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DEUTERIUM ISOTOPE EFFECT OF PHENELZINE ON THE INHIBITION OF RAT LIVER MITOCHONDRIAL MONOAMINE OXIDASE ACTIVITY

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Abstract—Phenelzine is a suicide monoamine oxidase (MAO) inhibitor with antidepressant properties. The present study compares the inhibition of rat liver mitochondrial MAO by phenelzine and 1,1-dideuterated phenelzine and the metabolism of these drugs by that enzyme. Phenylacetaldehyde, which was measured by a high performance liquid chromatographic procedure, was found to be the major metabolite of phenelzine after incubation with MAO. The time–courses of aldehyde formation were non-linear due to the time-dependent inhibition of MAO. The reaction rate was reduced substantially when the hydrogen atom in the 1-carbon position was replaced by deuterium. The V_H/V_D value was 3.1, indicating a primary isotope effect. Such a substitution of deuterium in the phenelzine molecule did not affect significantly the initial reversible inhibition of MAO, which was determined by comparison of their K_i values. The irreversible inhibition, as estimated from IC_{50} values, however, was potentiated substantially by deuteration. These results support the notion that the irreversible inhibition of MAO activity by phenelzine proceeds via a phenylethyldiazene intermediate, which reacts with the enzyme to form a covalent adduct. An alternative pathway involving hydrogen abstraction from carbon-1 of phenelzine or via rearrangement of the diazine on the enzyme surface could occur to form a phenylethylidene hydrazine intermediate which would subsequently be hydrolyzed to phenylacetaldehyde. The reduction in the rate of phenylethylidene hydrazine formation due to the isotope effect could lead to the accumulation of phenylethyldiazene intermediate and thus potentiate the inhibition of MAO activity.

Phenelzine (2-phenylethylhydrazine) has been used as an antidepressant drug for many years [1]; the efficacy of this antidepressant has been assumed to be due to inhibition of the monoamine oxidase enzyme (EC 1.4.3.4, MAO). It inhibits both monoamine oxidase A and B irreversibly [2, 3]. When phenelzine is modified by substitution with deuterium for hydrogen at the 1-carbon position, a considerable potentiation of its effects, as measured by the accumulation of several biogenic amines in the striatum after both acute and chronic treatment, is observed [4–6]. Potentiation of acute behavioral effects has also been reported after deuterium substitution [7, 8]. This deuterium effect has been attributed to an enhancement of the central potency of phenelzine because of a slowing of its peripheral inactivation [4]. Phenelzine is known to be an MAO inhibitor as well as an MAO substrate [9, 10], and its major metabolite is phenylacetic acid, as observed in the urine of rats as well as humans [11]. Recently, 1,1,2,2-tetradeutero-β-phenylethylamine has been detected *in vivo* after administration of 1,1,2,2-tetradeutero-phenelzine [12]. It would be interesting to know whether β-phenylethylamine is the major intermediate metabolite with respect to phenylacetic acid formation from phenelzine. The mechanisms whereby phenelzine exerts its inhibitory effect on MAO have been proposed to be either via a phenylethylidene hydrazine ($RCH_2CH{=}N{-}NH_2$) intermediate [13] or via phenylethyldiazene ($RCH_2CH_2N{=}NH$) [14, 15]. This paper describes results of studies designed to assess the inhibition of MAO by phenelzine as well as to reveal the deuterium isotope effect *in vitro*. Our aim was to attempt to understand the mechanism-based inhibition process of this compound.

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MATERIALS AND METHODS

Materials. Phenelzine (2-phenylethylhydrazine), 5-hydroxytryptamine, yeast acetaldehyde dehydrogenase, β-nicotinamide adenine dinucleotide (β-NAD), β-phenylethylamine, phenylacetaldehyde and phenylacetic acid were purchased from the Sigma Chemical Co. (St Louis, MO). Deuterated phenelzine sulfate (1,1-dideutero-phenelzine and 1,1,2,2-tetradeutero-phenelzine) were provided by Dr B. A. Davis. All other chemicals are of analytic grade.

Preparation of rat liver mitochondrial MAO. Rat (Wistar males) liver mitochondrial fractions were prepared by differential centrifugation as previously described [16]. Mitochondrial membrane fragments were obtained by lysing the mitochondria in chilled distilled water followed by centrifugation at 105,000 *g* for 30 min. The membrane preparations were further washed twice by suspension in water centrifugation. Resultant pellets were homogenized in water by repeated ultrasonic disruption at 75 W peak envelope power for 20 sec using a needle probe tip (Braunsonic 1510, San Francisco, CA).

Assay of MAO activity. The enzyme activity was determined radioenzymatically as previously described [16]. Briefly, rat liver MAO preparations were incubated at 37° for 30 min in the presence of radioactive substrate (0.1 μCi, 1 Ci = 37 GBq) which had been diluted with unlabeled substrates to yield final concentrations of 2×10^{-4} M for 5-hydroxytryptamine and *p*-tyramine, and 1×10^{-5} M for β-phenylethylamine in a final volume of 200 μl of 0.05 M phosphate buffer (pH 7.5). The reaction was terminated by adding 250 μl of 2 M citric acid. The aldehyde products formed were extracted into 1 ml of toluene:ethyl acetate (1:1, v/v) of which 600 μl was transferred to a counting vial containing 10 ml Omnifluor counting fluid (New England Nuclear, Boston, MA). The radioactivity was assessed by liquid scintillation spectrometry (Beckman 7500, Irvine, CA). Product formation was shown to proceed linearly for the assay time under these conditions at the enzyme concentrations used.

Detection of the formation of phenylacetaldehyde and phenylacetic acid from phenelzine in vitro. Phenelzine was incubated with MAO under different conditions, and the phenylacetaldehyde and phenylacetic acid products were assessed by a high performance liquid chromatographic (HPLC) procedure as previously described with a slight modification [17] at ambient temperatures on a 250 × 4.6 mm i.d. Ultrasphere I.P. analytical column packed with C-18 spherical 5 μm particles (Beckman), and a 15 × 4.6 mm i.d. Brownlee MPLC RP-18 Spherei-5 guard column (Technical Marketing Associates, Calgary, Alberta, Canada) which was installed between the Waters WISP 710B automated sample injector (Millipore, Milford, MA) and the analytical column. The mobile phase consisting of 75 mM monobasic sodium phosphate, 1 mM sodium octylsulfate, 500 μM EDTA, and 23% acetonitrile with the final pH adjusted to 2.75 with phosphoric acid, was pumped through the column at 1.0 ml/min using a Waters 6000A solvent delivery system. The column eluent was monitored spectrophotometrically (Waters Lambda-Max model 481) at 215 nm. Signals from the detector were integrated by peak area using a Spectra-Physics integrator model SP 4290 (Spectra-Physics, San Jose, CA). The conversion of aldehydes to their corresponding acids was carried out when necessary by including yeast acetylaldehyde dehydrogenase (1.25 units/assay) and β-NAD (5.2 mM) in the enzyme reaction mixture.

RESULTS

Detection of phenylethylacetaldehyde as the deaminated product of phenelzine in vitro. After phenelzine or 1,1-dideutero-phenelzine had been incubated with the rat liver mitochondrial MAO, phenylacetaldehyde (RCHO) and the deuterated homologue (RC^2HO) were identified as the major metabolites by HPLC respectively. As can be seen in Fig. 1, the retention times were 12.8 and 14.2 min respectively for the mono-deuterated and the non-deuterated aldehyde products. These retention times of the deaminated products of phenelzines are consistent with those of authentic phenylacetaldehyde and deuterated phenylacetaldehyde, which was prepared from 1,1-dideuterated-β-phenylethylamine after deamination catalyzed by MAO. The aldehyde peaks were relatively broad, which is characteristic for aromatic aldehydes in the reverse phase HPLC system [18, 19].

The separation of these deuterated and non-deuterated phenylacetaldehydes using the above-described system was consistent with our earlier observations on the separation of deuterated and non-deuterated 3,4-dihydroxyphenylacetaldehydes (products of deamination of deuterated and non-deuterated dopamine) and benzaldehydes (from deuterated and non-deuterated benzylamines) by HPLC [19, 20]. The substitution of deuterium in the aldehyde group probably changed the polarity of these compounds, thus altering their hydrophobic interaction in the reverse phase chromatographic system.

Conversion of phenylethylacetaldehyde to phenylacetic acid. The identities of the aldehydes have been further substantiated by observation of the conversion of both the phenylacetaldehyde and deuterated phenylacetaldehyde to phenylacetic acid when yeast acetaldehyde dehydrogenase and β-NAD cofactor were included in the incubation mixture. A sharp peak with a retention time of 11.6 min represents phenylacetic acid (result not shown).

Deuterium isotope effect on the deamination of phenelzine. The formation of aldehyde products from phenelzine as catalyzed by MAO was only linear in the initial phase (Fig. 2A) and ceased after approximately 10 min, by which time the enzyme was inhibited completely (results not shown). The initial reaction rate (i.e. aldehyde formation) was seen to be reduced significantly when deuterium atoms were substituted at the 1-carbon position of phenelzine. The time–courses of the increase in the formation of the aldehyde products were consistent with the decreases of the amounts of phenelzine and deuterated phenelzine. The deuterium isotope effect in these reactions was also demonstrated by using different concentrations of enzyme (Fig. 2B). When a small quantity of phenelzine was incubated for a very short period of time with an excess of MAO, most of the phenelzine was converted to phenylacetaldehyde. The amount of phenelzine bound to MAO must therefore be rather small, which would be consistent with inhibition resulting from stoichiometric formation of an enzyme-inhibitor adduct [14].

Kinetic plots as well as the K_m and V_{max} values with regard to the deamination of phenelzine and 1,1-dideutero-phenelzine are shown in Fig. 3. The isotope effect for these substrates was assessed to be $V_H/V_D = 3.1$ and $(V/K)_H/(V/K)_D = 3.7$ respectively.

The deuterium isotope effects for the deamination of 1,1-dideutero-phenelzine and 1,1,2,2-tetradeutero-phenelzine were not different (results not shown), indicating that only the abstraction of hydrogen from the 1-carbon is involved in the deamination of phenelzine.

Effect of phenylacetaldehyde on MAO activity. MAO activity towards 200 μM *p*-tyramine as substrate was measured in the presence of different concentrations of phenylacetaldehyde. Concentra-

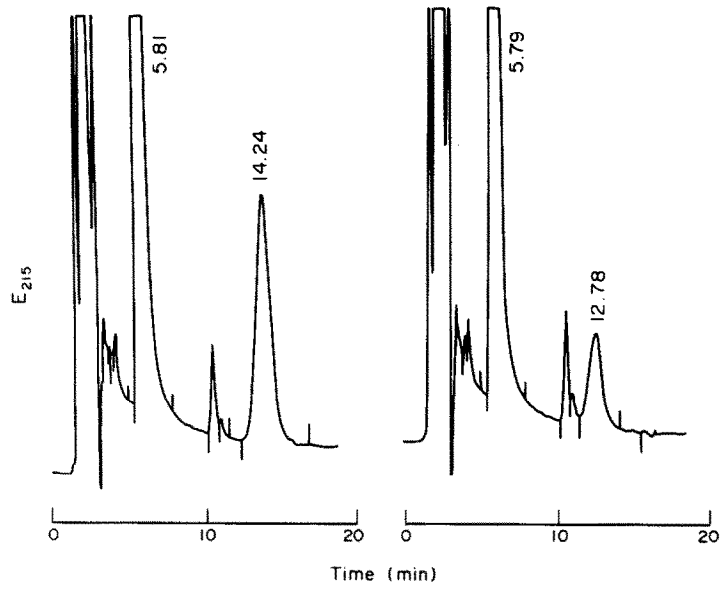


Fig. 1. High performance liquid chromatographic traces of phenelzine deaminated products as catalyzed by rat liver mitochondrial MAO. The enzyme (95 μg) was incubated at pH 7.4 with phenelzine (left panel) or 1,1-dideutero-phenelzine (right panel) in a total volume of 200 μl at 37° for 3 min. The reaction was terminated by addition of 500 μl of 0.1 N perchloric acid containing 0.25 mM sodium metabisulfite and 0.1 mM EDTA. After centrifugation, 50-μl aliquots of the supernatant fraction were subjected to HPLC analysis (see Materials and Methods). Peaks with the retention time of 5.8 min represent the unmetabolized phenelzine or deuterated phenelzine, and the peaks at 14.2 and 12.8 min are phenylacetaldehyde (RCH_2CHO) and deuterated phenylacetaldehyde (RCH_2C^2HO) respectively.

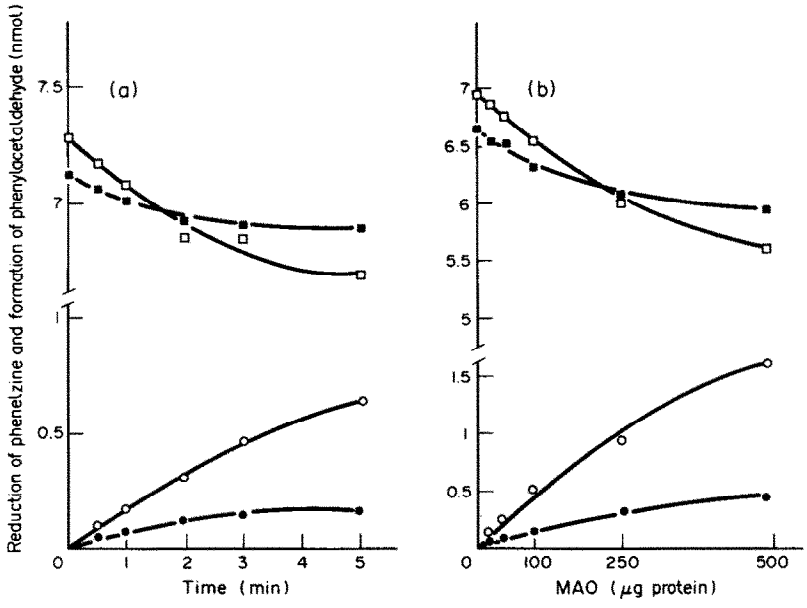


Fig. 2. Deamination of phenelzine and 1,1-dideuterated phenelzine, and the formation of corresponding phenylacetaldehyde products. (A) Initial velocities of the reduction of phenelzine (□) and 1,1-dideutero-phenelzine (■) and the production of pheylacetaldehyde (○) and deuterated phenylacetaldehyde (●) after incubation with MAO for 0–5 min under the same conditions as described in the legend to Fig. 1. (B) Effect of increasing concentrations of MAO on the conversion of phenelzine to aldehyde products. Different amounts of MAO (from 24 to 480 μg) were incubated with phenelzine and deuterated phenelzine for 3 min under the same conditions described above.

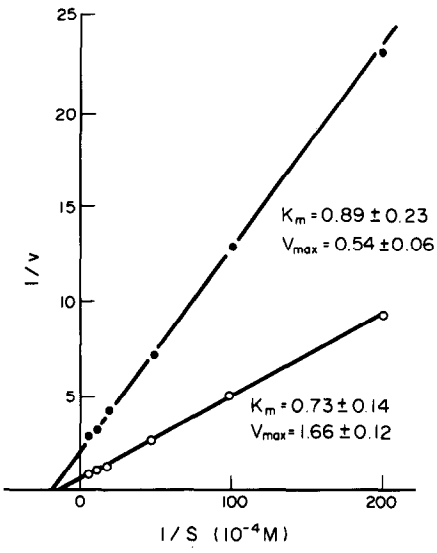


Fig. 3. Double-reciprocal plots for the deamination of phenelzine and 1,1-dideutero-phenelzine catalyzed by rat liver mitochondrial MAO. The assay conditions were similar to those described in the legend to Fig. 1, except that substrate concentrations were varied [v = velocity, nmol/mg protein/(min; S = substrate concentration]. The kinetic parameters with respect to deamination of phenelzine (○) and deuterated phenelzine (●) are indicated in the figure.

tions up to 0.1 mM exhibited no significant inhibitory activity towards MAO. Phenylacetaldehyde at a concentration higher than 1 mM could inhibit MAO activity, and the inhibition was reversible (unpublished data). The linearity of deamination of 25 μM β-phenylethylamine was unaffected by the inclusion in the assay mixture of the aldehyde-trapping semicarbazide at a concentration of 50 μM.

Initial reversible inhibition of MAO by phenelzine and deuterated phenelzine. The initial reversible phases of the inhibition of MAO-A and MAO-B (i.e activities towards 5-hydroxytryptamine and β-phenylethylamine respectively) by phenelzine and deuterated phenelzine were determined in each case by starting the reaction by adding the enzyme to substrate-inhibitor mixtures. Short incubation times (2.5 min) were used to ensure that no significant irreversible inhibition of the enzyme by the inhibitor occurred.

The reversible inhibition of MAO activity towards both MAO-A and MAO-B substrates by both phenelzine and 1,1-dideutero-phenelzine is illustrated in Fig. 4. In all cases the inhibition was competitive with K_i values (μM) as indicated in Fig. 4. The K_i values were obtained from the secondary plots of the slopes of the graphs against the inhibitor concentrations according to a previously described procedure [21]. The differences in K_i values for phenelzine and deuterated phenelzine with regard to the same substrate were small, indicating that the affinities of MAO for both phenelzine and deuterated phenelzine are similar.

Irreversible inhibition of MAO by phenelzine and 1,1-dideuterated phenelzine. The time–course of the irreversible inhibition of MAO by both phenelzine homologues was assessed. Mitochondrial enzyme preparations (5 mg/ml) were mixed with inhibitors to produce the inhibitor concentrations from 2 to 50 μM, and incubated at 10°. Aliquots were withdrawn at different times (0–15 min) and added to an enzyme assay mixture to measure the remaining enzyme activities. The irreversible inhibition proceeded rather rapidly so that reliable data concerning the half-life of the enzyme activities during inhibition by phenelzine were difficult to obtain. Complete inhibition was reached in less than 10 min. The percentage of inhibition after a 10-min preincubation of the enzyme with the inhibitors is plotted against phenelzine concentration in Fig. 5. The IC_{50} values were 17 and 3.5 μM for phenelzine and 1,1-dideuterated phenelzine respectively. Deuteration clearly potentiated the effect of phenelzine in inhibiting MAO activity. The results of the formation of deuterated and non-deuterated phenylacetaldehyde from their corresponding phenalzine homologues under the same conditions of incubation are also shown in Fig. 5. The relative MAO activity appears to be negatively correlated to the amount of acetaldehyde formed. Inclusion of 50 μM semicarbazide in the enzyme–phenelzine reaction mixture had no significant effect on the rate of inhibition (results not shown).

DISCUSSION

The major metabolite found in rat urine after *in vivo* administration of phenelzine is phenylacetic acid [10]. MAO is involved in this metabolic conversion [9]. The results presented here demonstrate that MAO can convert phenelzine *in vitro* to phenylacetaldehyde. The phenylacetaldehydes are then easily converted to phenylacetic acid *in vivo* by the action of aldehyde dehydrogenase. Clearly phenelzine acts as a k_{cat} (suicide) inhibitor; it first reacts reversibly with the enzyme to form a non-covalent complex and then subsequently a reaction within this complex occurs to form an irreversible, covalently-bound, enzyme–inhibitor complex [22]. The reaction between phenelzine and MAO can thus be represented by the following equation:

$$E + I \leftrightarrow EI \leftrightarrow EI^* \begin{matrix} \nearrow E - I \\ \searrow E + \text{Product} \end{matrix}$$

where EI represents the initial non-covalent complex and EI^* represents an activated species which can either react to form the irreversibly inhibited enzyme ($E - I$) or to release the products.

That the phenylacetaldehyde product itself might be involved in the inhibition of MAO activity has been ruled out, since even at rather high concentrations, it did not inhibit MAO. In addition, when semicarbazide, which can interact with the aldehyde groups to form an inactive hydrazone, was included in the MAO reaction mixtures, the rate of deamination of neither phenelzine nor β-phenylethylamine was affected.

It has been suggested that the mechanisms of inhibition may be preceded by formation of either a phenylethylidene hydrazine [13] or a phenylethyldiazene intermediate [14, 15] so forming C(4a)-phenyl-4a,5-dihydroflavine adducts. Both of these

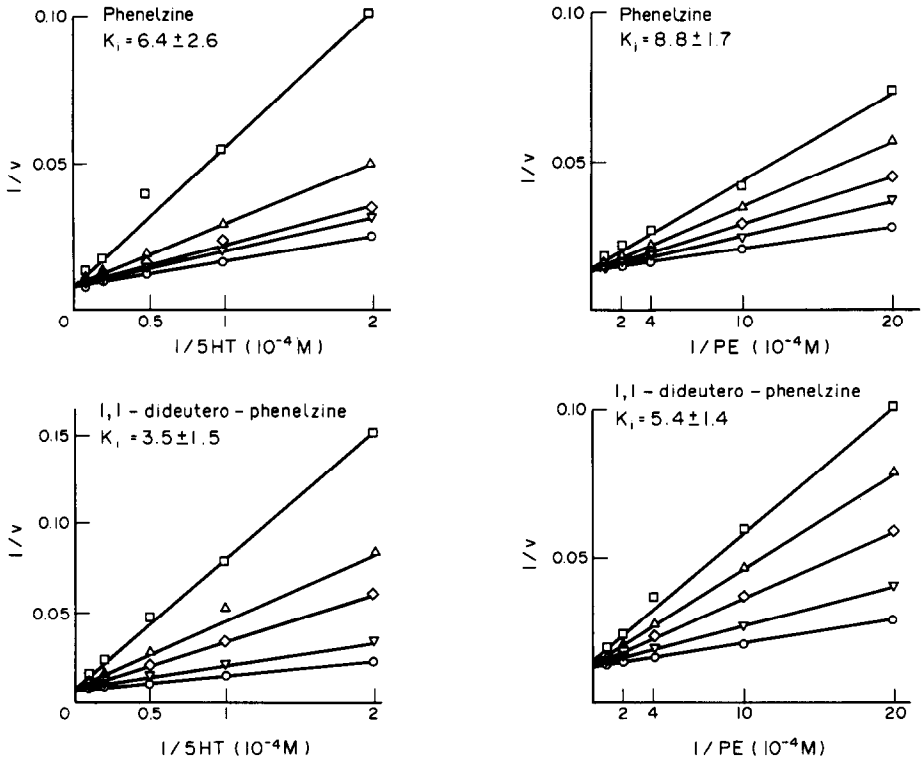


Fig. 4. Double-reciprocal plots of the inhibition of the deamination of 5-hydroxytryptamine (left panels) and β-phenylethylamine (PE, right panels) by phenelzine and 1,1-dideutero-phenelzine. Data are means ± SE for determinations of three experiments assayed at pH 7.4 and 37°. Protein concentrations at assay were in each case 0.45 mg/ml. Five substrate and four inhibitor concentrations [i.e. 20 μM (□), 10 μM (△), 5 μM (◇), 2 μM (▽) and no inhibitor control (○)] were used. In all cases data were plotted as $1/v$ against $1/S$ for calculatint K_m and V_{max} and slope values. K_i values (μM), the dissociation constants of the EI complex (k_{-1}/k_1), were calculated from replots of slope against inhibitor concentration (results not shown). The correlation coefficients of the regression lines used in the calculations were greater than 0.9 in all cases.

possible intermediate compounds are highly reactive and would be difficult to detect.

Substitution at the 1-carbon position with deuterium can potentiate significantly the inhibitory effect on MAO *in vivo* [4, 6]. It has also been demonstrated repeatedly that administration of deuterated phenelzine can cause an elevation in brain amine levels and a reduction in corresponding acid metabolites to an extent greater than that caused by non-deuterated phenelzine [4–6, 8]. The findings reported here concerning the *in vitro* deuterium isotope effect are consistent with those obtained in the *in vivo* studies. The affinity of phenelzine towards rat liver MAO was not changed significantly following deuterium substitution, and it is clear that cleavage of hydrogen from the 1-carbon is not involved in the binding of phenelzine to the enzyme. The extent of irreversible inhibition, as calculated from the IC_{50} values, however, was greatly potentiated following substitution on the 1-carbon atom with deuterium.

The observation of a primary deuterium isotope effect, (V_H/V_D = 3.1) in the formation of phenylacetaldehyde indicates removal of hydrogen from carbon-1 to be the rate-limiting step in the process. Thus, phenylethylidine hydrazine would be formed as an intermediate. Its hydrolysis to form phenylacetaldehyde would occur readily, and these data cannot distinguish whether such hydrolysis occurs on the enzyme surface or after release of the phenylethylidine hydrazine. However, kinetic studies have suggested that the former might be the case [23].

The increased inhibitory potency resulting from deuteration of phenelzine at carbon-1 indicates that abstraction of hydrogen from that carbon, leading to phenylethylidine hydrazine formation, is not involved in the irreversible inhibition of the enzyme. Since substrate oxidation has been shown to be involved in this process [14, 15], it would be reasonable to postulate that this step involves removal of hydrogen from the hydrazine group of phenelzine to form phenylethyldiazine, which was identified as the inhibitory species by Patek and Hellerman [14]. Thus, formation of phenylethyldiazine and phenylethylidine hydrazine would be competing reactions occurring at the enzyme surface. Such a reaction pathway is shown in Scheme 1.

Phenylethylidene hydrazine might be formed directly by hydrogen removal from phenelzine or by rearrangement of phenylethyldiazine. Both of these reactions involve hydrogen removal from carbon-1 and deuteration at that position would increase the inhibitory potency by decreasing the reaction flux through that pathway resulting in an increased accumulation of the diazine.

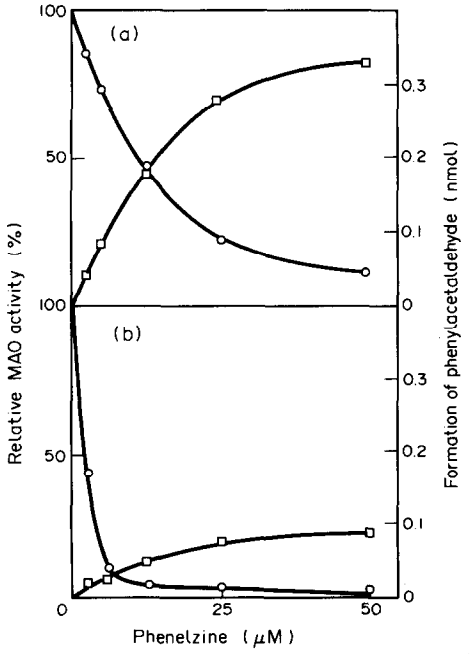
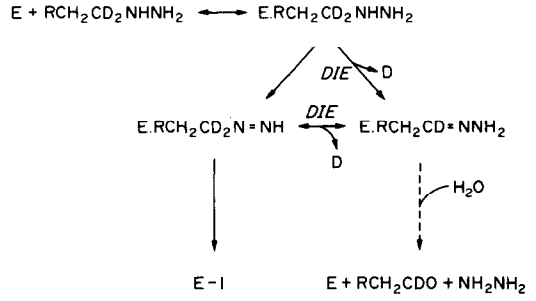


Fig. 5. Effects of phenelzine and 1,1-dideutero-phenelzine on MAO activity, and the formation of phenylacetaldehyde and deuterated phenylacetaldehyde. MAO activity (○) towards β-phenylethylamine (1×10^{-5} M) was measured radiometrically at pH 7.4, 37°, for 3 min in the presence of phenelzine (A) or deuterated phenelzine (B). Under the same conditions the formation of phenylacetaldehyde or deuterated phenylacetaldehyde (□) was determined by HPLC analysis.



R = ⬡ ; D = deuterium; DIE = deuterium isotope effect

E-I = covalent enzyme - inhibitor adduct

Scheme 1. Possible pathways in the oxidation of 1,1-dideutero-phenelzine by monoamine oxidase.

The possibility that some dissociation of the diazine product from the enzyme occurs cannot be excluded. This might then rearrange in solution to form phenylethylidine hydrazine, which would be hydrolyzed to the aldehyde. The rate of the former process might be expected to show a deuterium isotope effect, but the final extent of aldehyde formation would be independent of deuteration. Since no material that might correspond either to the diazine or to phenylethylidine hydrazine was observed in the HPLC studies, and diazine released must have been converted to the aldehyde during the time taken to prepare and run samples on HPLC. Thus, release of that product, if it occurs, would not contribute to the kinetic deuterium isotope effect observed in the present work. The mechanism by which the diazine reacts with the enzyme to form covalent adducts appears to be complex. One site of reaction is the enzyme-bound flavin, and the mechanism proposed for this by Kenney *et al.* [15] would not be expected to be affected by deuterium substitution at carbon-1. However, alternative reaction sites with amino acid groups on the enzyme may also be involved [15] and, since these have not been characterized, it is not possible to say whether those inhibitory reactions might show deuterium isotope effects.

Recently, it has been demonstrated that the administration of 1,1,2,2-tetradeutero-phenelzine to the rat causes 1,1,2,2-tetradeutero-β-phenylethylamine to appear in urine and various tissues [12]. It is not yet known how this reaction occurs. When phenelzine was incubated *in vitro* with MAO, phenylacetaldehyde was the major product and β-phenylethylamine was not detected. This, however, does not rule out totally the possibility that MAO may still be involved. For example, β-phenylethylamine is an MAO-B substrate with very high affinity; if it were formed from phenelzine, it would be deaminated very quickly by MAO to produce the same product, phenylacetaldehyde. Clearly, the conversion of phenelzine to β-phenylethylamine, if it does occur, would not be related to the inhibition of the enzyme by phenelzine, because the cleavage of the N—N bond should not be affected by deuterium substitution at the 1-carbon position. The conversion of [^{14}C]phenelzine to [^{14}C]phenylacetic acid in rats could only be partially blocked following pretreatment with the MAO inhibitor pargyline [10], so that there may be other pathways responsible for converting phenelzine to β-phenylethylamine.

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