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INHIBITION OF PURIFIED RAT LIVER MITOCHONDRIAL MONOAMINE OXIDASE BY *TRANS*-2-PHENYL-CYCLOPROPYLAMINE

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Abstract—The inhibition of purified rat liver mitochondrial monoamineoxidase by *trans*phenylcyclopropylamine was studied. Both the enzyme activity and its inhibition by tranylcypromine were optimal at pH 7·0 and I_{50} value was found to be 2×10^{-7} M. A short period of incubation of the enzyme with the drug caused appreciable inhibition even at very low inhibitor concentration. When the substrate and inhibitor were added simultaneously higher concentrations of inhibitor were required for producing marked inhibition. The inhibition was both competitive and irreversible and no restoration of enzyme activity occurred even after prolonged dialysis. When both the substrate and inhibitor were added simultaneously the degree of inhibition was found to decrease progressively with increasing concentrations of substrate. No such protective action of the substrate could be observed when the enzyme was preincubated with the inhibitor before addition of the substrate. Deamination of various monoamines was not inhibited to the same extent by tranylcypromine. Ultraviolet absorption spectra of the purified enzyme at pH 7·0 showed a peak at 410 mμ which disappeared leaving only a shoulder at 415 mμ in the presence of tranylcypromine.

THE inhibition of monoamine oxidase (monoamine: O_2 oxidoreductase (deaminating), E.C. 1.4.3.4) by cis- and trans-2-phenylcyclopropylamine* has been studied both *in vivo* and *in vitro*.[1–5] Maass *et al.*[1] observed that the *in vitro* inhibition of MAO by tranylcypromine was non-competitive whereas Barbato and Abood,[6] using a partially purified MAO preparation and Zeller and co-workers[3, 4] employing intact and solubilized mitochondria as the enzyme source found the inhibition to be irreversible and competitive. When phenyl-4-butylamine, however, replaced tyramine as substrate the inhibition could be reversed.[4] This observation has not been confirmed by Green and Sawyer[7] who found on the contrary that the inhibition of MAO by tranylcypromine was irreversible on liver and brain MAO. Zeller and co-workers[3] also noted that prolonged dialysis of the enzyme preparation exposed to the drug resulted in the recovery of a small fraction of enzyme activity. Inhibition of MAO by tranylcypromine was found useful in differentiating MAO from diamine oxidase.[8, 9] Horita and McGrath[10] observed harmine to afford very little protection against *in vivo* inhibition of MAO by tranylcypromine.

The above studies indicate that tranylcypromine is one of the most potent non-hydrazine inhibitors of MAO. In view of the current availability of a method for the

* The following abbreviations are used: *trans*-PCP (tranylcypromine), *trans*-2-phenylcyclopropylamine; MAO, monoamine oxidase.

preparation of a highly purified MAO from rat liver mitochondria[11] the mechanism of MAO inhibition by tranylcypromine was reinvestigated and the relevant results are summarized in this communication.

EXPERIMENTAL PROCEDURE

Purification of MAO

Purification of MAO from rat liver mitochondria was achieved as described previously.[11] The active fractions obtained after rechromatography on DEAE cellulose were pooled and used. The average specific activity of the pooled samples was 200–250-fold higher than that of the original liver homogenate whereas the degree of purification of the peak fraction was about 350-fold. The pooled active fractions were concentrated either by lyophilisation or by osmosis.[12] A dialysis casing containing the active eluate was surrounded by dry powdered sucrose. Due to rapid diffusion of water into the outer space that occurred during osmosis a 3 to 4-fold concentration of the fraction was achieved within 1 hr. The dialysis tubing was then washed with water to remove adhering sucrose and the contents dialysed for 20 min against 0·01 M phosphate buffer pH 7·0 and the dialysate used as such in inhibition studies.

Assay of MAO activity

The standard incubation mixture consisted of 0·05 M phosphate buffer pH 7·0 and appropriate enzyme solution corresponding to ~100–200 μg protein and 0·01 M tyramine in a final volume of 2 ml. All concentrations given pertain to the complete reaction mixture. In general the inhibitor was incubated with the enzyme for 15 min at 38° before the substrate was added unless stated otherwise. Exposure to the inhibitor was followed by an incubation period of 30 min to complete the reaction. In all the experiments air was used as the gas phase. MAO activity was determined by measuring the ammonia formed by deamination of substrate and was estimated by direct nesslerization as reported previously.[11] Alternatively the enzyme activity was assayed by measuring the aldehyde formed according to Green *et al.*[13]

Estimation of aldehyde

The amount of aldehyde formed was determined from a standard curve prepared in the following way. Aliquots of freshly distilled benzaldehyde were added to the complete reaction mixture used for enzyme assay in the aldehyde method[13] from which both enzyme and substrate were omitted and incubated at 38° for 30 min in the presence of 0·0125 M semicarbazide. The semicarbazone so formed was then converted to the corresponding 2:4-dinitrophenyl-hydrazone. The remaining steps were the same as described by Green *et al.*[13] The colour was read at 450 mμ against a reagent blank and optical density readings (E_{450}) were plotted against concentrations of benzaldehyde. This curve was also used when amines other than benzylamine were used assuming that the variation in colour intensity produced by the various hydrazones was negligible. Both the optical density readings as well as the calculated aldehyde values are presented.

All values of ammonia and aldehyde are corrected against enzyme blanks. Protein concentrations were determined by the method of Warburg and Christian[14] using bovine plasma albumin as standard. Light absorption was measured in a Beckman spectrophotometer model DU using either corex glass or silica cuvettes of 1 cm light path.

RESULTS

Optimal pH for inhibition. Purified rat liver mitochondrial MAO was found to be inhibited by tranylcypromine optimally at pH 7·0 which was also the optimal pH for the enzyme activity (Fig. 1).

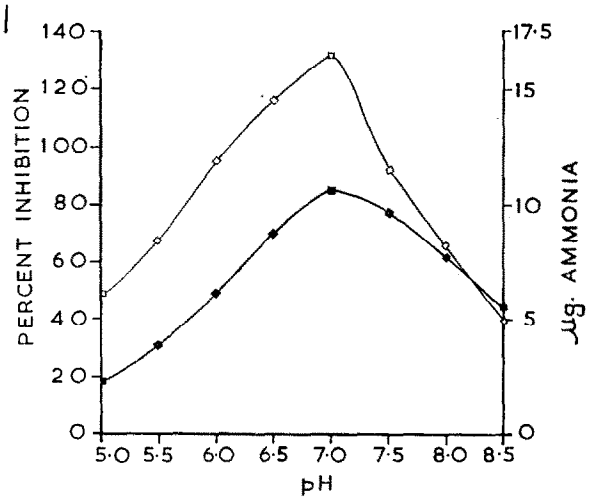

FIG. 1. Influence of pH on *trans*-PCP inhibition of purified MAO of rat liver mitochondria. The systems with or without *trans*-PCP (5×10^{-7} M) were first incubated for 15 min at 38° followed by the addition of tyramine (0·01 M) and activity determined after a further period of 30 min incubation.

□ — □ pH activity curve in absence of *trans*-PCP
■ — ■ per cent inhibition by *trans*-PCP

Effect of varied concentrations of tranylcypromine. Table 1 shows the inhibition of purified MAO by different concentrations of tranylcypromine. About 88 per cent inhibition of the enzyme at 10^{-6} M concentration of tranylcypromine was observed. The I_{50} value was found to be about 2×10^{-7} M.

TABLE 1. EFFECT OF DIFFERENT CONCENTRATIONS OF *TRANS*-PCP ON MAO INHIBITION

Systems	Ammonia formed (μg)	Percentage inhibition
Control	11·5	—
Control + *trans*-PCP (1×10^{-4} M)	0·4	97
Control + *trans*-PCP (1×10^{-5} M)	1·0	91
Control + *trans*-PCP (1×10^{-6} M)	1·4	88
Control + *trans*-PCP (5×10^{-7} M)	3·0	74
Control + *trans*-PCP (2×10^{-7} M)	5·2	55
Control + *trans*-PCP (1×10^{-7} M)	8·2	29
Control + *trans*-PCP (5×10^{-8} M)	9·6	17

The systems with or without *trans*-PCP were first incubated for 15 min at 38° followed by the addition of 0·01 M tyramine and incubated for a further period of 30 min. Other details are given in the text.

Effect of preincubation period on the degree of inhibition. At very low concentrations of the drug a minimum period of incubation with the enzyme was found necessary for the catalytic activity to be appreciably inhibited (Table 2). Thus 20–30 min of preincubation caused complete inhibition of MAO with 10^{-6}M tranylcypromine. However at higher concentrations of tranylcypromine (10^{-4} M), the enzyme was markedly inhibited even when the substrate and inhibitor were added simultaneously (not shown in the table). With 10^{-6} M *trans*-PCP, inhibition was negligible when both the substrate (0·01 M) and inhibitor were added simultaneously.

TABLE 2. EFFECT OF VARIATION OF TIME OF PREINCUBATION ON INHIBITION OF MAO BY *TRANS*-PCP

Time of preincubation (min)	Control systems μg Ammonia formed	Experimental systems with *trans*-PCP (1×10^{-6} M)	
		μg Ammonia formed	Percentage inhibition
0	11·0	10·8	2
5	11·0	7·5	32
10	12·0	5·8	52
15	12·0	3·6	70
20	12·0	0·5	96
30	11·5	0·0	100

After 10 min incubation at 38° of all the systems, *trans*-PCP was added to the appropriate tubes and, preincubated for a further period as indicated in the table. The control systems were also similarly preincubated. To each set of control and inhibitor-containing tube 0·01 M tyramine was added at the end of the preincubation period as specified in the table. After a further period of 30 min incubation ammonia was determined. Other details are given in the text.

Irreversible and competitive nature of inhibition of trans-*PCP.* The irreversibility of MAO inhibition by *trans*-PCP is illustrated in Table 3. No restoration of enzyme activity occurred even after dialysis for 4 or 24 hr. The inhibition was also competitive as shown in Fig. 2. Since the enzyme was inhibited by higher concentrations of substrate as well[15, 16] the data was subjected to graphical treatment according to Dixon[17] in order to elucidate the nature of inhibition.

TABLE 3. IRREVERSIBILITY OF MAO INHIBITION BY *TRANS*-PCP

Systems	Ammonia formed (μg)	After dialysis for 4 hr NH_3 formed (μg)	After dialysis for 24 hr NH_3 formed (μg)
Control	8·0	8·0	8·0
Control + *trans*-PCP (1×10^{-6} M)	0·0	0·0	0·0

Enzyme activity of both the systems were determined after 30 min preincubation with or without *trans*-PCP (1×10^{-6} M) and after dialysis for 4 and 24 hr against 0·01 M phosphate buffer pH 7·0. Other details are given in the text.

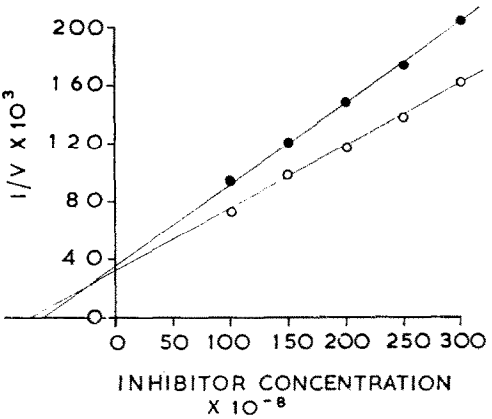

FIG. 2. Inhibition of purified MAO of rat liver mitochondria by *trans*-PCP (method of Dixon[17]) Substrate and inhibitor added first and after 10 min, enzyme was added and activity determined after 30 min of incubation.

● — ● 0·005 M tyramine
○ — ○ 0·01 M tyramine

Influence of varied concentrations of substrate on the inhibition of MAO by trans-*PCP.* Table 4 shows the effect of varied concentrations of tyramine on the *trans*-PCP inhibition of MAO when added simultaneously or preincubated with the enzyme for 15 min. When both substrate and inhibitor were added simultaneously, the degree of inhibition decreased progressively with increasing concentration of tyramine. No such protective action of the substrate could be observed when the enzyme was preincubated even with very low concentration of the inhibitor.

TABLE 4. EFFECT OF SUBSTRATE CONCENTRATION ON MAO INHIBITION BY *TRANS*-PCP

Tyramine concentration (final)	Substrate and inhibitor added simultaneously			Preincubated with inhibitor for 15 min		
	Ammonia formed (μg)			Ammonia formed (μg)		
	Control	*trans*-PCP (2×10^{-6} M)	Percent inhibition	Control	*trans*-PCP (2×10^{-7} M)	Percent inhibition
0·01 M	11·0	7·5	32	12·0	5·8	52
0·005 M	10·6	5·3	50	11·5	5·0	57
0·001 M	8·5	1·5	82	9·8	4·6	53

In the set of experiments on the left-hand side of the table substrate with or without inhibitor were first incubated for 10 min followed by the addition of enzyme. In the experiments on the right-hand side of the table enzyme with or without inhibitor were first incubated for 15 min followed by the addition of substrate. After a further period of 30 min incubation ammonia was determined. Other details are given in the text.

Effect of trans-*PCP on the deamination of various monoamines. Trans*-PCP did not affect the deamination of various monoamines to the same extent (Table 5). With serotonin, tyramine and adrenaline as substrates, maximum inhibition (57, 51 and 50 per cent respectively) occurred, while with tryptamine and benzylamine the inhibition was 35 and 41 per cent respectively.

TABLE 5. INHIBITION OF *TRANS*-PCP ON THE OXIDATIVE DEAMINATION OF VARIOUS MONOAMINES BY MAO

Substrates (0·01 M)	Control systems		Experimental systems with *trans*-PCP (2×10^{-7} M)		Percent* inhibition
	E_{450}	Aldehyde formed (μg)	E_{450}	Aldehyde formed (μg)	
Tyramine	0·190	6·50	0·090	3·20	51·0
Tryptamine	0·140	5·00	0·095	3·25	35·0
Serotonin	0·152	5·25	0·064	2·25	57·0
Adrenaline	0·112	4·00	0·056	2·00	50·0
Benzylamine	0·125	4·25	0·070	2·50	41·0

The complete reaction mixture contained about 100 μg of enzyme protein in 0·05 M phosphate buffer and preincubated with or without *trans*-PCP (2×10^{-7} M) for 15 min at 38° and then 0·0125 M semicarbazide (neutralised) and substrates (0·01 M) were added. At the end of 30 min incubation the reaction mixture was taken for aldehyde assay by the method of Green *et al.*[12]

* Calculated on the basis of aldehyde values.

Ultraviolet absorption spectra of purified enzyme and inhibitor. The u.v. absorption spectra of the enzyme with or without *trans*-PCP are represented in Fig. 3. The enzyme alone showed a peak at 410 mμ. In the case of the enzyme inhibitor system, there was a shouldering off of the peak at 415 mμ although the total absorption over the whole range of wavelength employed (200–500 mμ) was greater in the enzyme inhibitor system than with enzyme alone. 10^{-4} M Tranylcypromine had appreciable absorption only in the region of 200–300 mμ and there was no absorption in the region of 300–500 mμ

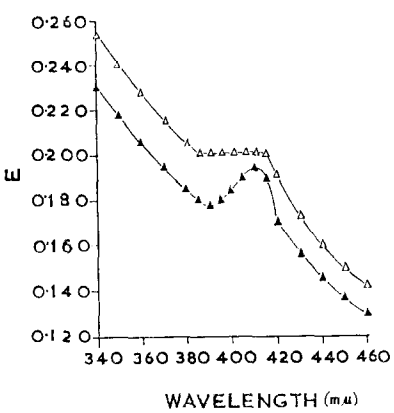

FIG. 3. Absorption spectra of purified MAO of rat liver mitochondria.
▲ — ▲ enzyme in 0·05 M phosphate buffer pH 7·0 incubated at 38° for 30 min.
△ — △ enzyme in 0·05 M phosphate buffer pH 7·0 incubated with (1×10^{-4} M) *trans*-PCP a 38° for 30 min. Enzyme protein 0·70 mg/ml.

DISCUSSION

The results obtained with purified rat liver mitochondrial MAO indicate pH 7·0 to be the optimum for both enzyme activity and its inhibition by tranylcypromine. Barbato and Abood[6] observed two peaks in the pH-activity curve of partially purified beef liver mitochondrial MAO, one at pH 7·0 and the other at pH 8·0 while in the

presence of tranylcypromine the pH-activity curve showed only one maximum at pH 7·0. Davison[18] reported intact mitochondrial enzyme to have an optimal pH of 8·0 and regarded the inhibition at pH 8·0 as complete inhibition. Subsequently Davison[19] found the optimal pH of MAO to be about 7·3. A detailed study on the various preparations of crude and purified rat liver mitochondrial MAO showed the optimal pH to be 7·0 in all cases.[16]

In view of the fact that a minimum period of preincubation is necessary to demonstrate an appreciable degree of inhibition, a time-dependent reaction between MAO and *trans*-PCP presumably reaching completion within 20–30 min is suggested. The interaction between MAO and *trans*-PCP is also reported not to be influenced by the presence of oxygen as in the case of iproniazid.[4, 6] In accordance with the results of earlier workers[3, 6, 20] tranylcypromine inhibition of MAO was found in the present study to be competitive and irreversible.

When substrate and inhibitor were added simultaneously there was competition between them for the active site on the enzyme. A protective action of high substrate concentration against tranylcypromine inhibition was observed when both substrate and inhibitor were added simultaneously. Similar results have been reported by Zeller and Sarkar.[4]

The u.v. absorption data presented indicates the possibility of a complex formation between MAO and *trans*-PCP. From studies *in vivo*, Horita and McGrath[10] concluded that transcyclopropylamine is an irreversible type of inhibitor although structurally it resembled amphetamine and ephedrine. The binding of the inhibitor with the enzyme was so strong that even after prolonged dialysis for 24 hr, the inhibition was complete. Zeller *et al.*[3] observed some activation of the enzyme on prolonged dialysis after exposure to the drug. Tranylcypromine has presumably a very high affinity for MAO and marked inhibition of the enzyme occurs even at 10^{-7} M concentration. I_{50} value approached 2×10^{-7} M in the present experiments and similar value has been reported with crude enzyme preparations.[4, 20]

The u.v. absorption spectra of the enzyme showed a peak at 410 mμ which is characteristic for pyridoxal phosphate.[21, 22] The possibility of any contaminating material in the purified enzyme preparation, employed in these experiments, giving such an absorption peak cannot be ruled out at present. However the peak changes to a shoulder in the presence of tranylcypromine and it would appear that the absorption of light by the drug itself is not interfering with such shouldering of the curve at 415 mμ. The nature of a complex formation if any, needs further elucidation.

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