C for 60 min, was cooled to 0 °C and was centrifuged at 100,000 × g for 30 min to remove the microsomal protein. The supernatant was lyophilized, the residue was extracted with acetone (20 ml), and the crude spin-adduct thus obtained was purified by the procedure described for the chemically generated species. Because the POBN was more difficult to remove, however, the chromatography on the Merck Lobar column was carried out twice with the biological sample.

The electronic absorption spectra of the chemically and metabolically obtained spin-adducts were recorded on a Hewlett-Packard HP

8450A spectrophotometer. The corresponding mass spectra were determined by direct probe insertion into a Kratos MS-25 instrument under electron impact conditions (70 eV).

Isolation and Characterization of the Phenelzine Prosthetic Heme Adduct—Phenelzine·HCl in water (60 mg/kg dose) was administered by intraperitoneal injection to 18 male Sprague-Dawley (250–300 g) rats after pretreatment with sodium phenobarbital (80 mg/kg/day) for 4 days. The rats were decapitated 3 h later and their livers were removed after perfusion with cold saline solution. The livers were homogenized and extracted with 5% H₂SO₄ in methanol (100 ml/liver) as described before in other studies (18). The crude zinc-complexed porphyrin fraction thus obtained was subjected twice to thin layer chromatography on silica gel plates (2000 μ thick) developed with 3:1 (v/v) CHCl₃/acetone. The red fluorescent fraction isolated from the plates was purified further by high pressure liquid chromatography on a Whatman 10-PAC column (4.6 × 250 mm) eluted at 3 ml/min with a 20-min linear 0–100% gradient of methanol into 1:1 hexane:tetrahydrofuran. The effluent was monitored with a variable wavelength detector set at 590 nm. The zinc complex of the porphyrin adduct eluted with a retention time of 8 min. The zinc was then removed by treatment with acidic methanol (18) and the metal-free porphyrin was chromatographed with the same elution protocol except with the detector set at 510 nm. The metal-free porphyrin eluted as a single peak with a retention time of 3 min. The field desorption mass spectrum of the metal-free porphyrin was obtained on an AEI MS-902 instrument as reported for other porphyrin adducts (18). The porphyrin adduct (approximately 400 μ g) was converted to its zinc chloride complex (19) before a 1 mM solution in 99.8% deuterated chloroform was prepared for NMR studies. NMR spectra were obtained on a custom built 240 MHz FT NMR spectrometer in San Francisco and on a Nicolet NT-360 FT NMR instrument at the University of California (Davis) NMR Facility. The electronic absorption spectra were recorded in methylene chloride on a Hewlett-Packard HP-8450A spectrophotometer.

Hemoglobin—The incubation of phenelzine with human oxyhemoglobin, monitored with an oxygen electrode, was carried out as described previously for other hydrazines except at a temperature of 37 °C (20). The attempt to isolate a prosthetic heme adduct from incubations of hemoglobin with phenelzine was carried out as reported for phenylhydrazine, except that the mixture was incubated for 24 h prior to workup (20).

RESULTS

Phenelzine Metabolites—Phenelzine was incubated with hepatic microsomes from phenobarbital-pretreated rats, the incubation mixture was extracted with hexane, and the extract was analyzed by gas chromatography. A comparison of chromatograms from phenelzine incubations carried out with and without NADPH reveals the formation of four, possibly five, metabolites (Fig. 1). Three of these, ethyl benzene (*peak A*), phenylacetaldehyde (*peak D*), and 2-phenylethanol (*peak F*), have been unambiguously identified by gas chromatographic and mass spectrometric comparison with authentic samples. The substances responsible for *peaks C* and *E* have not been identified.

Loss of Cytochrome P-450 Caused by Phenelzine and Benzyl Hydrazine—Incubation of phenelzine or benzyl hydrazine with hepatic microsomes results in time-dependent loss of cytochrome P-450 (Fig. 2). Little cytochrome P-450 is lost in control incubations without hydrazines, but some enzyme loss is observed in the absence of NADPH (Fig. 2). Virtually all of the cytochrome P-450 vulnerable to destruction by phenelzine succumbs within the first 15 min, whereas enzyme loss caused by benzyl hydrazine continues throughout the incubation period.

Metabolic Formation of Free Radicals from Phenelzine—The microsomal metabolism of phenelzine in the presence of a spin-trap (POBN) gives rise to a stable EPR signal (Fig. 3). The EPR signal is not observed in the absence of NADPH, but it is observed, albeit at a lower intensity, when NADPH is present but the microsomes are first denatured by immersion in boiling water (Fig. 3). NADH sustains radical forma-

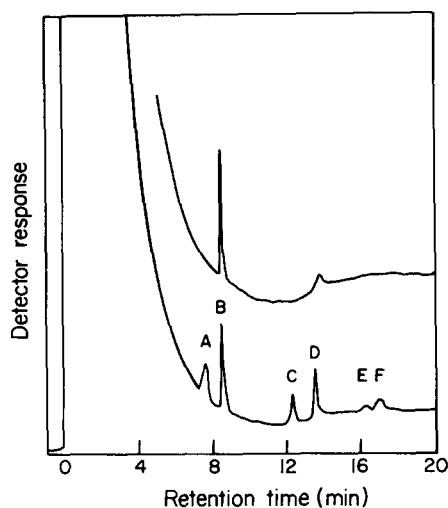


FIG. 1. Gas chromatographic analysis of the metabolites extracted by hexane from incubations of phenelzine with hepatic microsomes in the presence and in the absence (*inset tracing*) of NADPH. Peak B is due to the propylbenzene internal standard. The *inset* was recorded at a more sensitive instrument setting. The column oven temperature rose from 50–200 °C at a rate of 15 °C/min during the chromatographic run. Procedural details are given under "Experimental Procedures."

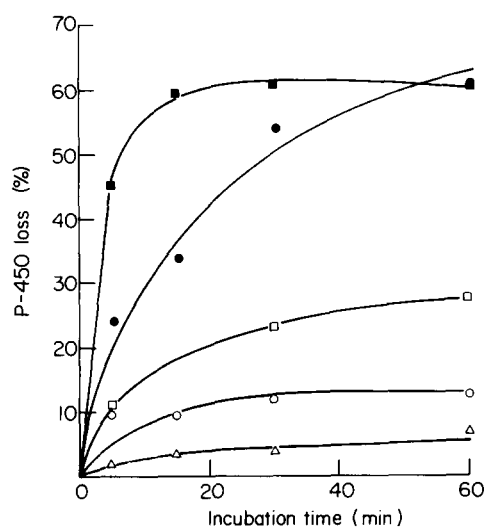


FIG. 2. Destruction of hepatic microsomal cytochrome P-450 by phenelzine and benzyl hydrazine as a function of incubation time. Enzyme loss caused by phenelzine (5 mM) in the presence (■) and absence (□) of NADPH; enzyme loss caused by benzyl hydrazine (5 mM) in the presence (●) and absence (○) of NADPH; and enzyme loss in the absence of hydrazine substrates (Δ).

tion less effectively than NADPH (Table I). No radicals are observed in the absence of phenelzine. Identical results are obtained after 30 rather than 60 min of incubation except that the EPR signal intensity is lower in all of the experiments (not shown).

The concentration of spin-trapped radical under various incubation conditions has been calculated from the height of the low field central EPR peak (Table I). The factor used to convert peak height into radical concentration was determined after isolation and characterization of the spin-trapped radical (see below). The concentration of the trapped radical (Fig. 4) rises steeply during the first 2–3 min of incubation and levels off at approximately the same value regardless of whether the initial phenelzine concentration is 1, 5, or 10 mM.

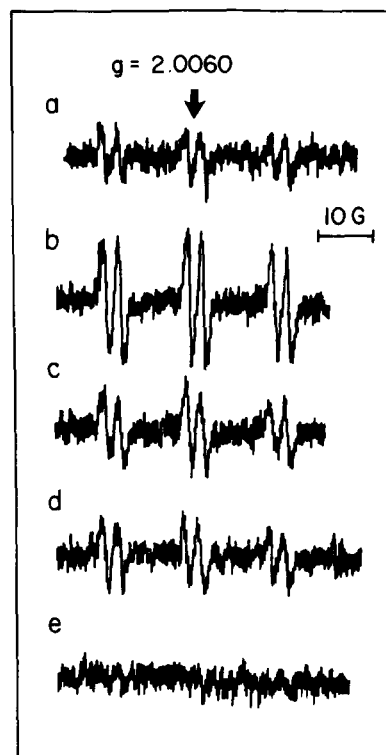


FIG. 3. EPR spectra of incubations of phenelzine (5 mM) with hepatic microsomes in the presence of POBN. *a*, control incubation with boiled microsomes; *b*, normal incubation; *c*, normal incubation after bubbling CO through the incubation mixture for 2 min; *d*, normal incubation also containing 0.1 mM SKF 525A; *e*, incubation without NADPH (the same result is obtained if phenelzine instead of NADPH is omitted). The spectra were obtained after 60 min of incubation at 37 °C. Identical, but weaker, EPR patterns are obtained after 30-min incubation. Instrument settings: microwave power, 10 milliwatts; modulation amplitude, 1.0 G; time constant, 1.0 s; scan time, 0.5 h; scan range, 200 G; gain, 6.2×10^5 .

TABLE I
Concentration of the phenelzine spin-trapped radical under various incubation conditions

Incubation conditions ^a	Concentration of radical adduct μM	Per cent of control
Normal	4.8	100
Boiled microsomes	2.2	47
+CO	3.2	67
+SKF 525A (0.1 mM)	3.6	76
+Catalase (0.1 mg/ml)	4.8	100
+Superoxide dismutase (0.1 mg/ml)	4.6	97
–NADPH + NADH (1.0 mM)	2.0	67
–NADPH	ND ^b	
–Phenelzine	ND	
–POBN + PBN (50 mM)	ND	

^a 60-min incubation period.

^b ND, not detectable.

A second, slower, phase of radical accumulation is then observed. The rate of the second phase depends directly on the concentration of phenelzine. The EPR signal intensity is attenuated in the presence of carbon monoxide or SKF 525A (Fig. 3). A significant concentration of the radical accumulates after 30 min with boiled microsomes. The higher activity of intact microsomes, the preference for NADPH over NADH,

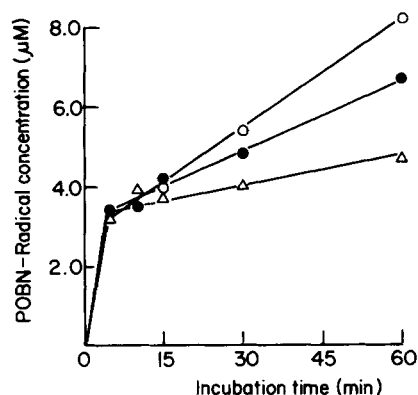


FIG. 4. Effect of phenelzine concentration and time on the concentration of the POBN-radical adduct. \circ , 10 mM; \bullet , 5 mM; and Δ , 1 mM. The phenelzine radical adduct concentration was calculated from the height of its EPR signal (see text).

the inhibition by carbon monoxide and SKF 525A, the failure to observe an EPR signal with PBN as the spin trap (Table I), and, finally, the inactivation of cytochrome P-450 by prosthetic heme alkylation (see below), suggest that cytochrome P-450 is one of the catalysts involved in radical generation. The failure of PBN as a radical trap supports this conclusion because PBN, an effective trap for 2-phenylethyl radicals, is in addition a good inhibitor of cytochrome P-450 (21, 22). The lack of inhibition by catalase and superoxide dismutase (Table I) argues against an important role in radical formation for the superoxide and hydrogen peroxide accessible to these exogenously added enzymes.

Identification of the POBN Radical Adduct Obtained with Phenelzine—The 2-phenylethyl radical formed by CuCl_2 -catalyzed oxidation of phenelzine at pH 10 was trapped with POBN and the adduct was purified by silica gel column chromatography. The purification sequence developed for the chemically generated radical was used, in turn, to isolate and purify the POBN radical adduct formed in the microsomal metabolism of phenelzine. The chemically and biologically obtained adducts, as judged by their chromatographic properties and by their EPR, electronic absorption, and mass spectra, are identical. The EPR spectra (in H_2O) of the pure POBN adducts are characterized by the following constants (Fig. 5): $a_N = 15.73$, $a_H = 2.75$, $g = 2.006$. The corresponding values in benzene under nitrogen are $a_N = 14.41$ and $a_H = 2.68$. The electronic absorption spectra of the adducts (Fig. 6, CHCl_3) exhibit a maximum at 283 nm (extinction coefficient $19,000 \text{ M}^{-1} \text{ cm}^{-1}$), whereas the maximum in hexane is at 290 nm (extinction coefficient $11,000 \text{ M}^{-1} \text{ cm}^{-1}$) and in methanol at 271 nm (extinction coefficient $22,000 \text{ M}^{-1} \text{ cm}^{-1}$). The chromophore of the adduct thus closely resembles that of the *N*-oxide of pyridine itself, which has a maximum at 276 nm (extinction coefficient $15,136 \text{ M}^{-1} \text{ cm}^{-1}$) in CHCl_3 (23), at 282 nm (extinction coefficient $10,000 \text{ M}^{-1} \text{ cm}^{-1}$) in hexane (24), and at 263 nm (extinction coefficient $13,800 \text{ M}^{-1} \text{ cm}^{-1}$) in methanol (23). The λ_{max} of the POBN adduct is 6–8 nm higher in each solvent than that of pyridine *N*-oxide, as expected from the presence of a substituent at position 4. The electronic absorption spectra of the POBN adducts differ substantially from the spectrum of POBN, which has a maximum at 355 nm with a broad shoulder on the shorter wavelength side (Fig. 6). The chemically and biologically obtained adducts have identical electron impact mass spectra with molecular ions at m/e 298 ($M - 1$) and high mass fragmentation peaks at m/e 283 ($M - 15$), 242 ($M - 57$), and 212 ($M - 87$) (Fig. 7). The molecular ion is that expected for the POBN-trapped 2-phenylethyl radical if a hydrogen atom is lost in the ionization

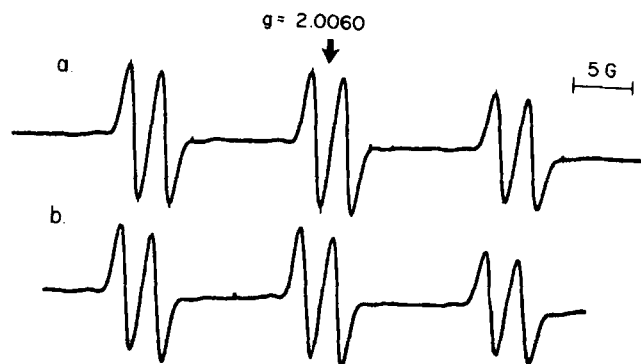


FIG. 5. EPR spectra of the 2-phenyl ethyl-POBN radical adduct isolated and purified from (a) CuCl_2 -catalyzed decomposition of phenelzine at pH 10 and (b) microsomal metabolism of phenelzine. The spectra were taken in 0.1 M phosphate buffer (pH 7.4). Instrument settings: microwave power, 10 milliwatts; modulation amplitude, 1 G; time constant, 0.3 s; scan time, 16 min; scan range, 100 G; gain, 1.5×10^4 for a and 8×10^3 for b.

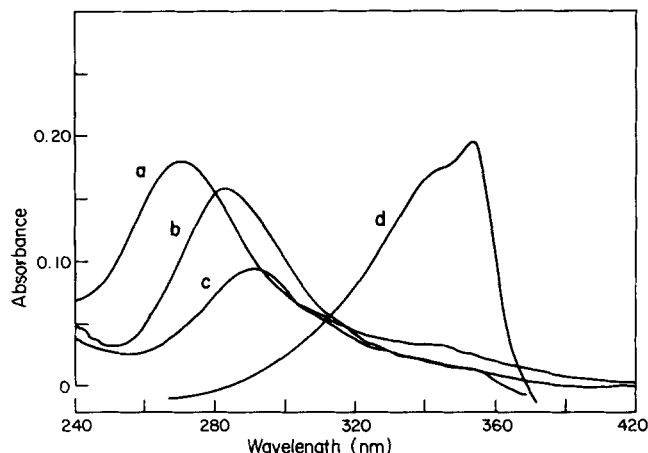


FIG. 6. Ultraviolet spectra of the 2-phenyl ethyl-POBN radical adduct in (a) methanol, (b) CHCl_3 , and (c) hexane. The spectrum of POBN (d) in CHCl_3 is given for comparison.

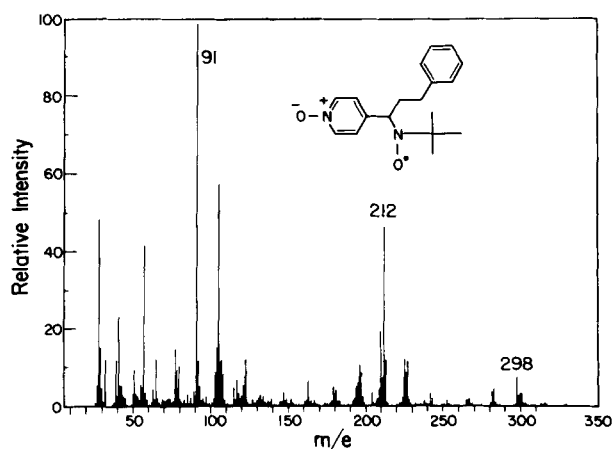


FIG. 7. Electron impact mass spectrum of the 2-phenyl ethyl-POBN radical adduct (inset structure) isolated and purified from a phenelzine microsomal incubation. The adduct formed by CuCl_2 -catalyzed decomposition of phenelzine has an identical mass spectrum.

process. The spectroscopic data uniquely identify the trapped species as the 2-phenyl ethyl radical.

Structure of the Phenelzine Prosthetic Heme Adduct—The inactivation of cytochrome P-450 by phenelzine was reported

by Muakkassah and Yang (16) to be accompanied by hepatic discoloration. We have confirmed this observation in phenobarbital-induced rats treated with phenelzine (60 mg/kg) and have succeeded in extracting and purifying the hepatic pigment. The *etio*-type porphyrin spectrum of the metal-free pigment in CHCl_3 has a Soret band at 416 nm and peaks of decreasing intensity at 512, 546, 592, and 652 nm. The zinc chloride complex of the green porphyrin (in CHCl_3) exhibits a Soret band at 434 nm and additional peaks at 548, 594, and 634 nm. The pattern and positions of the peaks in both spectra are characteristic of an *N*-alkyl protoporphyrin IX derivative (19, 25, 26). The Soret band of the zinc complex does not have a shoulder on the long wavelength side, a feature pertinent to the differentiation of the possible porphyrin isomers (see below). The only peaks in the field desorption mass spectrum of the green porphyrin are a monoprotonated molecular ion at m/e 695 and its isotopic satellites at m/e 696 and 697. The peak at m/e 695 is that expected for a structure derived by addition of a 2-phenyl ethyl moiety ($M_R = 105$) to the dimethyl ester of protoporphyrin IX ($M_R = 590$). The electronic absorption and mass spectra support the postulate that the isolated porphyrin is the dimethyl ester of *N*-(2-phenylethyl)protoporphyrin IX. This structural formulation is confirmed by a 240 MHz NMR study of the zinc-complexed porphyrin. The signals in the NMR spectrum of the zinc complex, their multiplicity, and the structural residues responsible for their presence, are summarized in Table II. It is clear from the NMR spectrum that one of the possible isomers of the porphyrin predominates, but a minor isomer (10–15% of the total sample) is also present. The data in the table are for the principal isomer. The protons of the parent protoporphyrin IX framework have been assigned by analogy with the corresponding signals of other *N*-alkylated protoporphyrin IX derivatives (19, 26, 27). The identity of the *N*-alkyl moiety itself has been confirmed by NMR decoupling experiments. Irradiation of the *ortho* phenyl protons at 5.305 ppm results in collapse of the triplet due to the *meta* protons at 6.508 ppm to a doublet. The residual coupling of the *meta* protons is with the *para* proton at 6.612 ppm. Irradiation of the triplet at -0.196 ppm, a signal assigned to the protons on the methylene group bearing the phenyl ring, leads to collapse of the triplet at -4.925 ppm to a singlet. The latter signal is

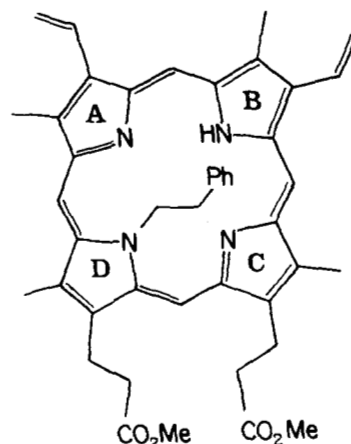


FIG. 8. Structure of the dimethyl esterified hepatic porphyrin isolated from rats treated with phenelzine.

therefore due to the protons on the nitrogen-bound methylene group. The phenyl ethyl structural fragment is confirmed by these relationships.

Four regioisomers that differ in the nitrogen that bears the substituent are possible for the dimethyl ester of *N*-(2-phenylethyl)protoporphyrin IX. The absence of a long wavelength shoulder on the Soret band of the zinc-complexed porphyrin (25–27), the presence of one multiplet for the two internal vinyl protons and of another for the terminal vinyl protons, and the presence of four distinct multiplets for the eight methylene protons on the two propionic acid side chains (19, 26) fixes the *N*-alkyl group on either pyrrole ring C or D (the ring D-alkylated product is shown in Fig. 8).

The reaction of oxyhemoglobin (1.8×10^{-5} M in heme) with phenelzine (1 mM) at 37 °C, measured by the rate of oxygen consumption (20), is slow. The rate constant derived from the equation $d\text{O}_2/dt = k[\text{HbO}_2 \text{ heme}] [\text{hydrazine}]$ is $k = 5.4 \text{ M}^{-1} \text{ s}^{-1}$ for phenelzine, compared to $k = 102 \text{ M}^{-1} \text{ s}^{-1}$ for phenyl hydrazine. This result agrees with our previous finding (at 20 °C) that ethyl hydrazine is a very poor substrate for oxyhemoglobin (20). When the electronic absorption spectrum of the hemoprotein was monitored as a function of time, only minor changes were observed after 24 h of incubation (not shown). No prosthetic heme adduct was found when a 24-h hemoglobin-phenelzine incubation was worked up by the usual procedure (20).

DISCUSSION

Phenelzine is converted *in vivo* and by rat liver homogenates to 2-phenylacetic acid (28, 29), by mitochondrial monoamine oxidase to the hydrazone of 2-phenylacetaldehyde (30), and by the cytosolic fraction of rat and human liver to the *N*-acetyl derivative (31). Only a very small fraction of phenelzine is excreted in man in forms that retain the hydrazine function (32). Our observation that phenelzine is oxidized by hepatic microsomes to 2-phenylacetaldehyde is consistent with the *in vivo* data because aldehydes are metabolic precursors of acids. Phenelzine, in addition, gives rise to ethylbenzene. A radical pathway is demonstrated for the formation of this hydrocarbon metabolite, which could derive from either a radical or an anion intermediate (7, 33), by our successful spin-trapping of the phenyl ethyl radical during the metabolic oxidation of phenelzine.

The interpretation of spin-trapping experiments hinges critically on definitive identification of the trapped radical. Spin-trapped radicals usually cannot be unambiguously identified from their EPR spectra because the signal patterns are

TABLE II

NMR signal assignments for the chlorozinc dimethyl ester of *N*-(2-phenylethyl)protoporphyrin IX

Proton(s)	No. of protons	Position	Multiplicity ^a
		ppm	
Meso	1	10.255	s
Meso	1	10.240	s
Meso	1	10.204	s
Meso	1	10.166	s
Internal vinyl	2	8.074–8.265	m
<i>p</i> -Phenyl	1	6.627	t ($J = 7$ Hz)
<i>m</i> -Phenyl	2	6.508	t ($J = 7$ Hz)
Terminal vinyl	4	6.079–6.538	m
<i>o</i> -Phenyl	2	5.305	d ($J = 7$ Hz)
Propionate methylenes	4	4.093–4.240	m
		4.303–4.367	m
Methyl and methoxyl	18	3.367, 3.550	s (six)
		3.593, 3.633	
		3.585, 3.641	
Propionate methylenes	2	3.252	t ($J = 7$ Hz)
Propionate methylenes	2	2.816	t ($J = 7$ Hz)
Benzylic methylene	2	-0.196	t ($J = 8$ Hz)
<i>N</i> -Methylene	2	-4.925	t ($J = 8$ Hz)

^a Signal multiplicity: s, singlet; d, doublet; m, multiplet; t, triplet. The coupling constants are given in parentheses.

primarily determined by properties of the spin-trap and not of the trapped radicals. We have recently reported the isolation from microsomal incubations and mass spectrometric characterization of POBN-trapped ethyl radicals (21), an approach that circumvents the ambiguities inherent in identification of spin-trapped radicals exclusively from their EPR spectra. The same unambiguous approach has been used here to identify the 2-phenyl ethyl radical (Fig. 7). The isolation and characterization of metabolically formed radicals places spin-trapping methodology on a firm structural footing and promises to greatly expand its biological utility.

Cytochrome P-450 and the FAD-containing amine oxidase are the microsomal enzymes now known to catalyze the oxidation of alkyl hydrazines, although the role of cytochrome P-450 is complicated because it is subject to inhibition by the metabolically formed products (15, 16, 34, 35). The cofactor requirements, inhibitor studies, and inactivation by prosthetic heme alkylation implicate cytochrome P-450 as a participant in the metabolism of phenelzine, but it is clear from the fact that radical formation continues at a reduced rate after cytochrome P-450 inactivation is complete that cytochrome P-450-independent processes are also important. The identity of the non-P-450 microsomal constituents involved in the metabolism of phenelzine to carbon radicals, however, remains unclear. It appears unlikely that traces of hemoglobin in the twice-washed microsomes contribute significantly to phenelzine metabolism because, as we show here, phenelzine is not only a very poor substrate for hemoglobin but also does not detectably alkylate the hemoglobin prosthetic heme group. The slow formation of carbon radicals with boiled microsomes probably results from autooxidative reactions of phenelzine with heme and other metal complexes (36, 37).

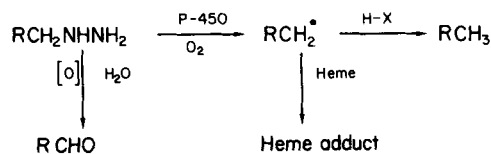
The unmasking of a carbon radical during the microsomal metabolism of phenelzine (16) (Fig. 2), phenyl hydrazine (15), and presumably other hydrazines results in inactivation of cytochrome P-450. The prosthetic heme of the hemoprotein is converted by reaction with the phenelzine-derived 2-phenyl ethyl radical into a porphyrin identified as *N*-(2-phenylethyl)protoporphyrin IX (Fig. 8). This structure formally arises by addition of the radical to one of the nitrogens of the heme framework, although the actual details of the reaction remain ill defined. The radical may react directly with a nitrogen of heme but may also react first with the iron atom to give a σ -bonded alkyl-iron complex (Scheme II). The for-

mation of *N*-phenylheme in the reaction of hemoglobin with phenyl hydrazine, a process tied to catalytic liberation of the phenyl radical by the hemoprotein (20, 38), provides a precedent for the intermediacy of an iron complex in heme alkylation (20, 39, 40).

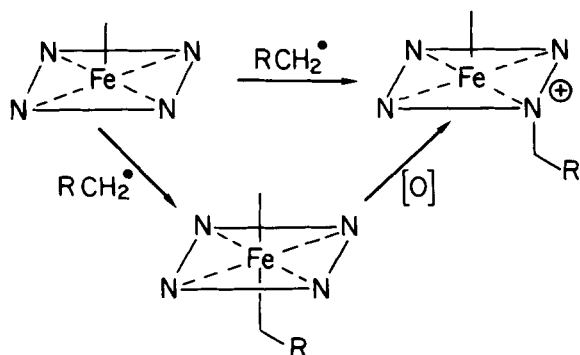
Acknowledgments—We thank Dr. David E. Kerr for the demonstration that heme adducts do not arise in the reaction of phenelzine with hemoglobin, and Dr. Conrad Wheeler for mass spectrometric identification of the phenelzine metabolites. Access to the mass spectrometers of the Biomedical, Bio-Organic Mass Spectrometry Resource provided by Dr. A. Burlingame, and to EPR spectrometers provided by Drs. L. Packer and A. Quintanilha (University of California, Berkeley) and Dr. T. Singer (San Francisco Veterans Administration Hospital) is gratefully acknowledged.

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SCHEME I



SCHEME II

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J. Biol. Chem. 1983, 258:8623-8629.

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