Brit. J. Pharmacol. (1965), **24**, 245–252.

THE INITIAL INACTIVATION OF PHENELZINE BY A MONOAMINE OXIDASE-LIKE SYSTEM *IN VITRO* AND *IN VIVO*

BY

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(*Received June* 18, 1964)

With the first demonstration of monoamine oxidase inhibition by a hydrazine derivative (iproniazid) by Zeller, Barsky & Berman (1955), a significant number of new compounds with similar anti-monoamine oxidase activity has been reported. Among these has been phenelzine (phenethylhydrazine, Nardil), a compound closely related to the natural substrate of monoamine oxidase, phenethylamine. These two compounds differ only by the presence of an extra amino group on the end of the side chain of the phenelzine molecule. Otherwise, phenelzine has the structural qualifications of a substrate of monoamine oxidase. However, phenelzine has been shown to possess the ability to inhibit monoamine oxidase irreversibly.

In an earlier report we described the marked difference in tissue reactivity between pheniprazine (α-methylphenethylhydrazine) and iproniazid. The former agent was inactivated rapidly when exposed to brain homogenates, and this process was non-enzymic in nature since boiled homogenate was equally effective (Horita, 1963). Several other compounds of related structure also had this property, and phenelzine was expected to exhibit a similar interaction since it did share several other properties with pheniprazine (Horita & McGrath, 1960a; Horita, 1961). However, phenelzine proved to be an exception in that liver homogenate was more effective than brain in destroying its anti-monoamine oxidase activity. Further investigations of this unexpected observation indicated that the inactivation of phenelzine by liver homogenates, unlike that of pheniprazine, occurs via an enzyme pathway. The present report describes studies of this phenomenon both *in vitro* and in the intact animal. It also presents evidence that this inactivation involves monoamine oxidase or a similar system, and that phenelzine may act both as a substrate and inhibitor of the enzyme.

METHODS

Both *in vitro* and *in vivo* experiments employed liver tissues from male Sprague-Dawley rats weighing 100 to 250 g. The preparation of homogenates and mitochondria for these studies has been described in a previous report (Horita, 1963). The disappearance of phenelzine activity *in vitro* was demonstrated by first incubating 1 ml. of 10% liver homogenate with a final concentration of 5×10^{-5} M of phenelzine in 3 ml. of incubation mixture. This suspension was shaken at 37° C for various lengths of time, after which it was transferred to centrifuge tubes and centrifuged at 40,000 *g* for 30 min (0° C, Spinco model L ultracentrifuge). 1.5 ml. of the " supernatant " fluid (consisting of the soluble fraction and some microsomes) were added to

1 ml. of mitochondrial preparation equivalent to 300 mg of liver in 0.067 M-sodium phosphate buffer, *p*H 7.4, and 6 μmoles of 5-hydroxytryptamine creatinine sulphate. The final volume was 3 ml., and at zero time the concentration of phenelzine was theoretically 2.5×10^{-5} M. Monoamine oxidase and anti-monoamine oxidase activities were determined by measuring the residual 5-hydroxytryptamine content after incubation for 40 min at 37° C (Udenfriend, Weissbach & Brodie, 1958). For control purposes, tissue homogenates to which no phenelzine had been added were processed in an identical manner to determine the influence of supernatant fluid alone on the mitochondrial monoamine oxidase preparation.

In the experiment on the concentration-effect relationship (Fig. 2) the concentrations of phenelzine were varied in the pre-incubation mixture, but, after the 30-min incubation period, all concentrations were diluted with 0.067 M-sodium phosphate buffer (*p*H 7.4) so that the final theoretical concentration in the mitochondrial assay preparation was 2.5×10^{-5} M.

In the *in vivo* experiments rats were injected intraperitoneally with 20 mg/kg of phenelzine, and after various intervals the animals were killed. Their livers were removed rapidly and homogenized in 0.067 M-sodium phosphate buffer to a concentration of 33%. The homogenates were centrifuged at high speeds, and the supernatant fluids were assayed for anti-monoamine oxidase activity on the mitochondrial preparation as described above. In those instances where the rats were first treated with another monoamine oxidase inhibitor before phenelzine, the initial drug [pargyline (MO-911), 20 mg/kg; tranylcypromine (SKF-385), 5 mg/kg; or pheniprazine, 10 mg/kg] was administered 17 to 19 hr before phenelzine. This procedure produced a prolonged inhibition of liver monoamine oxidase but permitted sufficient time to elapse so that any free residual inhibitor would no longer be present in the supernatant fraction of the homogenate. This was indicated by the absence of anti-monoamine oxidase activity in the supernatant fluids upon their assay on the mitochondrial monoamine oxidase system.

RESULTS

Analysis of the supernatant fluid of rat liver homogenates (10%) which had been previously incubated with phenelzine (5×10^{-5} M) exhibited a rapid loss of anti-monoamine oxidase activity compared with controls which were not exposed to the homogenate. Within 10 min, less than 20% of the original anti-monoamine oxidase activity was left, but beyond this period of incubation no further loss of anti-monoamine oxidase activity occurred. When lower concentrations of phenelzine were employed there was essentially complete loss of the anti-monoamine oxidase activity in the supernatant fluid. The ability

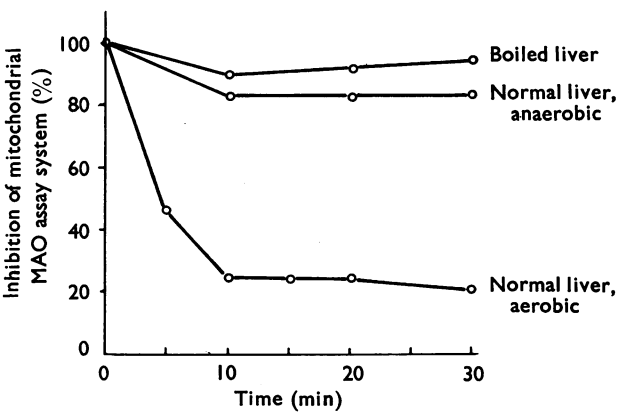


Fig. 1. The inhibition of phenelzine activity (initial concentration, 5×10^{-5} M) upon incubation with rat liver homogenate (10%). The inactivation process is abolished under anaerobic pure nitrogen conditions or when boiled tissue is used. Each point represents the average value of three determinations. MAO=monoamine oxidase.

of liver homogenate to inactivate phenelzine was abolished or attenuated in an anaerobic pure nitrogen environment or when the liver homogenate was boiled before incubation (Fig. 1). The effect of phenelzine concentration on its inactivation was also determined. In these experiments several concentrations of phenelzine were first incubated with liver homogenate for 30 min, after which the appropriately diluted supernatant fluid was assayed for anti-monoamine oxidase activity. The details of the procedure are described in Methods. Concentrations of phenelzine up to 5×10^{-5} M were effectively inactivated by the liver homogenate, but above this concentration phenelzine disappearance decreased, and at 10^{-3} M was completely absent (Fig. 2). Larger amounts of tissue homogenates were capable of inactivating higher concentrations of phenelzine, but a 10% homogenate was found to be most suitable for these studies. Excessive amounts of homogenates resulted in the degradation of phenelzine by other non-enzymic pathways as described earlier (Horita, 1963).

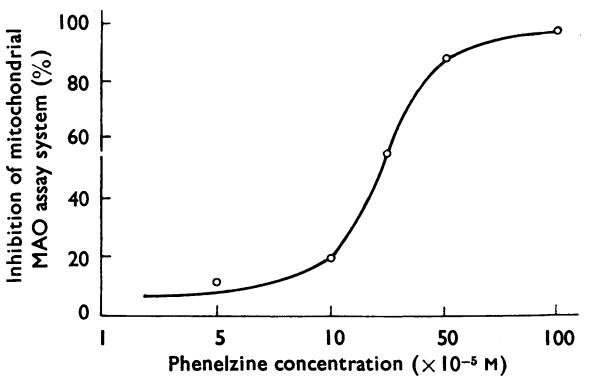


Fig. 2. The influence of concentration of phenelzine on its inactivation by rat liver homogenate. The concentrations ($\times 10^{-5}$ M) given are those in the mixture before incubation. For analysis of the residual anti-monoamine oxidase (MAO) activity on the mitochondrial preparation, all supernatant fluids were diluted to represent a final theoretical concentration of 2.5×10^{-5} M (see Methods).

The property of phenelzine inactivation by liver homogenates sufficiently resembled the enzymatic deamination of various amines by monoamine oxidase to warrant an investigation on the possible role of phenelzine as a substrate of this enzyme. For this purpose several other inhibitors of monoamine oxidase were employed. As shown in Table 1, liver homo-

TABLE 1

INHIBITION OF PHENELZINE INACTIVATION BY LIVER HOMOGENATES PREPARED FROM RATS TREATED WITH MONOAMINE OXIDASE INHIBITORS

10% liver homogenates were prepared from rats treated with the designated drugs 16 to 18 hr before killing (except for SKF-525A, where the interval was 30 min). 1 ml. of the homogenates was incubated with phenelzine (5×10^{-5} M) for 30 min at 37° C. The mixtures were then centrifuged as described in Methods, and aliquots of the supernatant fluids were assayed for anti-monoamine oxidase activity on the mitochondrial monoamine oxidase assay system. Each value is expressed as percentage inhibition and represents a single experiment

Treatment	Dose (mg/kg)	Anti-monoamine oxidase activity remaining in supernatant fluid
No drug	—	18, 26, 15, 15, 11
Pargyline	20	70, 92, 90, 65
Tranylcypromine	5	70, 90, 96, 79
Pheniprazine	10	72, 71, 65
SKF-525A	50	15, 9

genates prepared from rats previously treated with pargyline, tranylcypromine or pheniprazine 16 to 18 hr before killing possessed a much lower capacity to inactivate phenelzine. It was necessary to treat the animals for this long period, especially with pargyline and tranylcypromine, in order to assure complete removal of excess free inhibitor from the tissues. However, it should be emphasized that these doses of inhibitors were sufficient to produce and maintain complete monoamine oxidase inhibition in the animals. After 30 min of incubation with 5×10^{-5} M-phenelzine, control homogenates demonstrated less than 20% residual anti-monoamine oxidase action, while homogenates prepared from inhibitor-treated rats contained sufficient phenelzine to exert a 70 to 90% inhibition of the mitochondrial assay system. Compound SKF 525A (the 2-diethylaminoethyl ester of 2,2-diphenylvaleric acid), a potent inhibitor of the microsomal drug metabolizing enzyme, administered 30 min before killing the rat, proved to have no effect on the ability of liver homogenates to act on phenelzine.

The disposition of phenelzine in the intact rat liver was also examined. Doses of 20 mg/kg were administered intraperitoneally to rats, and at various intervals their livers were removed, homogenized and centrifuged. The supernatant fluid was then assayed for anti-monoamine oxidase activity. Residual phenelzine activity disappeared rapidly, for within 15 min after administration the supernatant fluid contained sufficient concentrations to inhibit only 10 to 15% of the assay system (Fig. 3). It was only at the first 5 to 10 min after

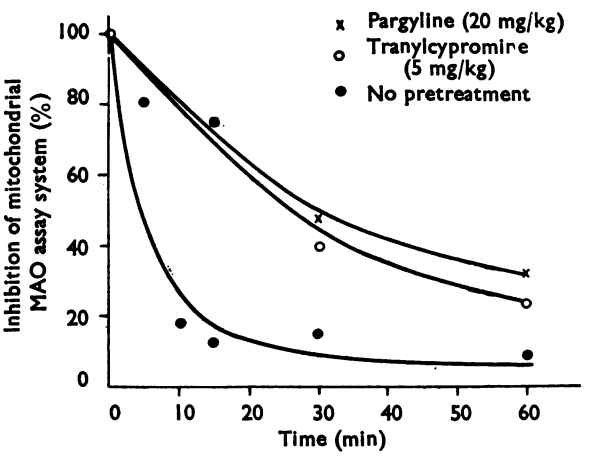


Fig. 3. The disappearance of anti-monoamine oxidase (MAO) activity from livers of rats given 20 mg/kg of phenelzine under several conditions. In those animals first treated (17 to 19 hr beforehand) with other monoamine oxidase inhibitors, the disappearance of phenelzine activity was markedly slowed. Each point represents the average value from three animals.

phenelzine administration that significantly higher activities (over 50% inhibition) were found. If the animals were treated with other irreversible monoamine oxidase inhibitors some 17 to 19 hr before administration of phenelzine, then the anti-monoamine oxidase activity curve of their liver supernatant fluid decreased at a much slower rate. For example, 15 min after phenelzine administration in rats treated with pargyline or tranylcypromine there remained over 70% of anti-monoamine oxidase activity, which was far more than was seen in the control animals (15%). Even 60 min after phenelzine the liver supernatant fluid

contained enough activity to inhibit the assay preparation by 30%. Again, it should be mentioned that the above treatment of rats with the irreversible inhibitors resulted in the prolonged inhibition of monoamine oxidase when tissues were measured by the usual *in vitro* techniques. The *in vivo* disappearance of phenelzine activity in inhibitor-treated rats, therefore, presumably involves other mechanisms (enzymic and/or nonenzymic) of inactivation or excretion.

A similar picture was seen when phenelzine was administered twice within a period of 2 hr. The first injection resulted in the appearance and rapid disappearance of anti-monoamine oxidase activity in the liver supernatant fraction. After the first 30 min only negligible quantities of phenelzine activity were present. Upon administering the second dose of 20 mg/kg, the same rapid peak of anti-monoamine oxidase activity occurred, but the rate of decline was greatly attenuated. For instance, at the 15- and 30-min periods after the first injection of phenelzine, the anti-monoamine oxidase activity of the liver supernatant fluid was 15 and 16%, respectively, while the corresponding periods after the second dose amounted to 65 and 42%, respectively (Fig. 4).

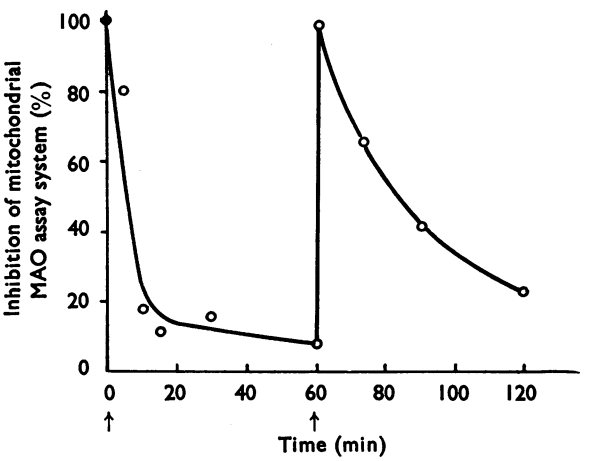


Fig. 4. The alteration in the rate of disappearance of phenelzine activity from livers of rats given two doses of 20 mg/kg of phenelzine within a 1-hr period (at arrows). The second injection resulted in a much slower rate of disappearance of anti-monoamine oxidase (MAO) activity as measured on the mitochondrial assay system. Each point represents the average value from three animals.

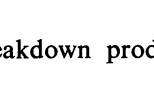
Homogenates of several other tissues were also examined for their ability to inactivate phenelzine. Rat heart, brain and kidney homogenates also possessed this property but were not as active as liver. Guinea-pig liver homogenate also was extremely active, showing even greater phenelzine inactivating powers than did corresponding amounts of rat liver.

DISCUSSION

The earlier finding that the hydrazine compound, pheniprazine, interacted nonenzymically with brain tissue to result in the loss of anti-monoamine oxidase activity of the compound suggested to us that other related agents might also possess this property. Unlike pheniprazine, however, phenelzine displayed a greater reactivity with liver than with brain homogenates, and this interaction appeared to be enzymic in nature. These findings prompted

us to investigate the possible mode by which phenelzine was being inactivated by liver homogenates, and to determine whether a similar pathway might be involved *in vivo*.

The results of this study strongly suggest that monoamine oxidase or a similar system is involved in the initial inactivation of phenelzine both *in vitro* and in the intact animal. This is a rather paradoxical finding, for phenelzine is well known as a potent irreversible inhibitor of the enzyme. Yet, from the structural point of view, it may be a substrate of such a system, for it does fulfil the qualifications as described by Blaschko (1952). Phenelzine possesses a mono-substituted ethylamine group, and the ethylene side chain is unsubstituted. Just as β-phenyl*iso*propylamine (amphetamine) does not serve as a substrate for monoamine oxidase, the α-methylated derivative of phenelzine (pheniprazine) also is not inactivated before exerting its inhibitory action. Although not discussed in the results, we have found that the compound γ-phenylpropylhydrazine may also resemble phenelzine in exhibiting the substrate property before inhibiting the enzyme, further supporting the structure-activity relationship of the substrate property of the arylalkylhydrazines.

The nature of the action of this enzyme system on phenelzine is at present unknown. Whether phenelzine inactivation results in the formation of phenylacetic acid and hydrazine or phenethylamine and ammonia has not yet been determined. Monoamine oxidase is known primarily as a deaminating enzyme, but with *N*-substituted compounds, such as *N*-methylphenethylamine, methylamine is the breakdown product. The  type linkage appears necessary for monoamine oxidase activity. If, in fact, monoamine oxidase were the enzyme involved, this would suggest that, in this case of phenelzine inactivation, the pathway involves a dehydrazination rather than a deamination of the molecule. The findings of Leverett, Leeson & Dubnick (1960) that the injection of [^{14}C]-phenelzine to mice resulted in the urinary excretion of [^{14}C]-phenylaceturic acid also supports this assumption. If such were the case, this would appear to be the first description of a dehydrazination mechanism for monoamine oxidase.

With increasing concentrations, phenelzine inactivation by liver homogenate decreased, and at 10^{-3} M essentially no disappearance of anti-monoamine oxidase activity occurred. The nature of this phenomenon was not investigated in this study, but the recent findings of Smith, Weissbach & Udenfriend (1963) and of Kory & Mingioli (1964) may be of importance in its explanation. These authors demonstrated that the anti-monoamine oxidase activity of the hydrazine compounds was exerted only after their conversion to an active volatile compound which is presumably not a hydrazine (Kory & Mingioli, 1964). It is therefore conceivable that the inhibition of monoamine oxidase by phenelzine also occurs by transformation to another substance. The inactivation of phenelzine by the monoamine oxidase or similar system, however, most likely occurs on the intact molecule. When higher concentrations of phenelzine are used more of the inhibitory substance will be formed, thus rapidly inhibiting the degradation of the phenelzine molecule. Although such a hypothesis is purely speculative, it is one possible explanation for the observed results.

The physiological significance of this pathway of inactivation of phenelzine *in vivo* is questionable, since it occurs only while the enzyme is active. However, it is possible that when small doses are administered the enzyme may inactivate a sufficient amount of the phenelzine to limit its pharmacological and biochemical actions. With repeated administra-

tions, greater enzyme inhibition would result and greater pharmacological activity from the subsequent doses may be expected. The progressive increase in the vasopressor activity of repeated injections of phenelzine in the anaesthetized dog (Chessin, Dubnick, Leeson & Scott, 1959) may be a consequence of such a mechanism. In any case the role of a monoamine oxidase-like enzyme in inactivating phenelzine is at most limited, and other pathways of elimination must be present. The nonenzymic inactivation process described for pheniprazine also appears to act on phenelzine, for when higher concentrations of tissue are employed, even in the presence of other monoamine oxidase inhibitors, phenelzine activity was seen to disappear from the supernatant fractions of brain and liver homogenates. Also, in the intact animal, even after inhibition of liver monoamine oxidase by other inhibitors, phenelzine activity disappeared from liver supernatant fluid within several hours, although this proceeded at a much slower rate than from control animals. These results support the belief that, while monoamine oxidase or a related enzyme is influential in initially inactivating phenelzine, other mechanisms of greater activity are also involved in its eventual detoxication.

The present study, therefore, has presented evidence for the participation of such a system in the initial inactivation of phenelzine *in vitro* and *in vivo*. Probably the strongest evidence for the hypothesis that monoamine oxidase may be the enzyme has been the fact that phenelzine inactivation was inhibited by the monoamine oxidase inhibitors. All of the monoamine oxidase inhibitors used demonstrated the ability to block this process. This finding with the non-hydrazine inhibitors is especially important, since they have little resemblance in chemical or biological activity to the hydrazine compounds. As far as is known no other enzyme system is inhibited in common by pargyline, tranylcypromine and pheniprazine in the doses used. We have also compiled certain other information which further adds to the above hypothesis. These include the findings that the inactivating system is insensitive to cyanide (as is monoamine oxidase), is found in the mitochondrial fraction of liver tissue, and is most active in tissues where monoamine oxidase activity is highest. A final test which strengthens the monoamine oxidase hypothesis is the fact that liver homogenates prepared from rats which had been treated with harmaline and phenelzine 16 to 18 hr before killing showed the ability to inactivate phenelzine. Since harmaline protects against the long-acting inhibitors (Horita & McGrath, 1960b), this may be expected if in fact monoamine oxidase is involved in the inactivation of phenelzine.

The final proof of this hypothesis will be found only when purer enzyme preparations can be used. Present plans in this laboratory include such an approach, and it is hoped that more information concerning the nature of monoamine oxidase action can be gained from such investigations.

SUMMARY

1. Supernatant fluids of rat liver homogenates which had been incubated with phenelzine showed a rapid loss of anti-monoamine oxidase activity. This inactivation was abolished by boiling or by incubation under nitrogen, indicating that it involved an enzymatic mechanism.

2. Liver homogenates from rats treated 16 to 18 hr previously with other irreversible monoamine oxidase inhibitors also lowered the usual rate of phenelzine inactivation. It is postulated that phenelzine acts as a substrate before it inhibits monoamine oxidase or a similar enzyme.

3. Intraperitoneal injection of phenelzine into intact rats led to anti-monoamine oxidase activity in the liver supernatant fluid. This activity fell rapidly over 15 to 30 min. Treatment with other irreversible inhibitors of monoamine oxidase 17 to 19 hr before phenelzine greatly decreased the rate of disappearance of phenelzine activity in the liver supernatant fluid.

4. It is concluded that monoamine oxidase or a similar enzyme is involved in the initial inactivation of phenelzine, probably by dehydrazination. Inhibition of the enzyme prevents this process, and greatly decreases the rate of inactivation of phenelzine both *in vitro* and *in vivo*.

The author wishes to thank Mrs M. Van Sloten for her competent technical assistance. Phenelzine sulphate (Nardil) was provided by the Warner-Lambert Research Institute, Morris Plains, N.J., U.S.A., research affiliate of Warner-Chilcott Laboratories. This investigation was supported in part by a P.H.S. research grant (MH-02435-05,06) from the National Institute of Mental Health, Public Health Service, U.S.A.

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