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Free Radical Pathways in the *In Vitro* Hepatic Metabolism of Phenelzine

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SUMMARY

The *in vitro* metabolism of phenelzine (2-phenylethylhydrazine) by phenobarbital-pretreated rat liver microsomes yields ethylbenzene, 2-phenylethanol, 2-phenylacetaldehyde, benzaldehyde, benzylalcohol, and toluene as metabolites. Isotopic studies demonstrate that the oxygen atom of 2-phenylethanol derives from molecular oxygen and that this alcohol is not produced by reduction of 2-phenylacetaldehyde. The rates of destruction of cytochrome P-450, accumulation of spin-trapped 2-phenylethyl radicals, and formation of ethylbenzene and 2-phenylethanol are the same for [1,1-^{2}H]-2-phenylethylhydrazine as for the undeuterated substrate. Small primary isotope effects are observed, however, for the formation of 2-phenylacetaldehyde ($k_H/k_D>1$) and benzaldehyde ($k_H/k_D<1$). Synthetic 2-phenylethylhydroperoxide is converted by liver microsomes to the same alcohol and aldehyde metabolites. The results indicate that the metabolism of phenelzine by rat liver microsomes proceeds primarily via the 2-phenylethyl radical.

Phenelzine (2-phenylethylhydrazine), a monoamine oxidase inhibitor employed clinically as an antidepressant (1), was found in early clinical work to strongly potentiate the activities of coadministered drugs (2, 3). This synergistic activity derives from the ability of phenelzine to inhibit the metabolism and, therefore, elimination, of agents with which it is coadministered (4–6). The inhibition of drug metabolism (6–8), linked by Muakkassah and Yang (7) to inactivation of cytochrome P-450 enzymes, was shown in our previous study to involve oxidation of phenelzine by cytochrome P-450 to a species that alkylates the prosthetic heme group of the enzyme (9). The alkylated prosthetic group was identified as *N*-(2-phenylethyl)protoporphyrin IX. Spin-trapping studies furthermore established that the microsomal oxidation of phenelzine produces a flux of 2-phenylethyl radicals (9). These radicals may be responsible for the enzyme destruction and for the carcinogenic and hepatotoxic properties of phenelzine (10, 11).

Phenelzine is extensively metabolized, as shown by the finding that man only excretes a small fraction of the drug in forms that retain the hydrazine function (12), but the pathways responsible for its metabolism remain vague. Qualitative studies have shown that phenelzine is converted by monoamine oxidase to 2-phenylacetaldehyde (13), by cytosolic fractions of rat and human liver to the *N*-acetyl derivative (14), by rat liver homogenates to 2-phenylacetic acid (15), by rat liver microsomes to phenylacetaldehyde and ethylbenzene (9), and *in vivo* to phenylacetic acid (16, 17) and 2-phenylethylamine (17). Deuteration of the nitrogen-substituted carbon potentiates the *in vivo* behavioral and biochemical effects despite the fact that it does not increase the *in vitro* activity of phenelzine as a monoamine oxidase inhibitor (18, 19). The increased pharmacological activity caused by deuterium substitution therefore appears to be related to changes in the metabolism or distribution of phenelzine rather than to changes in its intrinsic activity. The present investigation was undertaken to evaluate the importance of the free radical pathway in the metabolism of phenelzine and to determine the influence of deuterium substitution on the metabolic profile. The results indicate that the microsomal metabolism of phenelzine proceeds primarily via free radical pathways.

Experimental Procedures

Materials. Phenelzine was obtained from ICN Pharmaceuticals (Plainview, NY). NADP, glucose 6-phosphate, DETAPAC, hydrogen peroxide, and glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Co. (St. Louis, MO); sodium phenobarbital was from J. T. Baker Chemical Co. (Phillipsburg, NJ); POBN was from Aldrich Chemical Co. (Milwaukee, WI); and 99.8% ^{18}O-labeled molecular oxygen was from MSD Isotopes (St. Louis, MO).

Instrumental methods. Proton NMR spectra were obtained in deuterated chloroform on a Varian FT-80 NMR spectrometer. Chemical shifts are given in ppm relative to an internal tetramethylsilane

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ABBREVIATIONS: POBN, α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone; DETAPAC, diethylenetriaminepentaacetic acid; HPLC, high pressure liquid chromatography.

standard. Gas-liquid chromatographic analyses were carried out on a Varian model 2100 packed column instrument or on a Hewlett-Packard model 5890 capillary gas chromatograph. Both were equipped with flame ionization detectors and Hewlett-Packard 3390A integrators. A Kratos MS-25 instrument in line with a Varian model 3700 gas chromatograph was used for gas chromatography-mass spectrometry. The chromatograph was equipped with a DB-1 column programmed to rise from 50° to 150° at 5°/min. A Varian E-104 EPR spectrometer was employed to monitor the concentration of spin-trapped radicals.

Synthesis of [1,1-^{2}H]-2-phenylethanol. Lithium aluminum deuteride (2 g, 0.0476 mol) was added to 80 ml of stirred dry tetrahydrofuran under a nitrogen atmosphere in a three-neck, 250-ml flask equipped with a reflux condenser. Ethyl phenylacetate (15.5 g, 0.0942 mol) in 10 ml of dry tetrahydrofuran was then added dropwise to the stirred suspension (the mixture refluxed as the ester was added). The suspension was allowed to stir at room temperature for an additional 6 hr after the refluxing stopped. The reaction was carefully quenched by slowly adding a 1:1 (v/v) mixture of tetrahydrofuran and water. Cold water and 10% H_2SO_4 were then added to dissolve the precipitated aluminum salts, and the mixture was extracted with diethyl ether. Drying the combined extracts over anhydrous sodium sulfate and removal of the solvent under vacuum yielded 10.23 g (87.5% yield) of a clear, pale yellow liquid: ^{1}H-NMR 7.27 (m, 5H, phenyl protons), 2.82 (s, 2H, $PhCH_2$—), and 1.83 ppm (s, D_2O exchangeable). The deuterium content by mass spectrometric analysis was greater than 95%.

Synthesis of [1,1-^{2}H]-2-phenylethylhydrazine. Chlorotrimethylsilane (31.3 ml, 26.8 g, 0.246 mol) and sodium iodide (49.3 g, 0.329 mol) were added to a solution of [1,1-^{2}H]-2-phenylethanol (10.2 g, 0.082 mol) in 150 ml of reagent grade acetonitrile and the resulting mixture was stirred at 70° for 90 min. The mixture was cooled to room temperature and was then poured into 600 ml of 10% sodium thiosulfate solution to remove the excess chlorine. A yellow precipiate formed. The mixture was extracted with ether. Solvent removal from the combined ether fractions (filtration was required after partial solvent removal) yielded 8.36 g (44%) of clear, pale yellow liquid: ^{1}H-NMR 7.25 (m, 5H, phenyl protons) and 3.15 ppm (s, 2H, $PhCH_2$—).

The deuterated iodide (8.36 g, 0.036 mol) was stirred 4 hr with 40 ml of hydrazine monohydrate (**caution:** *carcinogen*) under a nitrogen atmosphere in a flask shielded by aluminum foil from light. Extraction with ether, drying of the combined ether layers (sodium sulfate), and removal of the ether on a rotary evaporator provided 3.35 g (67.3% crude yield) of a pale yellow oil: ^{1}H-NMR, 7.24 (m, 5H, phenyl protons), 2.96 (s, $NHNH_2$), and 2.79 ppm (s, 2H, $PhCH_2$—). The overall crude yield from ethyl phenylacetate is 25.9%. The hydrochloride salt was prepared by dropwise addition of a saturated ether solution of hydrogen chloride to an ether solution of the deuterated phenelzine. Recrystallization of the precipiated salt from methanol yielded 1.75 g of pale yellow crystals.

Synthesis of 2-phenylethylhydroperoxide. Freshly distilled pyridine (17 ml, ~200 mmol) was added dropwise to a cooled (ice bath) mixture of methanesulfonyl chloride (7.75 ml, 11.45 g, 0.1 mol) and either 2-phenylethanol or [1,1-^{2}H]-phenylethanol (12.21 g, 0.1 mol). The rate of addition was such that the temperature of the solution did not rise above 5°. The final reaction mixture was poured into 60 ml of ice-cold 10% HCl and was extracted twice with 40 ml of diethyl ether. The combined ether extracts were washed sequentially with water and saturated aqueous sodium bicarbonate before they were dried over potassium carbonate. Removal of the ether on a rotary evaporator yielded a turbid yellow oil that was purified by vacuum distillation. 2-Phenylethylmesylate (4.64 g, 23.1% yield) was thus obtained: ^{1}H-NMR 7.26 (m, 5H, phenyl protons), 4.39 (t, 2H, —CH_2O—), 3.02 (t, 2H, $PhCH_2$—), and 2.79 ppm (s, 3H, —SO_2Me).

A solution of 2-phenylethylmesylate (0.50 g, 2.5 mmol) in 5 ml of methanol was cooled to 0° before 0.567 ml of 30% hydrogen peroxide solution (5 mmol) followed by 160 μl of 50% KOH was added. The mixture was stirred for 24 hr at room temperature before it was cooled to 0° and 5 ml of 50% KOH were added. The mixture was extracted twice with diethyl ether (5 ml) and the organic layers were set aside. The aqueous layer was recooled to 0°, neutralized to pH 7 with concentrated HCl, and reextracted twice with ether (5 ml). The two sets of ether extracts were combined and washed twice with 25% KOH (5 ml). The aqueous layer was cooled to 0°, neutralized with concentrated HCl, and extracted with ether. The ether was added to 1 ml of glass-distilled water and the ether was allowed to evaporate. The peroxide activity of the turbid solution that was obtained was assayed by a standard procedure (20), and the identity of the peroxide was confirmed by mass spectrometry: m/z 138 (M^+), 122 ($M^+ - O$), 120 ($M^+ - H_2O$), 105 ($M^+ - HO_2$), 92 ($M^+ - CH_2O_2$) and 91 ($M^+ - CH_2O_2H$).

Metabolism of phenelzine by microsomal cytochrome P-450. Hepatic microsomes were prepared from 250–300-g male Sprague-Dawley rats after intraperitoneal injection of sodium phenobarbital (80 mg/kg/day) once a day for 4 days (21). Standard 10-ml incubation mixtures contained the following in 0.1 M phosphate buffer (pH 7.4): cytochrome P-450 (~3 nmol/ml), DETAPAC (1.5 mM), NADP (0.5 mM), glucose 6-phosphate (3 mM), glucose-6-phosphate dehydrogenase (1 unit/ml), $MgCl_2$ (2 mM), KCl (150 mM), and phenelzine (5 mM). The mixtures were incubated at 37° in a reciprocating water bath for the time indicated in the text and were terminated by immersion in an ice-water mixture. The incubation mixtures were centrifuged to remove precipitated protein and known concentrations of internal standards (3-phenylpropanol or *n*-propylbenzene) were added to the supernatants. The supernatants were extracted with 5 ml of HPLC-grade hexane and the extracts were analyzed by gas-liquid chromatography on a 6-foot glass column packed with 10% Carbowax 20M on 120/140 mesh Chrom Q or, in studies of toluene formation, by capillary gas-liquid chromatography on a 30-m DB-5 column. The chromatography columns in both systems were programmed to rise from 50° to 150° at a rate of 5°/min. Toluene eluted with the solvent from the packed column system but had a retention time of 6.07 min in the capillary column system. The quantitative metabolite yields were calculated from the chromatographic peak areas by comparison with standard curves prepared for each of the metabolites. Standard curves were constructed by correlating the peak areas obtained from known mixtures of the authentic compounds with the appropriate internal standard (3-phenylpropanol or *n*-propylbenzene). Selected samples were also analyzed by capillary gas chromatography-mass spectrometry.

Destruction of microsomal cytochrome P-450 by phenelzine. Quadruplicate 10-ml incubations were employed in each set of experiments to directly compare the NADPH-dependent destruction of cytochrome P-450 by phenelzine and [1,1-^{2}H]phenelzine. The microsomal mixtures, excluding NADPH and the substrate, were preincubated at 37° for 5 min before the reactions were started by adding the microsomal mixtures to vials containing the hydrazine substrate and NADPH. The reactions were quenched by saturation with carbon monoxide. The cytochrome P-450 concentrations were determined by difference spectroscopy on an Aminco DW-2A instrument, employing 100 mM^{-1} cm^{-1} as the molar extinction coefficient for the 450–490-nm absorbance difference between the dithionite-reduced CO-saturated sample and unreduced CO-saturated microsomes (22).

Chemical and biological generation of 2-phenylethyl radicals from phenelzine. Phenelzine-POBN spin adducts were generated chemically in 10-ml incubation mixtures containing 9 ml 0.05 M Na_2CO_3 (adjusted to pH 10), 1 ml of 1 mM $CuCl_2$, 39 mg (0.2 mM)of POBN, and 52 mg (5 mM) of phenelzine or its labeled analogue. The biological investigation of spin adduct formation was carried out in standard 10-ml rat liver microsomal incubations containing 5 mM phenelzine or [1,1-^{2}H]phenelzine. The incubations were stirred at room temperature rather than 37° and were protected by aluminum foil from light. Aliquots (25 μl) were periodically removed and examined by EPR spectroscopy. The concentration of radical adducts was taken to be proportional to the height of the central field signal.

Incorporation of molecular oxygen into metabolites. Approximately 100 ml of a microsomal suspension containing 3.5 nmol/ml of cytochrome P-450, 88 mg (5 mM) phenelzine, and the other components

of the standard incubation mixture, except for the NADPH-regenerating system, were placed in a 500-ml flask. The flask was equipped with valves leading to a vacuum pump, a nitrogen inlet, a balloon, and a 100-ml break-seal ampule containing 99.8% [$^{18}O_2$]-molecular oxygen. A flask containing the standard NADPH-regenerating system was also attached to the vacuum system. The system was alternately evacuated and filled with nitrogen six times. The break-seal of the $^{18}O_2$ ampule was then broken and the incubation was initiated by adding the NADPH-regenerating system to the microsomal mixture. The incubation was quenched with trichloroacetic acid and the resulting mixture extracted with hexane. The hexane extracts were analyzed by gas chromatography-mass spectrometry as described above.

Microsomal incubations with 2-phenylethylhydroperoxide. Standard microsomal incubations were carried out in duplicate with 0.1 mM 2-phenylethylhydroperoxide as the substrate. NADPH was added to initiate the incubations. The incubations were quenched at 0° and *n*-propylbenzene was added as an internal standard. The incubations were then extracted with hexane and the extracts were concentrated and analyzed by HPLC for the presence of benzaldehyde, phenylacetaldehyde, and 2-phenylethanol. These analyses were done by HPLC because the three products are formed by thermal decomposition of the hydroperoxide in the gas chromatograph. The analysis for benzyl alcohol, in contrast, was done by gas-liquid chromatography because it is not observed when the hydroperoxide itself is injected into the gas chromatograph. HPLC analyses were done on a normal phase Partisil-10 (Whatman) column eluted isocratically with 5% tetrahydrofuran/95% hexanes at a flow rate of 0.5 ml/min. The column effluent was monitored at 240 nm with a variable wavelength detector connected to a Hewlett-Packard 3390A integrator. Gas chromatography was carried out as already described.

Results

Microsomal metabolism of phenelzine. Six metabolites have been identified in incubations of phenelzine with liver microsomes from phenobarbital-pretreated rats (Fig. 1). Comparisons of the gas chromatographic retention times and mass spectra of the metabolites with authentic standards (not shown) identify the metabolites as toluene, ethylbenzene, benzaldehyde, phenylacetaldehyde, benzyl alcohol, and 2-phenylethanol. The formation of ethylbenzene, phenylacetaldehyde, and 2-phenylethanol has been reported (9), but toluene, benzaldehyde, and benzyl alcohol have not been identified previously as phenelzine metabolites. The rates of formation of four of these metabolites during the first 5 min of incubation are shown in Fig. 2. The metabolites, except for phenylacetaldehyde, are not formed in detectable amounts in the absence of NADPH under our incubation conditions. Phenylacetaldehyde, however, is generated in the absence of NADPH (45 nmol/10 min), albeit at a lower rate than in the presence of this cofactor (67 nmol/10 min).

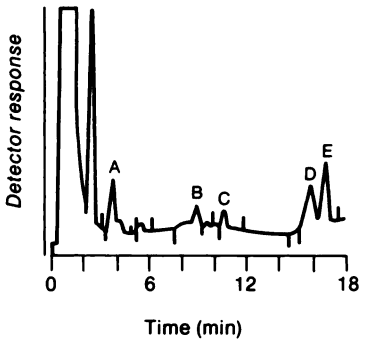


Fig. 1. Gas-liquid chromatographic analysis of the products formed in incubations of phenelzine with hepatic microsomes from phenobarbital-pretreated rats. The incubation and analysis conditions are given under Experimental Procedures. The identities of the peaks in the chromatogram are as follows: *A*, ethylbenzene; *B*, benzaldehyde; *C*, benzyl alcohol; *D*, phenylacetaldehyde; and *E*, 2-phenylethanol. Toluene, the sixth metabolite, eluted with the solvent in this system. Toluene analyses were therefore carried out by capillary gas-liquid chromatography as described in the text.

Microsomal metabolism of [1,1-^{2}H]phenelzine. The same metabolites are observed when [1,1-^{2}H]phenelzine is substituted for phenelzine, but deuterium substitution detectably alters the rates of formation of two of the four major metabolites (Fig. 2). The formation of ethylbenzene and 2-phenylethanol is not significantly altered by deuteration, but the formation of benzaldehyde is increased (Fig. 2B) and that of phenylacetaldehyde is decreased. The metabolic formation of benzaldehyde is thus subject to an inverse isotope effect, whereas that of phenylacetaldehyde is subject to a normal isotope effect.

Destruction of cytochrome P-450 by phenelzine and [1,1-^{2}H]phenelzine. The chromophore of cytochrome P-450, as reported previously (9), is rapidly destroyed when hepatic microsomes are incubated with phenelzine (Fig. 3). Maximum chromophore loss is observed within the first 10 min. The destruction of cytochrome P-450 by [1,1-^{2}H]phenelzine follows a virtually identical time course (Fig. 3), a result that clearly demonstrates the absence of a primary isotope effect in the destruction reaction.

Spin-trapping of enzymatically generated 2-phenylethyl radicals. Comparison of the rates of accumulation of spin-trapped radicals in microsomal incubations of phenelzine and [1,1-^{2}H]phenelzine shows that the 2-phenylethyl radical is formed at approximately equal rates from the two substrates (Fig. 4). Radical formation, as expected if the reaction depends on catalytic turnover of cytochrome P-450, levels off after about 5 min (Fig. 4), the time required to inactivate the enzyme (Fig. 3).

Incorporation of molecular oxygen. The incorporation of labeled oxygen into 2-phenylethanol in incubations carried out under an $^{18}O_2$ atmosphere shows that the hydroxyl group derives largely or exclusively from molecular oxygen. Mass spectrometric analysis of the 2-phenylethanol obtained in two independent experiments indicated that 49 and 31% of the oxygen, respectively, was ^{18}O-labeled. The less than quantitative incorporation of label presumably reflects dilution of the $^{18}O_2$ by residual $^{16}O_2$, but the alternative possibility that some of the label derives from water cannot be excluded. The unlabeled alcohol is not obtained by reduction of unlabeled phenylacetaldehyde because the alcohol derived from [1,1-^{2}H]phenelzine retains both deuterium atoms.

Microsomal metabolism of 2-phenylethylhydroperoxide. Incubation of 2-phenyethylhydroperoxide with liver microsomes in the presence of NADPH and its regenerating system results in the formation of phenylethanol, phenylacetaldehyde, benzyl alcohol, and benzaldehyde (Fig. 5). The same products are obtained with the 1,1-dideuterated analogue, but it has not been possible to reliably determine the kinetic effects of deuterium substitution due to the high variability in product yields.

Discussion

Two homologous sets of metabolites are formed when phenelzine is incubated with liver microsomes from phenobarbital-pretreated rats. The first set consists of ethylbenzene, 2-

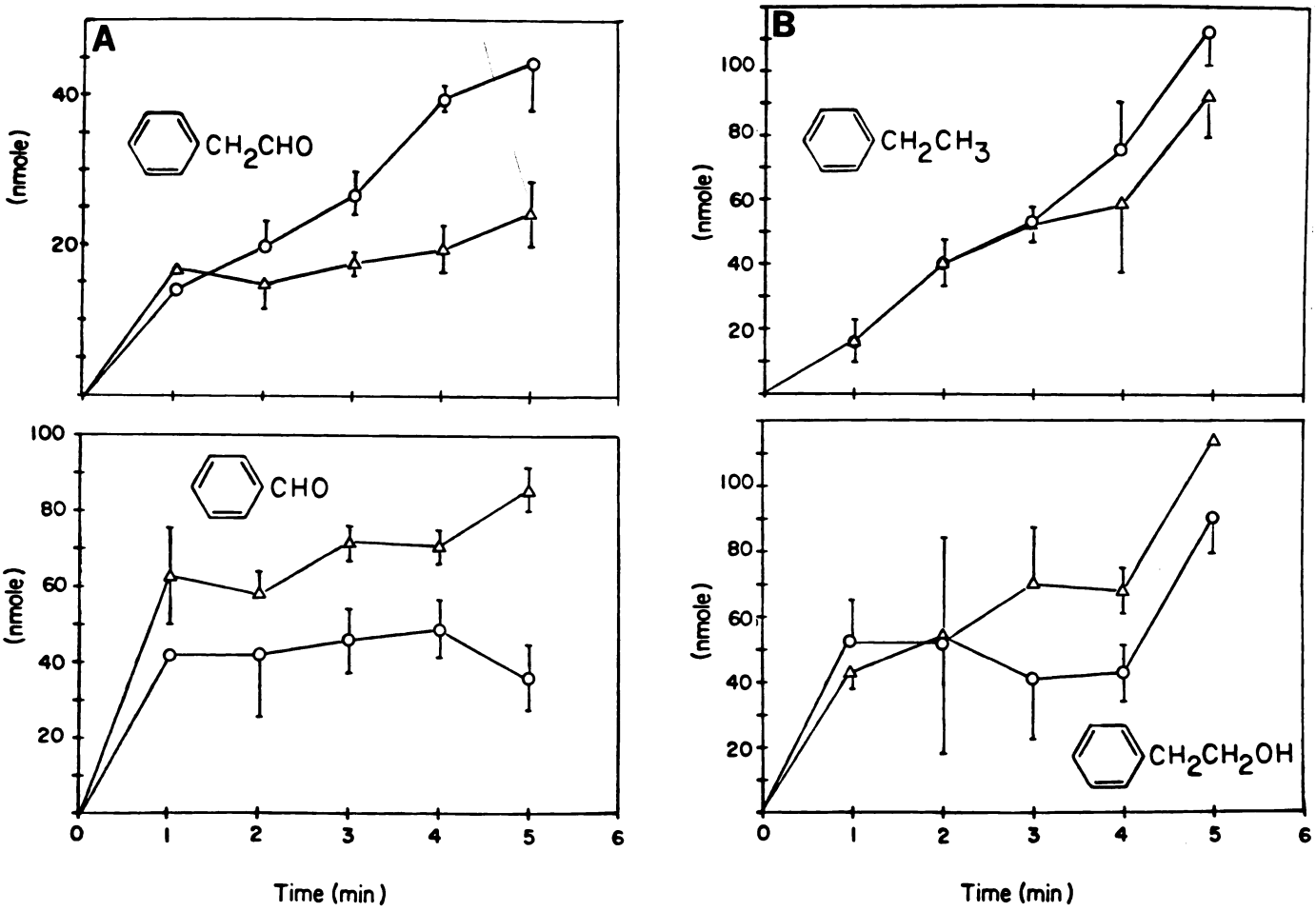


Fig. 2. Formation of the major metabolites from phenelzine (O) and d_2-phenelzine (Δ) as a function of time. The four metabolites (deuterated in the case of [1,1-^{2}H]phenelzine) are ethylbenzene, benzaldehyde, phenylacetaldehyde, and 2-phenylethanol (structures in the *insets*). The experimental protocol is given under Experimental Procedures. The values given are averages of three independent incubations. Standard deviations are indicated by the *vertical bars*.

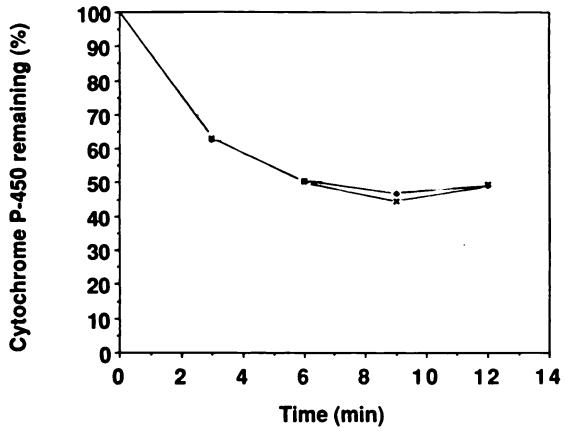


Fig. 3. Destruction of cytochrome P-450 by phenelzine (O) and [1,1-^{2}H] phenelzine (x). The protocol for these experiments is given in Experimental Procedures. The data points include small corrections for time-dependent chromophore losses observed in incubations with NADPH but no phenelzine. The concentration of cytochrome P-450 in these incubations was approximately 3 nmol/ml.

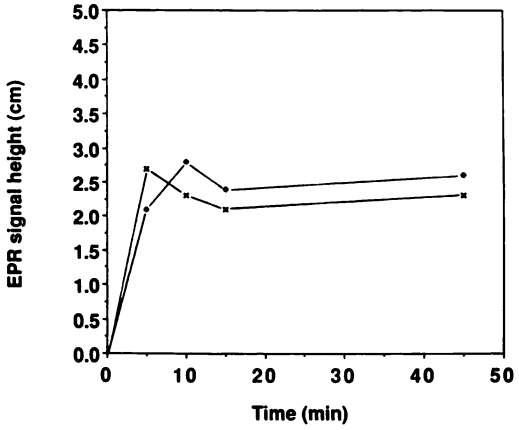


Fig. 4. Spin trapping of the 2-phenylethyl radical by POBN in microsomal incubations of phenelzine (O) and [1,1-^{2}H]-phenelzine (x). The EPR instrument parameters were as follows: microwave power (20 mW), modulation amplitude (2.0 G), time constant (0.250 sec), scan time (2 min), scan range (100 G), and gain (1.25×10^4).

phenylethanol, and phenylacetaldehyde, and the second of toluene, benzyl alcohol, and benzaldehyde. The first set thus consists of the hydrocarbon, alcohol, and aldehyde with the two-carbon side chain of phenelzine intact while the second set consists of the same three products with the side chain shortened by one carbon. To understand the microsomal metabolism of phenelzine it is consequently necessary to elucidate the mechanism by which the carbon-carbon bond of phenelzine

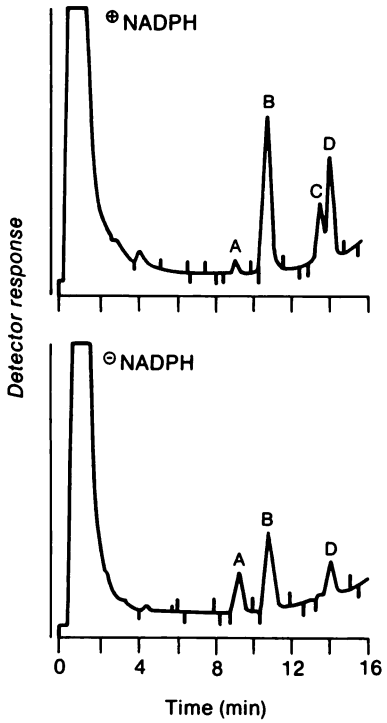
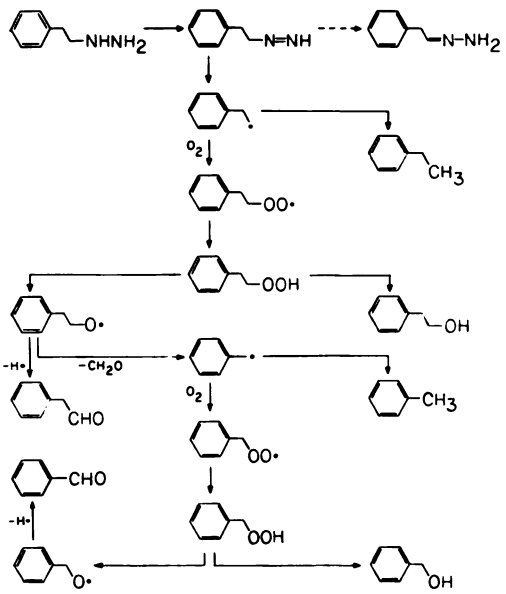


Fig. 5. Gas-liquid chromatographic analysis of the products formed in incubations of 2-phenylethylhydroperoxide with hepatic microsomes from phenobarbital-pretreated rats in the presence (⊕) and absence (⊖) of NADPH. The incubation and analysis conditions are given in the text. The metabolic products are: *A*, benzaldehyde; *B*, benzyl alcohol; *C*, phenylacetaldehyde; and *D*, 2-phenylethanol.

is cleaved and to determine how the hydrocarbon, alcohol, and aldehyde metabolites are obtained.

The two deuterium atoms of [1,1-^{2}H]phenelzine are quantitatively retained in the 2-phenylethanol product, a result that precludes derivation of the alcohol from phenylacetaldehyde. The finding that the oxygen of 2-phenylethanol derives primarily from molecular oxygen argues, furthermore, that the alcohol is not formed by addition of water to the 2-phenylethyl cation. A minor contribution from such a pathway cannot be ruled out, however, because some ^{16}O-alcohol is found in the product obtained under an $^{18}O_2$ atmosphere. The only route to 2-phenylethanol consistent with these results appears to be reaction of the 2-phenylethyl radical with oxygen followed by abstraction of a hydrogen atom to give 2-phenylethylhydroperoxide (Scheme 1). Heterolysis of the hydroperoxide or homolysis followed by hydrogen abstraction then yields the alcohol. Evidence exists that cytochrome P-450 promotes competitive heterolytic and homolytic cleavage of alkyl hydroperoxides (23–26), the former reaction leading to alcohols and the latter to carbon-carbon or carbon-hydrogen bond cleavage products (27). The reduction of 2-phenylethylhydroperoxide to 2-phenylethanol therefore probably reflects heterolytic scission of the oxygen-oxygen bond. Support for a hydroperoxide route to the alcohol is provided by the fact that hepatic microsomes generate 2-phenylethanol from authentic 2-phenylethylhydroperoxide (Fig. 5), by unambiguous spin-trapping of the 2-phenylethyl radical in microsomal incubations of phenelzine (Fig. 4) (9), and by the concomitant formation of ethylbenzene.

Three metabolic routes to phenylacetaldehyde are readily envisaged: (a) oxidation of 2-phenylethanol, (b) oxidation of the hydrazine to the hydrazone followed by hydrolytic release



Scheme 1. Proposed microsomal metabolism of phenelzine

of the aldehyde, and (c) elimination of a hydrogen after homolysis of the 2-phenylethylhydroperoxide oxygen-oxygen bond. The first alternative is ruled out by the finding that the aldehyde is not produced in significant amounts when 2-phenylethanol is incubated with hepatic microsomes, but the relative contributions of the latter two mechanisms are not readily differentiated. The hydrazone is probably the precursor of the aldehyde formed in the absence of NADPH because radical-derived products are not simultaneously generated. The hydrazone is also a precursor of the aldehyde in mitochondria because phenelzine is both a substrate and an inhibitor of monoamine oxidase (13). The origin of the aldehyde produced by the NADPH-dependent mechanism is more ambiguous. Efforts to determine the origin of the oxygen in the aldehyde, which would distinguish hydrolytic from oxidative mechanisms, were frustrated by rapid exchange of the aldehyde oxygen with oxygen from the medium. The isotope effect observed with 1,1-dideuterated phenelzine (Fig. 2) is of little assistance in this context because it is consistent with both mechanisms. The demonstration that phenylacetaldehyde is generated from 2-phenylethylhydroperoxide by hepatic microsomes (Fig. 5), however, clearly argues that a fraction of the phenylacetaldehyde derives from the hydroperoxide.

The formation of toluene, benzyl alcohol, and benzaldehyde points to elimination of the benzyl radical in the carbon-carbon bond cleavage reaction. The benzyl radical would be expected to give rise to the observed metabolites by reactions similar to those just described for the 2-phenylethyl radical. Thus, abstraction of a hydrogen by the benzyl radical yields toluene, whereas reaction of the radical with oxygen yields the hydroperoxide. The hydroperoxide, as before, is converted by heterolytic dioxygen cleavage to benzyl alcohol (see previous discussion), and by homolytic dioxygen cleavage to benzaldehyde (Scheme 1). No ambiguity exists here concerning the route to the aldehyde because a hydrazone cannot be invoked as an alternative precursor.

Extrusion of the benzyl radical in the reaction that cleaves the side chain is consistent with the pivotal position proposed for 2-phenylethylhydroperoxide in the microsomal metabolism

of phenelzine. Cytochrome P-450-catalyzed homolysis of the hydroperoxide, a reaction particularly associated with this enzyme (28), would yield the 2-phenylethoxy radical. The 2-phenylethoxy radical, as expected from the propensity of alkoxy radicals to β-elimination reactions (29), has actually been shown to fragment rapidly to the benzyl radical and formaldehyde (Scheme 1, Refs. 30–32). β-Elimination to give the benzyl radical is thermodynamically favored over hydrogen elimination to give phenylacetaldehyde (29). The microsomal formation of benzaldehyde from 2-phenylethylhydroperoxide is thus consistent with the expected chemical behavior of the hydroperoxide.

The small but detectable effects of deuterium substitution support the proposed metabolic scheme (Scheme 1). The time courses of product formation from phenelzine and [1,1-^{2}H] phenelzine show that deuteration decreases the formation of phenylacetaldehyde (normal isotope effect), increases the formation of benzaldehyde (inverse isotope effect), and has little effect on the formation of ethylbenzene and 2-phenylethanol. The *inverse* deuterium isotope effect on the formation of benzaldehyde, which indicates that deuterium substitution increases the substrate flux toward the benzyl radical, supports the proposal that elimination of a hydrogen from the 2-phenylethoxy radical competes with cleavage of the carbon-carbon bond. Deuterium substitution, as found, should slow down the hydrogen elimination reaction and thus channel more of the alkoxy radical into the carbon-carbon cleavage pathway. A normal isotope effect is found, as predicted by this model, for the formation of phenylacetaldehyde. The interpretation of this latter observation is not straightforward, however, because the aldehyde is generated by multiple pathways that are not differentiated by the isotope effect. The formation of ethylbenzene, as expected, is not subject to a measurable isotope effect because no deuterium bond is broken in the reaction. The absence of a deuterium isotope effect on the production of 2-phenylethanol is consistent with heterolysis but not homolysis of the hydroperoxide because homolysis should have given rise to a small inverse isotope effect analogous to that seen in the carbon-carbon bond cleavage reaction. Technical difficulties have prevented analysis of the effect of deuterium substitution on the other metabolites. The available isotope effect data fully support the proposed metabolic scheme. A more quantitative analysis of the isotope effect data is not warranted because the mass balance for the metabolism of phenelzine is not complete. The binding of phenelzine to macromolecules at sites other than the prosthetic heme of cytochrome P-450, in particular, has not been measured.

Catalytic turnover of phenelzine by cytochrome P-450 produces a flux of 2-phenylethyl radicals and results in covalent attachment of the 2-phenylethyl group to the prosthetic heme group (9). The 2-phenylethyl radical presumably derives from 2-phenylethyldiazene. If a competition exists within the active site of the enzyme between oxidation of the hydrazine to the diazene or the hydrazone, 1,1-dideuteration should increase the proportion of metabolic turnovers that yield the diazene and thus should increase the flux of 2-phenylethyl radicals. The shift from one to the other of two competing catalytic outcomes, or "metabolic switching," is well documented (e.g., Ref. 33). Deuterium substitution does not discernibly alter the accumulation of spin-trapped carbon radicals (Fig. 4), however, or the rate of loss of the cytochrome P-450 chromophore (Fig. 3). The fact that neither of these two processes is sensitive to deuterium substitution specifically argues (a) that the oxygen is not inserted by the enzyme into the carbon-hydrogen bond and (b) that catalytically generated nitrogen radical-cation intermediates do not partition between the diazene and the hydrazone. The data therefore support the postulate that the diazene is the precursor of all the metabolites.

The present results indicate that the 2-phenylethyl radical is the precursor of all the microsomal phenelzine metabolites except for a fraction of the phenylacetaldehyde. This radical is the progenitor of a family of secondary radicals that includes the 2-phenylethylhydroperoxy, 2-phenylethoxy, benzyl, benzylhydroperoxy, and benzyloxy radicals. The metabolism of phenelzine by hepatic microsomes therefore appears to proceed primarily via free radical pathways. Even though phenelzine may be metabolized by alternative mechanisms in other tissues or cell compartments, it is likely that the radical cascade associated with metabolism by cytochrome P-450 is related to the carcinogenic and hepatotoxic properties of phenelzine and other alkyl hydrazines. It is to be noted, in this context, that the carbon radical derived from phenelzine causes DNA strand-scission (34) and that the formation of hydrocarbon metabolites from other alkyl hydrazines, which implies the formation of carbon radicals, is well established (35, 36).

The microsomal metabolism of phenelzine, as shown by the present results, is relatively insensitive to deuterium substitution. Isotopic substitution modestly changes the rate of appearance of some of the metabolites without altering the rate of destruction of the cytochrome P-450 chromophore or the rate at which the parent phenylethyl radical is formed. The observed changes do not appear large enough to account for the elevated *in vivo* pharmacological activity of [1,1-^{2}H]phenelzine (18, 19). The basis for the *in vivo* pharmacological effect of deuterium substitution therefore remains obscure.

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