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# ANTAGONISM BETWEEN STEREOISOMERIC AMPHETAMINES, AMPHETAMINE DERIVATIVES AND OTHER MONOAMINE OXIDASE INHIBITORS*

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**Abstract**—An antagonism between stereoisomeric amphetamines, amphetamine derivatives and other monoamine oxidase inhibitors has been investigated. The monoamine oxidase activity of isolated rat liver mitochondria was measured manometrically and the values checked by spectrophotofluorometric estimation of tyramine substrate to show that the oxygen uptake reflects the true enzyme activity under present experimental conditions. It was also found that concentrations of amphetamine and an amphetamine derivative P-1882 (*p*-S-methylamphetamine), without effecting the enzyme activity, protected monoamine oxidase against inhibition by iproniazid, pheniprazine tranylcypromine, nialamide and P-1726 (*p*-trifluoromethylamphetamine). Another amphetamine derivative P-1726 was found to be a more potent and more persistent inhibitor of monoamine oxidase than either amphetamine or P-1882. On the other hand such low concentrations of P-1726 had no protective effect on the inactivation caused by other inhibitors. Further, with respect to both direct inhibition and protection against other inhibitors, D-amphetamine was more active than the corresponding L-isomer.

THE ENZYME monoamine oxidase (MAO) oxidatively deaminates many compounds having a terminal amino group, including 5-hydroxytryptamine (serotonin), and epinephrine and norepinephrine. It frequently has been suggested to be of biological importance in regulating the action of such substances[1, 2] but such a function has not yet been clearly demonstrated in any instance. The role of epinephrine and norepinephrine as a neurohumoral transmitting agent in the autonomic nervous system[3–5] has stimulated studies to determine the active centers of this particular enzyme and to gain further insight into the mode of action of the MAO inhibitors commonly known as psychotropic drugs. Studies of the nature of their enzyme inhibition have shown that *in vitro* MAO inhibitors like iproniazid and pheniprazine react relatively irreversibly with the active sites on the MAO to produce a "non-equilibrium" antagonism.[6] Amphetamine on the other hand showed reversible inhibition. Pratesi and Blaschko[7] have shown that the amine oxidase of guinea pig liver was more strongly inhibited by the dextrorotatory isomer of amphetamine than by the laevorotatory isomer. Recently Blaschko and Strömblad[8] have reported that the pharmacologically more active D-amphetamine is a stronger inhibitor of MAO from human brain, from human parotid and submaxillary glands. In order to gain further

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information on the nature of this inhibition and the reactive surface of MAO, the interaction between the long-acting inhibitors and the stereoisomeric amphetamines (short-acting inhibitors) and two amphetamine derivatives, P-1726 (*p*-trifluoromethylamphetamine) and P-1882 (*p*-S-methylamphetamine) was investigated, on the MAO activity of isolated rat liver mitochondria. It was found that concentrations of amphetamine and P-1882 having no effect on the MAO activity protected this enzyme against inhibition by other long-acting inhibitors. With respect to both direct inhibition and protection against the other inhibitors, D-amphetamine was more active than the corresponding L-isomer.

## MATERIALS AND METHODS

Adult rats weighing 200–250 g were killed by a blow on the head and decapitated. The livers were homogenized in ice-cold 0·25 M sucrose to give a final homogenate concentration of 10% (w/v). The mitochondria were isolated by the differential centrifugation method of Hogeboom *et al.*,[9] as modified by Hawkins.[10] These particles were washed three times with 0·25 M sucrose and suspended in the same concentration of sucrose so that 15 ml of the suspension was equivalent to 10 g of fresh liver.

### *Measurement of* MAO *Activity*

Standard Warburg manometric procedure was used to measure the rate of oxygen uptake as an assay of MAO activity.[11] Rat liver mitochondria in 0·5 ml sucrose suspension were incubated at 37° in phosphate buffer, 0·05 M, pH 7·4 in a total volume of 3 ml using tyramine, 0·01 M, L-norepinephrine, 0·01 M or 5-hydroxytryptamine 0·01 M as substrates, the concentrations all expressed as the final concentration in the flask. When used, amphetamine and amphetamine derivatives were added to the main vessel with the enzyme, whereas other inhibitors were added from a side arm 10–20 min prior to the addition of substrate.

In order to determine if the $O_2$ consumption of our experiments accurately reflected the activity of MAO, the oxidative deamination of tyramine was also assayed by measuring the disappearance of tyramine during the total experimental period. After the experiment, 5 ml of 10% trichloroacetic acid was added to each vessel and the precipitated proteins were removed by centrifugation. Suitable aliquots of the supernatant fluid were assayed for tyramine fluorometrically in an Aminco Bowman Spectrophotofluorometer using activating light of 275 m$\mu$ and measuring fluorescence at the maximum of 310 m$\mu$. Recoveries of tyramine added to mitochondrial preparations without incubation were quantitative.

In the dialysis experiments 4·0 ml of the mitochondrial suspensions, in a total volume of 8 ml, were incubated in test tubes at 37°. Amphetamine and its derivatives were incubated with the enzyme preparation for 15 min prior to the addition of other MAO inhibitors. After a total incubation period of 45 min, 3 ml aliquots from the total 8 ml were dialyzed against distilled water for varying lengths of time. After dialysis, the dialysed preparations were made up to a total volume of 8 ml and 2 ml aliquots were tested for their MAO activity.

## RESULTS

The results of the study of the inhibition of MAO by two enantiomorphs of amphetamine show that $3 \times 10^{-3}$ M D-amphetamine inhibited oxygen uptake by

50 per cent (45·8; 53·2; 51·6) as compared to 37 per cent (37·8; 38·7; 34·8) by the same concentration of L-amphetamine. Direct estimation of tyramine utilization gave values of 50 per cent (48·4; 51·8; 48·2) and 36 per cent (35·5; 38·0; 32·3) inhibition in these experiments. Same relation of D- and L-amphetamine inhibition was observed when the MAO activity was measured during oxidative deamination of L-norepinephrine and serotonin. With both isomers the degree of inhibition increased with the concentration of the inhibitor. Inhibition by amphetamines was unaffected by preincubation of the enzyme before the addition of substrate indicating that the amphetamines are in rapid equilibrium with the substrate and that the enzyme-inhibitor complex is readily dissociated.[6]

The nature of the inhibition by the two stereoisomers of amphetamine was evaluated by means of the conventional graphic method of Lineweaver and Burk.[12] Both isomers were found to be competitive inhibitors of MAO. The $K_1$ values (dissociation constants) were determined graphically[13] and were found to be $8{\cdot}5 \times 10^{-4}$ M for D-amphetamine and $1{\cdot}4 \times 10^{-3}$ M for L-amphetamine.

The inhibition of MAO by pheniprazine and the protection against hydrazine-induced inhibition by D-amphetamine are shown in Table 1. Pheniprazine (β-phenyl-

TABLE 1. PROTECTION OF PHENIPRAZINE-INDUCED INHIBITION OF MAO BY D-AMPHETAMINE

| Additions | Enzyme activity/60 min/500 mg | | | |
|---|---|---|---|---|
| | Oxygen uptake (μmoles) | % Inhibition | Tyramine utilized (μmoles) | % Inhibition |
| Control | 17·72 ±0·43 | — | 17·99 ±0·11 | — |
| D-amphetamine ($3 \times 10^{-5}$ M) | 17·79 ±0·48 | — | 18·03 ±0·25 | — |
| Pheniprazine ($1 \times 10^{-6}$ M) | 6·61 ±0·18 | 62·72 ±0·42 | 6·49 ±0·32 | 63·95 ±1·46 |
| D-amphetamine ($3 \times 10^{-5}$ M) + Pheniprazine ($1 \times 10^{-6}$ M) | 9·92 ±0·11 | 44·16 ±1·30 | 10·19 ±0·17 | 43·44 ±1·06 |

All values are mean of six experiments. Assay procedure and vessel contents are described in the text. Inhibition with S.E. was calculated on the basis of decrease in oxygen uptake and tyramine utilization. Figures in parentheses represent final concentration of the inhibitors. D-Amphetamine was initially present in the vessel with the enzyme preparation. Pheniprazine was added 15 min prior to the addition of tyramine ($1 \times 10^{-2}$ M).

isopropylhydrazine) possesses marked anti-MAO activity.[14] D-Amphetamine at a concentration of $3 \times 10^{-5}$ M was found to have no effect on MAO activity in the presence of $10^{-2}$ M tyramine as substrate. However, this concentration reduced the inhibition produced by pheniprazine when the latter was incubated with the enzyme for 15 min before adding tyramine. The pheniprazine inhibition was reduced comparably when amphetamine was added before or with, but not when added after the hydrazine. The degree of protection was unaltered when the amphetamine and pheniprazine were incubated together prior to their addition to the reaction

vessel. It can also be seen that equivalent amounts of tyramine were oxidized corresponding to oxygen uptake indicating the stoichiometric relationship between oxygen uptake and tyramine oxidation. Thus for every $\mu$mole of oxygen utilized, 1$\mu$ mole of tyramine was oxidized.

TABLE 2. STEREOSPECIFICITY OF AMPHETAMINE DURING PROTECTION AGAINST MAO INHIBITION IN ISOLATED RAT LIVER MITOCHONDRIA

| Inhibitors | % Inhibition | | |
|---|---|---|---|
| | Amphetamine absent | Amphetamine present ($3 \times 10^{-5}$ M) | |
| | | D-amphetamine | L-amphetamine |
| Iproniazid ($1 \times 10^{-5}$ M) | 51·08 ±1·08 | 31·60 ±0·59 | 40·5 ±0·49 |
| Pheniprazine ($2 \times 10^{-6}$ M) | 84·88 ±0·53 | 55·85 ±0·49 | 63·88 ±0·38 |
| Tranylcypromine ($6 \times 10^{-6}$ M) | 73·93 ±1·08 | 56·98 ±0·44 | 63·58 ±0·20 |
| Nialamide ($1 \times 10^{-4}$ M) | 62·0 ±0·53 | 50·58 ±0·76 | 60·30 ±0·25 |
| P-1726 ($6 \times 10^{-6}$ M) | 70·0 ±0·59 | 57·2 ±0·61 | 62·9 ±0·94 |

All values are mean of eight experiments. Figures in parentheses represent final concentration of the inhibitors. Details of the assay procedure and vessel contents are described in the text. Inhibition with S.E. was calculated on the basis of decrease in oxygen uptake during 45 min from rat liver mitochondria derived from 333 mg wet tissue. Amphetamine where added was initially present in the main vessel together with mitochondrial preparation. Iproniazid and nialamide were added 20 min and pheniprazine, tranylcypromine and P-1726 were added 15 min before adding tyramine ($1 \times 10^{-3}$ M).

The results shown in Table 2 show that D-amphetamine affords greater protection than the corresponding L-isomer against MAO inhibition by other well-known inhibitors i.e. iproniazid,[15] pheniprazine,[14] tranylcypromine,[16] nialamide[17] and P-1726.[17] Inhibition by all of these agents increased by preincubation of inhibitor and enzyme prior to the addition of substrate. The evaluation of the nature of the inhibition produced by these inhibitors in our enzyme system will be discussed in detail elsewhere.[17] Both isomers of amphetamine at a concentration of $3 \times 10^{-5}$ M were found to have no inhibitory effect on the MAO activity in rat liver mitochondria during oxidative deamination of tyramine. However, the degree of inhibition by the other agents was reduced when the enzyme preparation was preincubated with either D- or L-amphetamine. The protection afforded by D-amphetamine was always greater than that due to L-amphetamine.

In order to ascertain the greater protection by D-amphetamine than the corresponding L-isomer dialysis experiments were carried out using high concentrations of D- or L-amphetamine ($3 \times 10^{-3}$ M). The enzyme preparations were incubated with either amphetamines for 15 min after which other inhibitors were added for a total incubation period of 45 min. The suitable aliquots of these preparations were dialyzed against distilled water and their activity was measured accordingly as described earlier in the methods. The results of these experiments are shown in Table 3. We have consistently found a decrease in the enzyme activity on dialysis which was not observed when the dialysis was performed after incubation of the enzyme preparation with either of the

isomers of amphetamine. At present no attempts were made to explain this decrease in the enzyme activity. However, complete restoration of the activity after amphetamine inhibition was achieved on dialysis. The protection against the inhibition produced by other MAO inhibitors was found to be greater for D-amphetamine than for the L-isomer. These dialysis experiments were also able to show clearly the ability of

TABLE 3. DIALYSIS EXPERIMENTS SHOWING GREATER PROTECTION OF MAO INHIBITION BY D-AMPHETAMINE

| Additions | Enzyme activity $\mu$mole $O_2$/45 min/500 mg | | |
|---|---|---|---|
| | Amphetamine absent | Amphetamine present | |
| | | D-Amphetamine | L-Amphetamine |
| Control | 12·91 ± 0·59 | 14·69 ± 1·03 | 14·44 ± 1·01 |
| Iproniazid ($3 \times 10^{-5}$ M) | 4·64 ± 0·11 | 8·60 ± 0·15 | 6·44 ± 0·19 |
| Pheniprazine ($3 \times 10^{-6}$ M) | 1·26 ± 0·05 | 8·38 ± 0·25 | 6·73 ± 0·42 |
| Tranylcypromine ($3 \times 10^{-6}$ M) | 2·69 ± 0·18 | 7·13 ± 0·27 | 4·20 ± 0·07 |
| Nialamide ($3 \times 10^{-5}$ M) | 4·95 ± 0·26 | 9·11 ± 0·05 | 7·05 ± 0·07 |

All values are mean of eight experiments. Figures in the parentheses represent final concentration of the inhibitors. Details of the assay procedure and vessel contents are described in the text. Mitochondrial suspensions (4 ml) in a total volume of 8 ml were incubated with D- and L-amphetamine at a final concentration of $3 \times 10^{-3}$ M for 15 min prior to the addition of other MAO inhibitors. After a total incubation period of 45 min 3 ml aliquots were dialysed against 1 l of distilled water for 16–24 hr. Dialysed preparations were made up to the total volume of 8 ml and 2 ml aliquots were tested for enzyme activity, and values of oxygen uptake and S.E. are recorded using tyramine ($1 \times 10^{-2}$ M) as the substrate.

L-amphetamine to afford such protection against nialamide inhibition which was not clearly marked in other studies (Table 2). Identical values were obtained when the enzyme activity was measured by estimating the amounts of tyramine utilized under these experimental conditions.

The protection of MAO inhibition by iproniazid and nialamide by both isomers of amphetamine was also observed during oxidative deamination of serotonin under similar experimental conditions. In similar dialysis experiments D-amphetamine was able to afford greater protection than the corresponding L-amphetamine as was observed in experiments where tyramine was used as the substrate. The enzyme activity with serotonin was approximately 2/3 as compared with that of tyramine.

The ability of amphetamine to afford protection of MAO inhibition was found not to be dependent on its reversible nature of inactivation. This is explained on the basis of the studies dealing with the interaction of pheniprazine, a potent irreversible inhibitor with other moderately reversible inhibitors including iproniazid and nialamide. The results of these experiments as shown in Table 4 indicate that in dialysis experiments, unlike amphetamine, the inhibition due to pheniprazine could not be protected by either of these agents. Addition of these agents either together or before pheniprazine did not alter the degree of inhibition as indicated by a decrease in oxygen uptake and tyramine utilization.

The interactions between two amphetamine derivatives P-1882 and P-1726 and other long acting MAO inhibitors are shown in Table 5. It was found that concentrations of P-1882 having no effect on the enzyme activity were able to antagonize the inhibitions

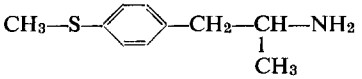
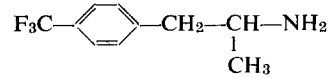
caused by pheniprazine, iproniazid and tranylcypromine. This protection, like the one caused by amphetamine, was only observed when P-1882 was incubated with the enzyme preparation prior to the addition of other inhibitor or when both these inhibitors were added at the same time. P-1726 was found to be more potent and considerably more persistent inhibitor of MAO than either amphetamine or P-1882.[17]

TABLE 4. DIALYSIS EXPERIMENTS SHOWING INTERACTION OF PHENIPRAZINE WITH IPRONIAZID AND NIALAMIDE

| Additions | Enzyme activity $\mu$mole $O_2$/60 min/500 mg | |
|---|---|---|
| | Pheniprazine absent | Pheniprazine present ($2 \times 10^{-6}$ M) |
| Control | 18·54 ± 0·41 | 2·04 ± 0·08 |
| Iproniazid ($3 \times 10^{-6}$ M) | 12·62 ± 0·17 | 1·94 ± 0·11 |
| Nialamide ($3 \times 10^{-6}$ M) | 12·37 ± 0·24 | 1·95 ± 0·19 |

All values are mean of eight experiments. Figures in the parentheses represent final concentration of the inhibitors. Details of the assay procedure and vessel contents are described in the text. Dialysis experiments were carried out as indicated in Table 3 where enzyme preparations were incubated with either iproniazid or nialamide prior to the addition of pheniprazine. Oxygen uptake during oxidation of tyramine ($1 \times 10^{-2}$ M) and S.E. are recorded.

TABLE 5. INTERACTION BETWEEN MAO INHIBITORS AND AMPHETAMINE DERIVATIVES

| Inhibitor | Inhibition (%) | | |
|---|---|---|---|
| | Control | P-1882 ($1 \times 10^{-5}$ M)  | P-1726 ($1 \times 10^{-6}$ M)  |
| Iproniazid ($3 \times 10^{-5}$ M) | 81·9 ±0·41 | 50·2 ±0·35 | 81·0 ±0·25 |
| Pheniprazine ($1 \times 10^{-6}$ M) | 44·8 ±0·53 | 30·0 ±0·41 | 45·8 ±0·32 |
| Pheniprazine ($3 \times 10^{-6}$ M) | 90·8 ±0·31 | 60·3 ±0·61 | 92·3 ±0·51 |
| Tranylcypromine ($1 \times 10^{-5}$ M) | 94·8 ±0·28 | 79·8 ±0·77 | 95·6 ±0·31 |

All values are mean of eight experiments. Figures in the parentheses represent final concentration of the inhibitors. Details of the assay procedure and vessel contents are described in the text. Amphetamine derivatives where added were initially present in the main vessel together with mitochondrial preparation. Inhibition with S.E. was calculated on the basis of the decrease in oxygen uptake during 45 min from rat liver mitochondria derived from 333 mg wet tissue. Pheniprazine, iproniazid and tranylcypromine were added 10 min prior to the addition of tyramine ($1 \times {}^{-2}$ M).

However, it was observed that the inhibition caused by P-1726 was antagonized by amphetamine (Table 2) where D-amphetamine showed greater protection than the corresponding L-isomer. Similar results were obtained when the dialysis experiments were carried out to show the effect of these amphetamine derivatives against the inhibition caused by pheniprazine. It was found that 80 per cent inhibition caused by

pheniprazine in these dialysis experiments was reduced to 50 per cent when the enzyme preparations were incubated with low concentrations of P-1882 ($1 \times 10^{-5}$ M) prior to the addition of pheniprazine. The other amphetamine derivative, P-1726 was unable to show any antagonism against pheniprazine under similar experimental conditions.

Horita and McGrath[18] have provided evidence for the protection of the enzyme MAO from the long acting inhibitory effects of pheniprazine by first treating the animal with harmine, a short acting reversible inhibitor. However, they were unable to demonstrate similar protection against tranylcypromine and proposed that tranylcypromine unlike pheniprazine acts by a different mechanism of action. Our present experiments *in vitro* consistently show protection against tranylcypromine by both isomers of amphetamine and amphetamine derivative (P-1882) as is observed with pheniprazine.

## DISCUSSION

Our present study deals with the interaction of various inhibitors on the MAO activity of isolated rat liver mitochondria. MAO activity as determined by oxygen uptake was found to reveal the true activity of the enzyme since for the oxidation of 1 $\mu$mole of tyramine equivalent amount of oxygen was consumed. It was therefore considered that cyanide and semicarbazide used during the assay of MAO [11]was not necessary. The striking antagonism between amphetamine or amphetamine derivative (P-1882) and other MAO inhibitors points to the possible existence of a close similarity of their site of action on enzyme molecule. Thus, the effect of amphetamine may indeed involve similar active centers or "receptor sites" that are particularly sensitive to the action of other MAO inhibitors. Our present results are in good agreement with those of Pletscher and Besendorf[19] where similar antagonism between harmaline, a reversible inhibitor, and iproniazid has been demonstrated. They found that treatment with harmaline 1 hr prior to the administration of iproniazid antagonized iproniazid induced increase in 5-hydroxytryptamine and norepinephrine contents in the brains of mice and rats. This antagonism, however, could not be observed when harmaline was administered 6–8 hr after treatment with iproniazid. The ability of amphetamine and P-1882 to protect MAO inhibition in our experiments *in vitro* was similarly lost when the enzyme preparations were incubated with other long acting inhibitors prior to the addition of amphetamine or P-1882. This can be explained by the fact that either these MAO inhibitors cause irreversible damage to the enzyme or that the hydrazine enzyme bond is very stable. On the basis of these observations it can be postulated that amphetamine blocks the active centers or "receptors" on the enzyme molecule reversibly and during this period they cannot be attacked by other agents any more. Our findings with regards to the ability of D-amphetamine to afford greater protection of MAO than the corresponding L-isomer is of interest because of what is known of their effects on the central nervous system of man and animals. A relationship between central stimulant action of amphetamine and inhibition of amine oxidase has been proposed by Mann and Quastel.[20] It has also been reported that the central stimulant activity of D-amphetamine was four times as active on the weight basis as the L-isomer.[21] Alles[22] has previously shown by subjective observations in man that D-amphetamine was four times as active as L-amphetamine. Furthermore, the

enzyme MAO was found to show no distinction between the geometrical isomers (*cis*- and *trans*-) of phenylcyclo-propylamine[23] since both these isomers show equal degree of enzyme inhibition. In contrast with these results Belleau *et al.*[24] have reported that MAO involved in adrenergic mechanism displays an absolute stereospecificity which is identical with liver MAO on account of the fact that a marked increase in the potency of sympathomimetic amines was produced by stereospecific deuterium substitution. These workers therefore postulated the involvement of such isotopic effects with MAO. Belleau *et al.*[25] have also observed a configuration dependent deuterium isotope effect in the enzymic oxidation of asymmetrically labeled tyramine. Thus MAO may be similar to alcohol dehydrogenase in exhibiting a degree of optical specificity.[26,27] Pletscher and Gey have similarly reported the stereospecificity of MAO inhibitors and suggested an optically active center for this enzyme.[28] Pratesi and Blaschko[7] also suggested that the presence of a center of asymmetry may confer different affinities (rate of oxidation or inhibition) on two enantiomorphs. In the present study the ability of D-amphetamine to cause greater protection than the corresponding L-isomer, against the inhibition caused by other long acting inhibitors, give further evidence regarding the presence of an optically active center on the enzyme and its stereospecificity. It could therefore be assumed that certain stereoisomeric factors are involved during protection by two enantiomorphs of amphetamine. Experiments with amphetamine derivatives, P-1882 and P-1726 have demonstrated the ability of P-1882 alone to afford protection similar to one produced by amphetamine. Although these two derivatives only differ in their para substitution in the phenyl nucleus of amphetamine (Table 5), P-1882 behaves entirely like the parent substance (amphetamine) being a rapidly reversible inhibitor. P-1726 on the other hand is a more potent inhibitor and is considerably more persistent due to its relatively irreversible nature.[6] These results point to the existence of primary and secondary centers on the enzyme molecule which are essentially involved in the formation of an enzyme-substrate or enzyme-inhibitor complex. The attachment of an inhibitor to the secondary centers in addition to the essential primary one through the amine moiety may lead to its greater inhibitory effects together with irreversibility in its nature of inactivation. In addition the fact that the percent inhibition was reduced while the enzyme was being incubated with amphetamine or P-1882 together with other long acting inhibitors, suggests that the former produce less complete inhibition of the primary and secondary enzyme sites. These observations could be explained by assuming that two or more points of attachments of the inhibitor to the enzyme are required for full inactivation of the active sites. However, further experimental evidence to add more light to this problem will be required before the validity of this statement can be fully established.

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