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THE MONOAMINE OXIDASE CATALYZED DEGRADATION OF PHENELZINE-1-^{14}C, AN IRREVERSIBLE INHIBITOR OF MONOAMINE OXIDASE—I

STUDIES *IN VITRO*

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Abstract—Exposure of phenelzine-1-^{14}C to a biotransformation system which included mitochondria isolated from homogenate of rat liver resulted in a metabolite with thin-layer chromatographic characteristics identical to those of authentic phenylacetic-1-^{14}C acid.

The conversion of phenelzine to phenylacetic acid was found to be: (1) relatively insensitive to preincubation of the bioconversion system with cyanide; (2) dependent upon the presence of O_2; (3) abolishing by boiling the mitochondrial component of the system; and (4) inhibited by preincubation of the biotransformation system with isocarboxazid, pheniprazine, tranylcypromine or pargyline.

These results provide the first substantial evidence for an irreversible inhibitor of monoamine oxidase (MAO) acting as a substrate of the enzyme, and the results suggest that degradation of the inhibitor is very likely accomplished by oxidative dehydrazination, a reaction mechanism previously unknown for MAO.

THE INVOLVEMENT of monoamine oxidase (MAO) in the regulation of tissue levels of endogenous monoamines has aroused considerable interest in defining the general characteristics of the enzyme and in exploring it as a possible target for pharmacological manipulation. After the demonstration of effective inhibition *in vivo* of MAO by iproniazid,[1] a hydrazine derivative, other hydrazines were tested for their ability to inhibit MAO. Phenelzine (β-phenylethylhydrazine; Nardil) proved to be a potent and irreversible inhibitor of MAO.[2]

In addition to being an irreversible inhibitor of MAO, the results reported herein indicate that phenelzine is also a substrate for MAO. Although MAO (EC 1.4.3.4, monoamine:O_2 oxidoreductase deaminating) is primarily recognized as a deaminating enzyme, phenelzine appears to be biotransformed mainly to phenylacetic acid suggesting that MAO catalyzes the removal of the hydrazine moiety from the molecule.

METHODS

Incubation. Mitochondria for use in the biotransformation system (Table 1) were prepared from homogenate of liver tissue from male Sprague–Dawley rats weighing 300–350 g according to the procedure described by Weinbach.[3] For every initial 30 g of liver tissue, 70 ml of phosphate buffer (0·067 M, pH 7·4) was used to resuspend the final pellet of washed mitochondria. The source of aldehyde dehydrogenase was a

TABLE 1. COMPOSITION OF THE BIOTRANSFORMATION SYSTEM

Amount (ml)	Component
1·0	Mitochondrial preparation
0·2	Aldehyde dehydrogenase preparation
0·5	NAD (13·5 μmoles in 0·067 M phosphate buffer, pH 7·4)
0·5	Nicotinamide (60 μmoles in 0·067 M phosphate buffer, pH 7·4)
0·3	0·067 M phosphate buffer (pH 7·4) or inhibitor (incubation period of 20 min)
0·5	Phenelzine-1-^{14}C-H_2SO_4 (0·15 μmole) (incubation period of 40 min)

high-speed supernatant fraction of guinea pig kidney homogenate according to the method of Lovenberg *et al.*[4]

The mitochondria and other components of the biotransformation system were incubated at 37° for 20 min (Dubnoff metabolic shaking incubator) with 0·3 ml of phosphate buffer (0·067 M, pH 7·4) or 0·3 ml of molar concentrations of KCN or inhibitors of MAO. After the initial incubation period, 0·15 μmole phenelzine-1-^{14}C-H_2SO_4 (final concentration = 5×10^{-5} M) was introduced into the flask containing the incubation mixture and the incubation was continued for an additional 40 min.

Some incubations were carried out in Warburg flasks under nitrogen. After placing the appropriate amount of phenelzine-1-^{14}C solution in the sidearm of the reaction vessel, nitrogen was flushed through the flask for approximately 1 min prior to sealing. Subsequent to preincubation as usual for 20 min, the vessel was tipped repeatedly to allow the phenelzine-1-^{14}C to mix with the other components of the system. The incubation was continued for 40 min.

In some instances the mitochondrial preparation was boiled for 30 sec prior to addition to the reaction flask. All other procedures were performed as usual.

Extraction. The number of reaction flasks used during a typical experiment was 16 and they were divided into four groups. Subsequent to the last period of incubation, the total contents of a group of flasks (12 ml) was emptied into a 125-ml pear-shaped separatory funnel containing 8 ml of 0·5 N HCl and 8 g of NaCl. Ten ml of ethyl ether was added to each of the four flasks, swirled and then added to the separatory funnel. Therefore, the total volume of ether added to each separatory funnel was 40 ml. After shaking the funnel and allowing the phases to separate and settle, the aqueous phase was drawn off and discarded. The ether phase was collected and then evaporated to a volume of 5 ml with the aid of a stream of nitrogen. Fifty-μl portions of the ether extract for counting or chromatographing were withdrawn as needed with a Hamilton microliter syringe.

In a few cases, phenylacetic-1-^{14}C acid (0·15 μmole) was added to the incubation flask in lieu of phenelzine-1-^{14}C and carried through the incubation and extraction procedures.

Chromatography. Sheets, 2·5 × 20 cm, of microfilaments of glass impregnated with alumina gel (ITLC type A, Gelman Instrument Co.) were spotted 4 cm from the bottom edge with 50-μl quantities of the solutions or extracts under investigation. After drying for 15 min, the sheets of chromatographic material were suspended in a Gelman chromatography tank (model 51325-1). Ten to 15 ml of the solvent was added to the upper trough of the chromatography tank and immediately thereafter

solvent was placed in the lower trough until it contacted the bottom edge of the chromatographic sheet. When the solvent had advanced 10 cm from the origin of application, the sheet was removed and dried.

To determine the location of radioactivity on chromatograms, the area between the origin and the solvent front was divided into twenty strips cut transversely to the direction of solvent movement. The strips (0·5 × 2·5 cm) were placed in vials containing 20 ml of 0·5% (w/v) PPO (2,5-diphenyloxazole) in toluene and the radioactivity was ascertained.

The three solvent systems employed were: (1) isopropanol:ammonium hydroxide: water (80:4:4); (2) chloroform:cyclohexane:glacial acetic acid (20:80:2); and (3) 95% (v/v) ethanol:water:25% (v/v) ammonium hydroxide (100:12:16). The ratios of the components of the systems are volume ratios.

Radioactivity measurements. The radioactivity of the various experimental samples was measured with a Mark I liquid scintillation computer (model 6860, Nuclear-Chicago Corp.). In addition to correction for background activity, samples were corrected for efficiency according to the channels ratio method.

Phenelzine-1-^{14}C-H_2SO_4 was a gift from Dr B. Dubnick and the Warner–Lambert Research Institute. The specific activity of the drug was 1·71 μc/mg and it was described as having a radiochemical purity of 99+ per cent. Two samples of phenylacetic-1-^{14}C acid were obtained from New England Nuclear Corp. Their specific activities were 1·7 mc/m-mole and 8·4 mc/m-mole. The radiochemical purity of phenylacetic-1-^{14}C acid was greater than 96·5 per cent.

RESULTS

Recovery of radioactivity in ether extracts. After incubation of phenelzine-1-^{14}C or phenylacetic-1-^{14}C acid with mitochondria isolated from liver tissue of the rat, the incubation mixture was acidified and then extracted with ether. The radioactivity of the ether extract was determined and the recovery calculated. When phenylacetic-1-^{14}C acid was carried through the incubation and extraction procedures, approximately 96 per cent of the label present was recovered in the ether extract (Table 2).

TABLE 2. EFFECT OF VARIOUS EXPERIMENTAL PROCEDURES ON THE RECOVERY OF THE RADIOACTIVE LABEL FROM PHENELZINE-1-^{14}C AFTER INCUBATION IN THE MITOCHONDRIAL SYSTEM*

Substance	Molar concn	No.	Incubation time (min)	Experimental	dpm present	dpm recovered	% Recovery
Phenylacetic acid-^{14}C	5×10^{-5}	3	40	—	2,298,000	2,201,000	95·8 ± 2·2
Phenelzine-^{14}C	5×10^{-5}	3	0	—	547,300	23,900	4·4 ± 0·2
Phenelzine-^{14}C	5×10^{-5}	13	40	—	547,300	464,560	84·9 ± 2·2
Phenelzine-^{14}C	5×10^{-5}	3	40	boiled mitochondria	547,300	81,833	15·0 ± 4·0
Phenelzine-^{14}C	5×10^{-5}	3	40	anaerobic	547,300	76,900	14·1 ± 2·8
Phenelzine-^{14}C	5×10^{-5}	3	40	KCN $5 \cdot 10^{-3}$ M	547,300	405,490	74·3 ± 2·6

* The substance indicated was incubated with the mitochondrial biotransformation system. The incubation mixture was acidified prior to extraction with ether. The ether extract was counted (dpm recovered) and the percentage recovery calculated. Dpm recovered is the mean value; percentage recovery is the mean value ± S.E.

Recovery of the radioactive label from phenelzine-1-^{14}C introduced into the incubation flask was dependent upon a period of incubation (Table 2). Only 4–5 per cent of the radioactivity present in the incubation vessel was recovered into ether with a 0-min incubation time, whereas nearly 85 per cent of the radioactive label could be recovered with a period of incubation of 40 min.

Boiling the preparation of mitochondria for 30 sec prior to addition to the reaction flask decreased the recovery of the label of phenelzine-1-^{14}C from the usual level of nearly 85 per cent to a value of approximately 15 per cent (Table 2).

The substitution of an atmosphere of nitrogen for the ambient air effected a large reduction in the conversion of phenelzine-1-^{14}C to a product or products capable of being extracted with ether from the acidified incubation mixture (Table 2). The experimental value of 14·1 per cent is considerably lower than the control of 84·9 per cent but it is higher than the value of 4·4 per cent representing extraction of unchanged phenelzine-1-^{14}C.

Preincubation of the isolated mitochondria with 5×10^{-3} M KCN for 20 min prior to the addition of phenelzine-1-^{14}C had a noticeable but not dramatic effect on the recovery of radioactivity in the ether extract. As shown in Table 2, the recovery of the radioactive label from phenelzine-1-^{14}C was slightly decreased from the control as the result of preincubation with this high concentration of KCN.

Before investigating the effects of other inhibitors of MAO on the recovery of the radioactive label of phenelzine-1-^{14}C incubated in the mitochondrial system, the concentration–effect relationship was established for pargyline (Eutonyl HCl), the most specific inhibitor of MAO. As shown in Table 3, the amount of radioactivity appearing in the ether extracts could be markedly reduced by including pargyline in the reaction flasks. Inasmuch as the reduction of about 55 per cent at a concentration of 10^{-4} M was only slightly larger than the outcome at 10^{-6} M, a similar decrease in ether-extractable radioactivity could be anticipated with reasonable concentrations of other inhibitors of MAO.

In addition to pargyline, three other inhibitors of MAO were tested. Isocarboxazid (Marplan), PIH (pheniprazine HCl; Catron), and tranylcypromine (Parnate sulfate) were selected because this particular combination provided two hydrazines (iso-

TABLE 3. EFFECT OF INHIBITORS OF MAO ON THE RECOVERY OF THE RADIOACTIVE LABEL OF PHENELZINE-1-^{14}C AFTER INCUBATION IN THE MITOCHONDRIAL SYSTEM*

Inhibitor	Molar concn	Ether-extractable radioactivity (% of control)	
			mean
Pargyline	10^{-7}	92, 96, 98	95
Pargyline	5×10^{-7}	72, 78, 80	77
Pargyline	10^{-6}	49, 51, 56	52
Pargyline	10^{-5}	45, 48, 52	48
Pargyline	10^{-4}	44, 45, 48	46
Isocarboxazid	10^{-4}	40, 45, 51	45
PIH	10^{-4}	33, 38, 41, 41	38
Tranylcypromine	10^{-5}	43, 45, 46, 46	45

The inhibitor was preincubated for 20 min with the biotransformation system. After the addition of phenelzine-1-^{14}C final concentration, 5×10^{-5}M), the incubation was continued for 40 min. The mixture was then acidified and extracted with ether. Each value in the table represents a single experiment.

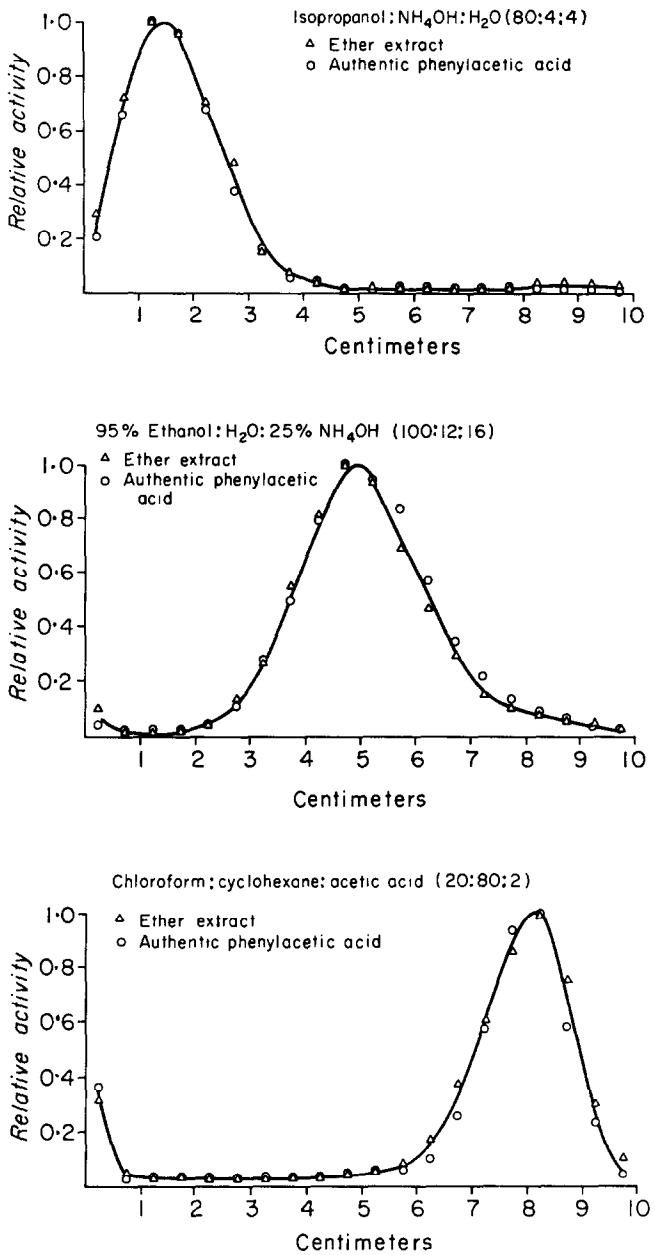

FIG. 1. Radiochromatograms of ether extracts of incubation mixtures exposed to phenelzine-1-^{14}C vs. authentic phenylacetic-1-^{14}C acid. Authentic phenylacetate or the ether extract was applied at the origin (0 cm) of the chromatographic sheet. The solvent was allowed to advance past the origin for 10 cm. The sheet of chromatographic material was dried prior to dividing the area between 0 and 10 cm into twenty equal strips. The radioactivity of each strip was determined as described in Methods. The strip with the highest radioactivity was assigned a relative activity of 1·0. The results of eighteen radiochromatograms (nine of ether extracts and nine of authentic phenylacetic acid) are summarized in this figure and, thus, each point is the average value from three experiments. The ratios of the components of the solvent systems are volume ratios.

carboxazid and PIH) and two nonhydrazines (pargyline and tranylcypromine). Isocarboxazid (10^{-4} M), PIH (10^{-4} M) and tranylcypromine (10^{-5} M) were also found to be capable of effecting a reduction in ether-extractable radioactivity (Table 3). Isocarboxazid and tranylcypromine decreased the conversion of phenelzine-1-^{14}C to a product or products extractable with ether from the acidified incubation medium to the same extent as the maximum achieved with pargyline. PIH, however, in a concentration of 10^{-4} M, showed a slightly greater effect, perhaps indicating that systems other than MAO were being influenced. In a preliminary trial, 10^{-5} M PIH had virtually no effect on the amount of radioactivity recovered in the ether extract.

Chromatographic profiles of ether extracts. Figure 1 shows graphically the close correspondence between the radiochromatographic distribution patterns of ether extracts from control experiments (horizontal row 3 in Table 2) and authentic phenylacetic-1-^{14}C acid. The ether extracts used in constructing Fig. 1 were obtained from three individual experiments. Thus, an ether extract from a particular experiment was chromatographed only once in each of the three solvent systems.

Phenelzine-1-^{14}C which had not undergone biotransformation migrated with the solvent front in the isopropanolol:NH_4OH:HOH (80:4:4) and the 95% ethanol:HOH:25% NH_4OH (100:12:16) systems, whereas it remained at the origin in the chloroform:cyclohexane:acetic acid (20:80:2) system.

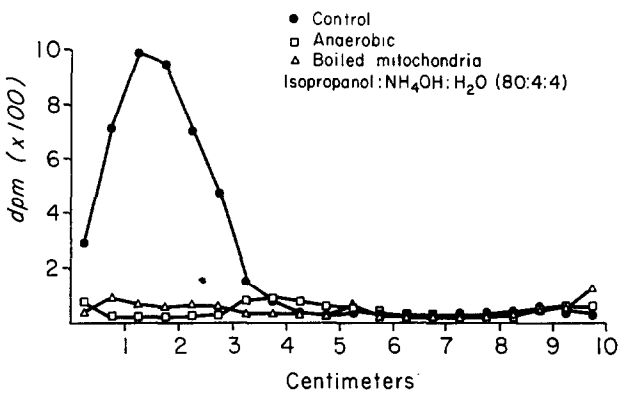

FIG. 2. Effect of boiling the mitochondrial preparation or substituting an atmosphere of nitrogen on the radiochromatographic profile of the ether extract. Equal volumes of ether extracts from the control or experimental were spotted at 0 cm and the solvent was allowed to advance for 10 cm. After the division of each sheet into twenty equal strips, the radioactivity of each strip was determined. The large peak toward the application origin in the control corresponds to the location of phenylacetic acid (see Fig. 1). Each control point is the mean value obtained from three radiochromatograms, while values for the boiled mitochondria and anaerobic curves are from single chromatograms. The ratios of the components of the solvent system are volume ratios.

The formation of phenylacetic acid from phenelzine was abolished, or nearly so, as the result of boiling the mitochondrial preparation prior to addition to the reaction vessel or by the substitution of an atmosphere of nitrogen for the ambient air (Fig. 2).

Inhibition of MAO decreased the formation of ether-extractable radioactive material to the extent of approximately 45 per cent of the control value (Table 3). The chromatographic distribution of these extracts is shown in Fig. 3. As a consequence of preincubation with an inhibitor of MAO, relatively more radioactivity was

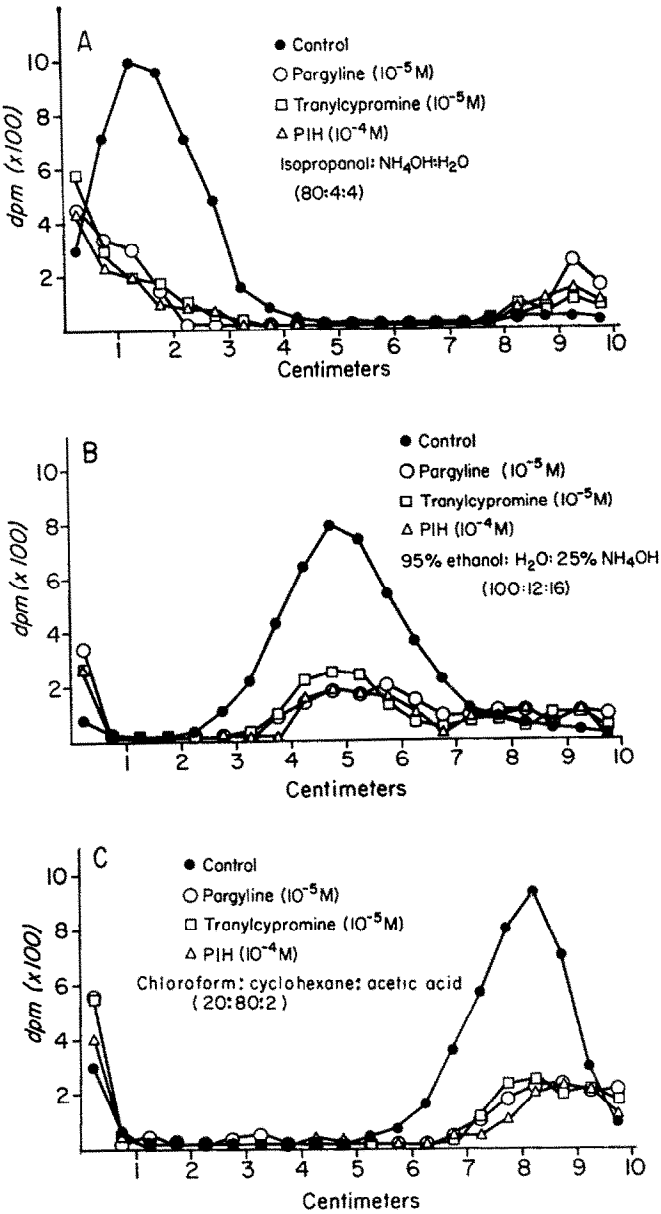

FIG. 3. Effect of inhibitors of MAO on the radiochromatographic distribution of ether extracts.

A. Equal volumes of ether extracts from the control or those preincubated with an inhibitor of MAO were spotted at 0 cm and the solvent was allowed to proceed for 10 cm. After the division of each sheet into twenty equal strips, the dpm of each strip was determined. Each control point is the average value obtained from three radiochromatograms, whereas values for each inhibitor of MAO are from single chromatograms. The ratios of the components of the solvent system are volume ratios. The large peak toward the application origin in the control corresponds to the location of phenylacetic acid (see Fig. 1).

B. Except for a different solvent system, conditions and procedures were identical to those of 3A. The large peak near the center of the chromatogram in the control corresponds to the location of phenylacetic acid (see Fig. 1).

C. Except for a different solvent system, conditions and procedures were identical to those of 3A. The large peak toward the solvent front of the chromatogram in the control corresponds to the location of phenylacetic acid (see Fig. 1).

found concentrated at the origins and toward the solvent fronts (Fig. 3A, 3B and 3C). This would seem to indicate the presence of a metabolite or metabolites of phenelzine other than phenylacetic acid. From the data of Fig. 3B, the amount of radioactivity falling under the phenylacetic acid peak (3–7 cm, inclusive) was ascertained. Whereas there are 3963 dpm under the phenylacetic acid peak in the control, the values obtained after chromatography of ether extracts from incubation mixtures preincubated with pargyline (10^{-5} M), tranylcypromine (10^{-5} M) or PIH (10^{-4} M) are 1048, 1141 and 799 respectively. Expressed as per cent of control, these values become 26, 29 and 20 per cent.

DISCUSSION

The results of this investigation support the supposition that phenelzine can be bioconverted via a reaction sequence catalyzed by MAO. MAO is associated to a large extent with the mitochondrial fraction of tissue homogenates[5] and thus, in accord with MAO as the enzyme involved, the formation of an ether-extractable product from phenelzine was markedly reduced by boiling the mitochondrial component of the incubation mixture (Table 2). Phenylacetic-1-^{14}C acid could not be found in ether extracts of incubation mixtures containing boiled mitochondria (Fig. 2). Inasmuch as the catalytic activity of MAO is dependent upon oxygen,[6] the pronounced dependency of the conversion process on the presence of oxygen further supports MAO as the catalytic enzyme (Table 2 and Fig. 2). The transformation of phenelzine into ether-extractable material was in effect insensitive to cyanide (Table 2). This observation agrees nicely with the curious insensitivity of MAO toward cyanide.[6] Further evidence for the involvement of MAO comes from investigations utilizing inhibitors of the enzyme. Preincubation of the biotransformation system with isocarboxazid, pheniprazine, tranylcypromine or pargyline effected a large reduction in the formation of phenylacetic-1-^{14}C acid from phenelzine-1-^{14}C (Table 3 and Fig. 3). The radiochromatographic identification of phenylacetic acid as a bioconversion product of phenelzine provides further evidence for MAO as the enzyme involved in the reaction. Phenelzine (β-phenylethylhydrazine) is the amino isostere of *N*-methyl-β-phenylethylamine. Thus, in a manner comparable to the MAO-catalyzed demethylamination of *N*-methyl-β-phenylethylamine to methylamine plus phenylacetic acid,[7] hydrazine and phenylacetic acid would be expected to result from an MAO-catalyzed biotransformation of phenelzine.

MAO belongs to the family of enzymes known as the amine oxidases. In addition to different preferential substrates and cellular localization, monoamine oxidases differ from other amine oxidases in their capacity for acting on secondary and tertiary amines, as well as primary amines, and in their resistance to inhibition by cyanide.[6] Phenelzine can be viewed as a secondary amine. Furthermore, the biotransformation of phenelzine was carried out by mitochondria, was resistant to cyanide and was inhibited by the rather specific nonhydrazine inhibitors of MAO tranylcypromine and pargyline. Tranylcypromine and pargyline are more specific than hydrazine inhibitors of MAO as evidenced by their lack of effect, even in high concentrations, on enzymes such as diamine oxidase, aromatic amino acid decarboxylase, amphetamine oxidase and choline oxidase.[8–10] For the preceding reasons, MAO, not one of the other amine oxidases (e.g. diamine oxidase, spermine oxidase, benzylamine oxidase), appears to be the enzyme catalyzing the breakdown of phenelzine.

The involvement of MAO in the formation of phenylacetic acid from phenelzine is both particularly interesting and seemingly paradoxical, since phenelzine is well recognized as an irreversible inhibitor of MAO. Zeller *et al.* have called attention to the similarity in structure between many hydrazine inhibitors of MAO and amine substrates of the enzyme.[11–13] This observation, plus the observation that the presence of substrate during incubation of MAO with a hydrazine inhibitor will protect the enzyme from inhibition, indicates that hydrazine inhibitors of MAO may react with the same site of the enzyme as the usual amine substrates.[14–16] This concept is strengthened by the fact that inhibition of MAO by hydrazines requires preincubation of the compound with MAO in the presence of oxygen.[15,17] Davison proposed that iproniazid might react in the presence of oxygen with the active site of MAO in a manner similar to the first stage in amine oxidation.[15] Dehydrogenation of the hydrazine was visualized as giving a substituted hydrazone, $(CH_3)_2C{=}NNHR$, which then reacted further with the enzyme's active center to form an irreversible complex. The discovery that phenelzine is dehydrazinated by MAO can be interpreted as supporting and extending the concept of Davison. Phenelzine is presumably dehydrogenated in a manner similar to the usual amine substrates of MAO. The resulting hydrazone might then undergo hydrolysis to form phenylacetaldehyde plus hydrazine. Phenylacetaldehyde would then be oxidized to phenylacetic acid. If phenelzine is handled by MAO in this way, how does phenelzine irreversibly inhibit the enzyme? One possibility is that MAO may be hydrazinated as a result of interaction with phenelzine. However, no substantial evidence is currently available to support or refute this idea.

In addition to phenelzine, some of the other aliphatic and aralkylhydrazines, such as benzylhydrazine and γ-phenylpropylhydrazine, appear to possess the structural features required to be substrates of MAO. Studies with ^{14}C-isocarboxazid labeled in the benzyl moiety have shown this compound to be converted to ^{14}C-benzylhydrazine, which in turn forms ^{14}C-benzoic acid prior to conjugation with glycine for excretion as hippuric acid.[18,19] The nature of the dehydrazination of benzylhydrazine to benzoate has not been investigated. With the exception of phenelzine and possibly a few other related substances, the reaction between hydrazines and MAO apparently does not reach fruition. Nevertheless, since irreversible inhibition of MAO by many hydrazines requires preincubation of the compound with MAO in the presence of oxygen,[15,17] these inhibitors may react with the active center of MAO in a manner analogous to the first stage in the oxidation of phenelzine.

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