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STUDIES OF MONOAMINE OXIDASES*

INHIBITION OF BOVINE BRAIN MAO IN INTACT MITOCHONDRIA BY SELECTIVE INHIBITORS

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Abstract—The inhibition of mitochondrial monoamine oxidase (MAO) from beef brain cortex by the selective inhibitors, clorgyline, harmaline, Deprenyl and pargyline, was compared using five substrates: serotonin (5-HT), β-phenylethylamine (PEA), tyramine, tryptamine and dopamine. Dose–response studies, consistent with the classification of MAO, types A and B, indicated that serotonin deamination was more sensitive to clorgyline and harmaline inhibition than was phenylethylamine. However, the curves for all substrates were double-sigmoidal, rather than being a single sigmoid curve for 5-HT and PEA. Deprenyl and pargyline did not exhibit any marked selectivity for inhibiting PEA deamination without prior preincubation of enzyme and inhibitor. The rate of inhibition was variable and was dependent upon the substrate, the nature of the inhibitor and the inhibitor concentration. Dual inhibitor studies, using the "type A" inhibitor, clorgyline, and the "type B" inhibitor, Deprenyl, together, resulted in almost complete MAO inhibition, regardless of substrate. Combining the two type A inhibitors, clorgyline and harmaline, or the two type B inhibitors, deprenyl and pargyline, resulted in inhibitions that were equal to or only slightly greater than the inhibition produced by a single inhibitor. These results suggested that there are at least two distinct sites in beef brain MAO from cortical mitochondria which may be interacting. The deamination of all substrates occurs at both sites.

There has been considerable interest in the evaluation of the multiple activities of MAO (monoamine: oxygen oxidoreductase, EC 1.4.3.4.) [1, 2]. Although the existence of separate multiple forms has not been definitely established, the variable characteristics of the enzyme observed with respect to substrate [3–5] have made the concept of multiple forms an attractive hypothesis. In particular, the classification on MAO into "A" and "B" types has received considerable acceptance [6–8]. The classification was derived from the work of Johnston [9] who described an abnormal double-sigmoid dose response of liver MAO to the inhibitor, clorgyline, when tyramine was used as substrate as opposed to serotonin which exhibited only a single-sigmoid curve. The double-sigmoid curve was taken to be indicative of two enzyme forms with different inhibitor sensitivities. The substrate-related sensitivity to several inhibitors has become a primary consideration in the designation of the two types of activity which have been found to occur in variable proportions in different organs [10, 11]. Nevertheless, there have been several reports where the enzyme properties do not agree *in toto* with the A–B concept as generally defined with respect to substrate and inhibitor specificities [12, 13].

Previous investigations from this laboratory [14, 15] have dealt with the characterization of MAO in intact, purified beef brain mitochondria and the effect of various parameters on activity with respect to substrate. Variations in certain properties such as pH optimum and response to anions were observed, but these appeared to follow a pattern along a substrate structural line rather than a possible A–B enzyme classification. The thermostability of the various deaminating properties was not clearly different so as to indicate the possibility of distinct molecular species. The differentiation of putative MAO forms remains of importance for a better understanding of neurochemical function and the present investigation describes some distinct characteristics of MAO inhibition in intact beef brain mitochondria which point to the complexity of this problem. Preliminary communication of some of these findings has been presented [16, 17].

* This article constitutes the fifth paper in a series entitled, "Studies of Monoamine Oxidases."

MATERIALS AND METHODS

Reagents. All radiochemicals: ([1-^{14}C]dopamine hydrobromide (6.28 mCi/m-mole), [1-^{14}C]β-phenylethylamine hydrochloride (7 mCi/m-mole), [2-^{14}C]-tryptamine bisuccinate (47.3 mCi/m-mole), [1-^{14}C]tyramine hydrochloride (9.20 mCi/m-mole) and [2-^{14}C]serotonin binoxalate (17.2 mCi/m-mole), and liquid scintillation chemicals were obtained from New England Nuclear Corp., Boston, MA. The cation resins, AG50W-X8 (200–400 mesh) and Amberlite CG-50 (100–200 mesh), were obtained from Bio-Rad Corp., Richmond, CA and A. H. Thomas Co., Philadelphia, PA, respectively. The following drugs were kindly supplied through the courtesy of the manufacturers as indicated: clorgyline hydrochloride (M & B 9302), May & Baker, Ltd., London, England, and pargyline hydrochloride ("Eutonyl"), Abbott Laboratories, North Chicago, IL. Deprenyl [(−)E-250] was a gift from Drs. J. Knoll and K. Magyar, Budapest, Hungary. Harmaline was purchased from Aldrich Chemical Co., Inc., Milwaukee, WI.

Methods. Mitochondrial fractions were prepared and MAO activity was assayed as previously described [14, 15]. Radiometric assays were used to measure the deamination of ^{14}C-labeled serotonin (0.5 mM), β-phenylethylamine (0.5 mM), tyramine (1.0 mM), tryptamine (0.2 mM) and dopamine (0.5 mM). The substrate concentrations used were those determined to be optimal for each substrate under the conditions of assay (0.05 M potassium phosphate buffer, pH 7.4; 20 min, 37°. Protein concentrations were determined by the method of Lowry *et al.* [18], using bovine serum albumin as standard.

Inhibition was measured with and without preincubation of the enzyme with inhibitor. For studies with preincubation, the enzyme was preincubated with the inhibitor at 37°, for the desired length of time. The reaction was then started by the addition of the substrate. In studies without preincubation, the enzyme was added to the reaction mixture containing substrate and inhibitor. The degree of inhibition was determined by comparison to control samples which were assayed under similar conditions as the inhibited samples. In the mixed inhibitor studies, the enzyme was preincubated with the inhibitors, singly or in combination, for 15–20 min at 37°, pH 7.4, prior to the addition of substrate. Equimolar concentrations of the inhibitors were present when used in combination.

In dialysis experiments, the enzyme was incubated with inhibitor for 30 min at 37° in pH 7.4 buffer. An aliquot was taken for activity measurements and the remaining fraction was dialyzed against 500 vol. of 0.05 M phosphate buffer, pH 7.4, at 4° for approximately 48 hr with several changes of buffer. After dialysis, activity was measured using standard assay procedures as previously described [15].

RESULTS

Differential inhibition of MAO. In order to study the A–B nature of MAO in beef brain cortex, the effects of the inhibitors said to be selective for the "A" form (clorgyline and harmaline) and the "B" form (Deprenyl and pargyline) were determined. The responses of beef brain MAO to clorgyline using five substrates are shown in Figs. 1 and 2. There was a distinct susceptibility of the enzyme with regard to the deamination of 5-HT (preferred substrate of A form), compared to PEA (preferred substrate of B form). The I_{50} values (molar inhibitor concentration required to inhibit enzyme activity by 50 per cent) for the overall deamination reaction were 7.5×10^{-9} M vs 1.8×10^{-6} M for 5-HT and PEA respectively. The deamination of the other substrates, tyramine, dopamine and tryptamine, said to be commonly deaminated by both A and B forms, was found to be intermediate in sensitivity to the inhibitor. Thus, the substrate susceptibility was in the order that might be expected under the A–B classification. However, the dose–response curves were double-sigmoidal for all substrates. Similar results were obtained with harmaline, although the inhibitor potency was slightly less than clorgyline with I_{50} values of 5.6×10^{-8} M and 4.6×10^{-6} M for 5-HT and PEA deamination respectively.

The differentiation observed in the inhibition of 5-HT and PEA deamination by Deprenyl and pargyline (Figs 3 and 4) and not as marked as with clorgyline and harmaline. The dose–response curves with pargyline appeared to be single-sigmoid curves for all substrates and with Deprenyl for 5-HT; however, the dose–response curves to Deprenyl of PEA deamination, as well as those of the "common" substrates (Fig. 5), showed a shoulder which might or might not be considered indicative of a double-sigmoid curve. In his description of the abnormal inhibitory properties of clorgyline, Johnston [9] described the general slope of the dose–response curves ($25S75 = pI_{25} - pI_{75}$, where pI_{25} and pI_{75} are equal to the negative logarithm of the inhibitor concentrations required to give 25 and 75 per cent inhibition, respectively) as generally being greater than 1 for the complex inhibition of two enzymes, due to the interpolation of a plateau region in the curve. The slopes observed here for clorgyline ranged from 3.45 to 3.90 for all substrates. For Deprenyl, the slope with 5-HT was 1.0, but slopes were shallower for the other substrates, in the order of 2.3 to 2.45.

Effect of preincubation of E + I *on MAO inhibition.* The rate of interaction of inhibitor with MAO affected the deamination of substrates unequally. This was evident from the dose–response studies which were performed with and without preincubation of

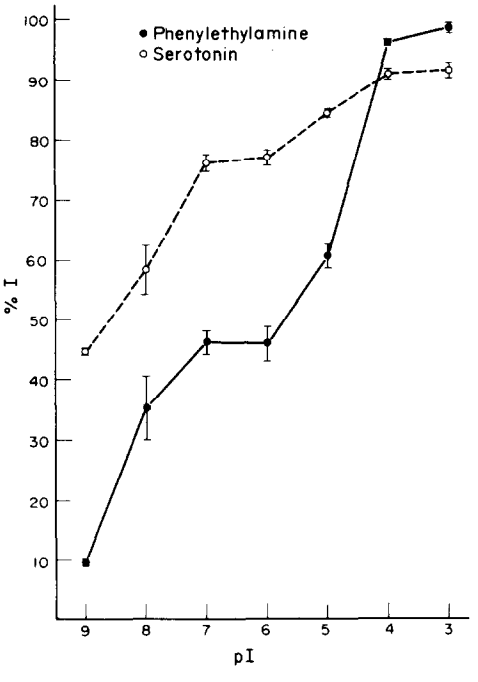

Fig. 1. Clorgyline inhibition of phenylethylamine and serotonin oxidation. Enzyme was preincubated with clorgyline for 15 min at 37° before the addition of substrate [(●——●) PEA; (○– –○) 5-HT] to start the reaction. Enzyme activity was determined radiometrically as described previously [14, 15]. Substrate concentrations were 0.5 mM for both PEA and 5-HT. Per cent inhibition was determined by comparison with control sample containing no inhibitor. The values obtained were plotted against the negative logarithm of the inhibitor molar concentration (= pI).

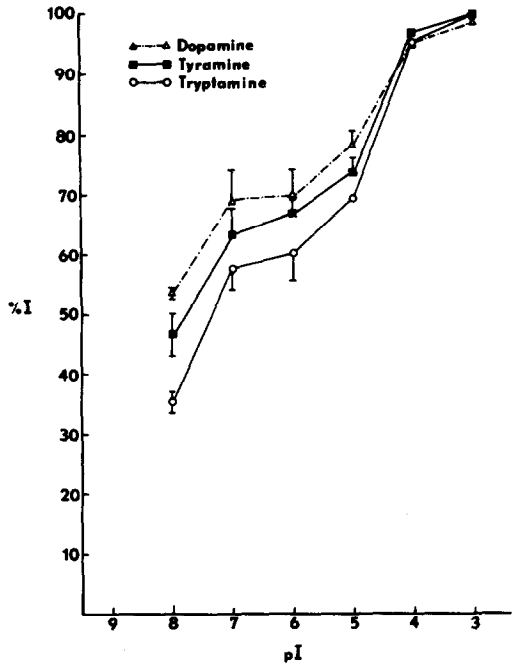

Fig. 2. Clorgyline inhibition of dopamine, tyramine and tryptamine oxidation. Same conditions as in Fig. 1, using (△-·-·△) dopamine, (■——■) tyramine, and (○——○) tryptamine as substrates.

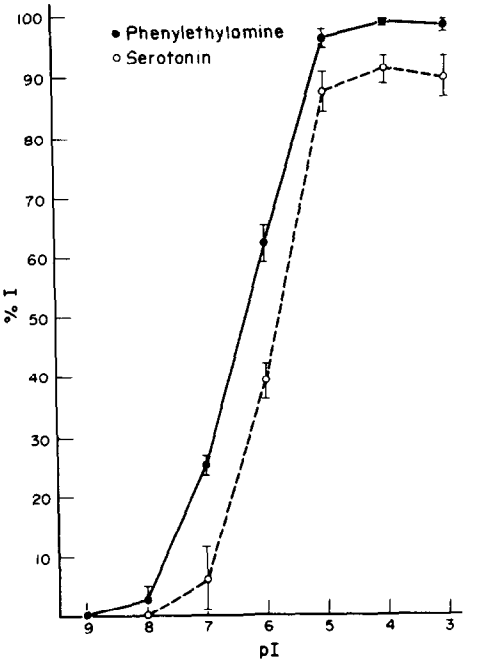

Fig. 4. Pargyline inhibition of phenylethylamine and serotonin oxidation. Same conditions as in Fig. 3.

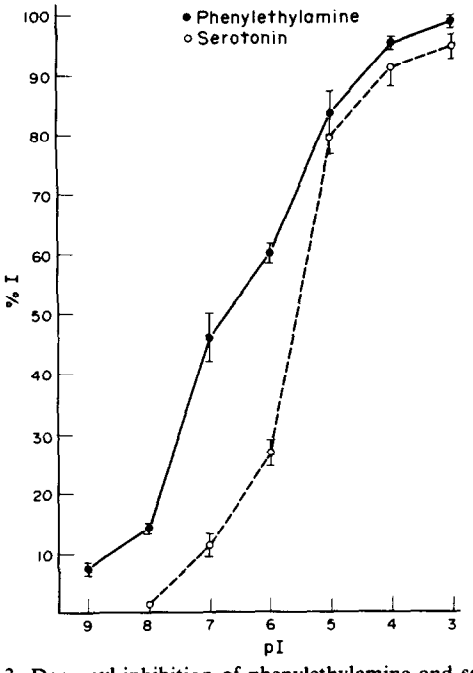

Fig. 3. Deprenyl inhibition of phenylethylamine and serotonin oxidation. Enzyme was preincubated with Deprenyl for 15 min at 37° before the addition of substrate, either PEA (●——●) or 5-HT (○- - -○), to start the enzyme reaction. Activity was determined radiometrically as previously described [14, 15].

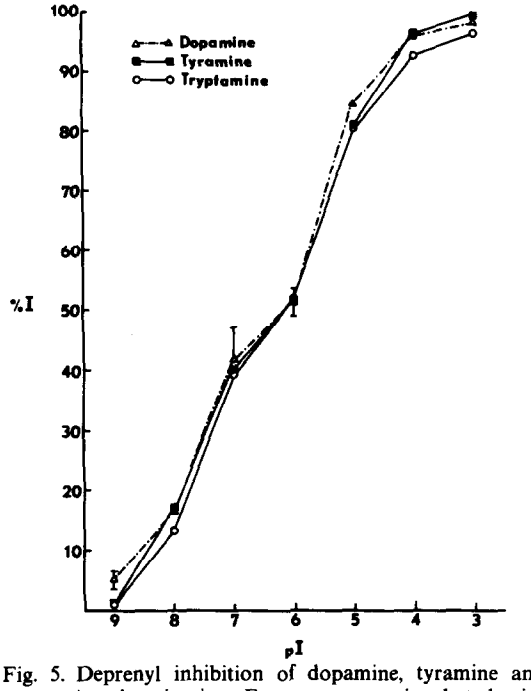

Fig. 5. Deprenyl inhibition of dopamine, tyramine and tryptamine deamination. Enzyme was preincubated with Deprenyl for 15 min at 37° before the start of the reaction by the addition of substrate [(△-·-·△) dopamine, (■——■) tyramine, and (○——○) tryptamine]. Activity was determined radiometrically as previously described [14, 15].

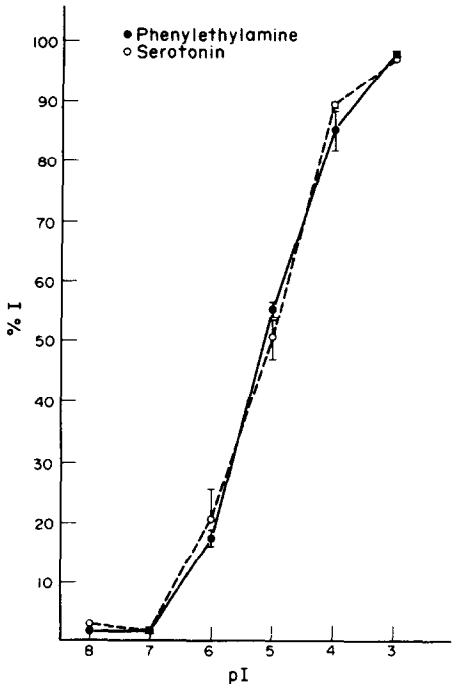

Fig. 6. Instaneous inhibition of phenylethylamine and serotonin deamination by Deprenyl. Enzyme was not preincubated with inhibitor. Enzyme reaction was started by addition of enzyme to inhibitor and substrate [(●——●) PEA; (○---○) 5-HT] and activity determined radiometrically as previously described [14, 15]. Control samples were assayed under identical conditions without inhibitor. pI = negative logarithm of molar inhibitor concentration.

$E + I$. In the case of Deprenyl, the dose–response curves of 5-HT and PEA deamination were superimposable in studies where the enzyme was not preincubated with the inhibitor prior to assay (Fig. 6). Individual rate studies using 5-HT and PEA as substrates indicated that three variables needed to be considered; namely, the substrate itself, the nature of the inhibitor and the inhibitor concentration (Fig. 7). Clorgyline had been reported to require no preincubation for development of inhibition [19]; however, the present studies showed that this was not true for all conditions. With moderately low inhibitor concentrations (10^{-7} and 10^{-8} M), approximately 10–20 min of preincubation was required for maximal inhibition of the deamination of all substrates. At higher concentrations of clorgyline, the deamination of PEA was not inhibited maximally until after 15 min of preincubation, whereas the inhibition with 5-HT seemed to require no preincubation. The studies with Deprenyl also showed some anomalies. Preincubation was required for maximal inhibition of PEA at low to moderate inhibitor concentrations from 10^{-8} to 10^{-5} M, whereas with high concentrations of Deprenyl (10^{-4} M), the inhibition of PEA deamination was not time dependent. As seen in Fig. 7b, the degree of inhibition of 5-HT deamination was not dependent on incubation times, at both high and low concentrations of inhibitor. At high concentrations of Deprenyl (10^{-4} M), the deamination of the other substrates also did not require preincubation for maximal inhibition. These variations in the effect of preincubation did not appear to be a consequence of reversible vs irreversible inhibition, since similar results were obtained with the reversible inhibitor, harmaline. Preincubation was necessary for maximal inhibition by harmaline at concentrations lower than 10^{-6} M (Fig. 8).

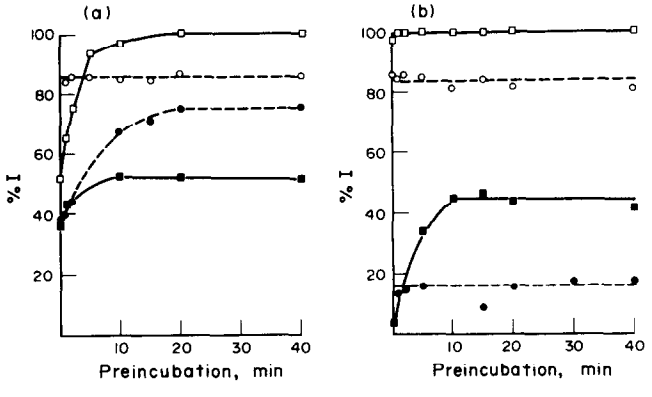

Fig. 7. Rate of inhibition of MAO in beef brain mitochondria by clorgyline and Deprenyl—Effect of inhibitor concentration. High concentrations (10^{-4} – 10^{-5} M) of inhibitor were preincubated with enzyme for indicated lengths of time at 37° before the start of the reaction by the addition of substrate [(○---○) 5-HT; (□——□) PEA]. Similarly, low concentrations (10^{-7} – 10^{-8} M) of inhibitor were preincubated with enzyme and inhibition of 5-HT (●---●) and PEA (■——■) oxidation was measured. Figure 7a shows results with clorgyline using 10^{-5} and 10^{-8} M clorgyline as the high and low concentrations for the inhibition of 5-HT oxidation and 10^{-4} and 10^{-7} M clorgyline for the inhibition of PEA oxidation. Figure 7b shows the results for Deprenyl inhibition using 10^{-4} M and 10^{-7} M as the high and low concentrations for the inhibition of both 5-HT and PEA oxidation. Activities were determined radiometrically as described previously [14, 15] and per cent inhibition was determined by comparison with control experiments in which enzyme was preincubated for the same length of time without inhibitor.

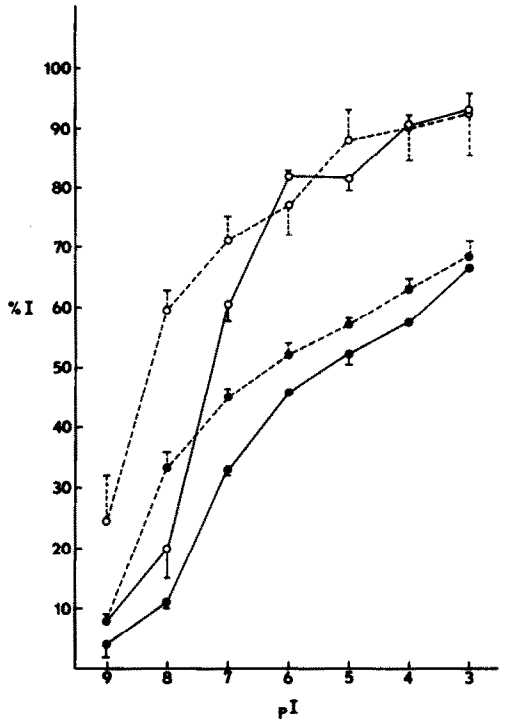

Fig. 8. Effect of preincubation on harmaline inhibition of serotonin and phenylethylamine deamination. The inhibition of 5-HT and PEA deamination by the reversible inhibitor, harmaline, was measured with and without preincubation of $E + I$, before starting the reaction with substrate. Preincubation time was 15 min for studies with preincubation. Key: (○---○) 5-HT deamination with preincubation; (○——○) 5-HT deamination without preincubation; (●---●) PEA deamination with preincubation; and (●——●) PEA deamination without preincubation.

On the other hand, studies with the tricyclic antidepressant, imipramine, which also inhibits MAO reversibly, yielded a constant inhibition, with and without preincubation, regardless of inhibitor concentration and substrate used (to be published).

Effect of dialysis. The reversibility of harmaline inhibition and the irreversibility of Deprenyl and clorgyline inhibition was confirmed by dialysis experiments (Table 1). After 48-hr dialysis of the inhibited enzyme, MAO activity was recovered in the harmaline-treated sample but not in the deprenyl- or clorgyline-treated samples. There was little difference in the recovery of enzyme activity irrespective of the substrate used: 5-HT, PEA and tyramine.

Mixed inhibitor studies. The inhibition of MAO activity in beef brain mitochondria obtained when a combination of two inhibitors was used simultaneously is shown in Tables 2 and 3. When two inhibitors are present at the same time, acting on the same system, the inhibition produced by both inhibitors together is defined by:

$$i_{1,2} = i_1 + i_2 - i_1 i_2$$

where i_1 and i_2 are the fractional inhibitions produced by each inhibitor separately [20]. In Table 2, the experimentally determined multiple inhibitions, $i_{1,2}$, for the combination of an A inhibitor, clorgyline, and a B inhibitor, Deprenyl, were compared to the theoretical, calculated values defined by the equation above. For all substrates, with the exception of 5-HT, the experimental values were greater than the theoretical values. With 5-HT, the experimental results were about equal to the calculated values. There was almost 100 per cent inhibition of MAO activity with A–B combination, indicating that the inhibitions produced by each inhibitor were essentially additive. In contrast, the combination of two A inhibitors or two B inhibitors (Table 3) did not result in total inhibition. The inhibition of 5-HT and PEA was not additive and found to be equal to or less than the calculated inhibition. With the A inhibitors, clorgyline and harmaline, the inhibition produced with the two inhibitors present was essentially the same as with either single inhibitor. The combination of B inhibitors, Deprenyl and pargyline, resulted in inhibitions slightly greater than inhibition with either inhibitor alone, but still less than the sum of the two inhibitors.

DISCUSSION

The studies presented here indicate that, with beef brain mitochondrial MAO, clorgyline and harmaline

Table 1. Reversal of MAO inhibition by dialysis*

Inhibitor	Substrate	Per cent inhibition Before dialysis	After dialysis	Per cent reversal of inhibition
Clorgyline (10^{-5} M)	5-HT	99.0	94.6	4.4
	PEA	85.4	87.1	0
	Tyramine	86.0	85.7	0.3
Deprenyl (10^{-4} M)	5-HT	81.2	80.8	0.5
	PEA	96.6	97.4	2.2
	Tyramine	99.8	94.7	5.1
Harmaline (10^{-5} M)	5-HT	83.5	0	100
	PEA	54.5	0	100
	Tyramine	68.5	0	100

* Dialysis experiments were carried out as described in Methods. Per cent inhibition was determined by a comparison of inhibited samples with control samples which were treated in the same manner as the inhibited samples but with no inhibitor present.

Table 2. Multiple inhibition of MAO in beef brain mitochondria using an A inhibitor and a B inhibitor*

Substrate	[I] (M)	Measured clorgyline inhibition (i_1)	Measured Deprenyl inhibition (i_2)	Measured mult. inhibition ($i_{1,2}$)	Calculated sum of ind. inhibition ($i_1 + i_2$)	Calculated theoretical mult. inhibition ($i_1 + i_2 - i_1 i_2$)
Serotonin	10^{-6}	76.7 ± 1.6 (10)	38.1 ± 2.7 (9)	85.0 ± 3.9 (5)	114.8	85.6
	10^{-7}	75.2 ± 1.9 (10)	14.6 ± 1.8 (12)	83.6 ± 4.4 (5)	89.8	78.8
Phenylethylamine	10^{-6}	50.5 ± 2.8 (9)	65.3 ± 2.1 (9)	98.6 ± 0.7 (4)	115.8	82.8
	10^{-7}	48.3 ± 2.0 (10)	50 ± 2.2 (11)	96.5 ± 1.5 (4)	98.3	74.1
Tyramine	10^{-6}	62.9 ± 5.2 (4)	55.9 ± 2.9 (5)	99.1 ± 0.5 (2)	118.8	83.6
	10^{-7}	59.9 ± 5.7 (4)	38.3 ± 2.2 (7)	98.6 ± 1.0 (2)	98.2	75.3
Tryptamine	10^{-6}	55.1 ± 6.2 (4)	58.3 ± 3.3 (5)	95.2 ± 4.2 (2)	113.4	81.3
	10^{-7}	48.5 ± 7.6 (4)	44.6 ± 3.7 (5)	91.5 ± 4.2 (2)	93.1	71.5
Dopamine	10^{-6}	64.8 ± 5.8 (4)	58.0 ± 3.8 (4)	99.6 ± 0.1 (2)	122.7	85.2
	10^{-7}	64.8 ± 5.2 (4)	42.1 ± 4.1 (5)	99.3 ± 0.7 (2)	106.9	79.6

* Inhibition is expressed as per cent or fractional inhibition using equimolar concentrations of the A inhibitor, clorgyline, and the B inhibitor, Deprenyl, for the multiple inhibition. Studies were carried out as described in Methods. Measured data are per cent inhibitions ± S.E.M., determined by comparison with control samples containing no inhibitor. Numbers in parentheses are the number of determinations; all determinations were done in duplicate for the inhibited samples and in triplicate for the controls.

were clearly more effective in inhibiting the oxidation of 5-HT than PEA, in agreement with the A–B model. It should be emphasized, however, that the clear preference ascribed to these inhibitors, in terms of a single-sigmoid curve, was not observed. Double-sigmoid or biphasic curves were observed for both 5-HT and PEA, in addition to the other substrates. This indicated that there may be two types of activities present in intact purified beef brain mitochondria differing in their sensitivities to clorgyline or harmaline which act on both 5-HT and PEA. Yasuhara [21] also found a biphasic dose–response curve for the inhibition of 5-HT oxidation by harmine, the unsaturated analogue of harmaline. Upon heat treatment, however, a single-sigmoid curve was obtained. Results were interpreted on the basis of two sites or forms, one of which was heat labile and the other heat stable. On the other hand, the B-type selective inhibitors, Deprenyl and pargyline, weakly differentiated between 5-HT and PEA oxidation by beef brain mitochondria, and this, only with preincubation of enzyme and inhibitor. In this respect, beef brain MAO appeared to respond similarly to MAO from rabbit tissues, which Squires [10] reported was not selectively inhibited by Deprenyl and pargyline, while showing a differential response to clorgyline and harmine.

The results presented here are in agreement with other recent reports [22, 23] which have shown that both A and B types of MAO can oxidize substrates which had been previously thought to be exclusive for one form or another. While there may be variations due to species and organs, Ekstedt [23] reported large differences in the K_m values of the two forms for the different amines. Since the PEA concentration used in the present experiments was relatively

Table 3. Multiple inhibition of MAO using two A or two B inhibitors*

Substrate	[I] (M)	Measured inhibition (i_1)	Measured inhibition (i_2)	Measured mult. inhibition ($i_{1,2}$)	Calculated sum of ind. inhibition ($i_1 + i_2$)	Calculated theoretical mult. inhibition ($i_1 + i_2 - i_1 i_2$)
Inhibition by A inhibitors, clorgyline (i_1) and harmaline (i_2)						
Serotonin	10^{-6}	76.7 ± 1.6 (10)	76.9 ± 5.0 (6)	75.1 ± 4.5 (3)	153.6	94.6
	10^{-7}	75.2 ± 1.9 (10)	71.0 ± 4.3 (6)	76.1 ± 1.7 (3)	146.2	92.9
Phenylethylamine	10^{-6}	50.5 ± 2.8 (9)	52.1 ± 1.7 (5)	57.3 ± 2.7 (3)	102.6	76.3
	10^{-7}	48.3 ± 2.0 (10)	45.2 ± 1.3 (6)	52.1 ± 0.6 (3)	93.5	71.7
Inhibition by B inhibitors, Deprenyl (i_1) and pargyline (i_2)						
Serotonin	10^{-6}	38.1 ± 2.7 (9)	48.6 ± 4.2 (5)	66.3 ± 1.2 (3)	86.7	68.2
	10^{-7}	14.6 ± 1.8 (12)	11.8 ± 2.8 (6)	22.1 ± 2.0 (5)	26.1	24.6
Phenylethylamine	10^{-6}	65.3 ± 2.0 (9)	69.7 ± 4.1 (6)	86.4 ± 2.1 (3)	135.0	89.5
	10^{-7}	50.0 ± 2.2 (11)	32.7 ± 3.1 (7)	52.7 ± 1.5 (4)	82.7	66.3

* Equimolar concentrations of inhibitors were used for multiple inhibitions. Measured data are per cent inhibition ± S.E.M. determined by comparison with control samples assayed without inhibitor. Studies were carried out as described in Methods. Numbers in parentheses are the number of determinations; all determinations were done in duplicate for the inhibited sample and in triplicate for the controls.

high (0.5 mM), this may in part account for the substrate preferences reported here for the beef brain MAO.

The rate studies with clorgyline and Deprenyl (Fig. 7) might be interpreted as a result of different rates of attack by the inhibitors on two distinct centers of oxidation: a fast-reacting center and a slow-reacting center, as suggested for the biphasic inhibition of rat liver MAO oxidation of tyramine by 5-phenyl-(-3-*N*-cyclopropyl)ethyl-amine-1,2,4-oxaldiazole (PCO) [24]. However, this does not fully explain all the ramifications of the present study with regard to the variations in rate occurring with the different inhibitors, inhibitor concentrations and various substrates. In general, all the inhibitors used showed some time dependency, but there also was instantaneous inhibition, the degree of which depended on the inhibitor concentrations. The present results, perhaps, might be better explained by assuming a two-step inhibition reaction occurring at two different centers [25].

$$E_A + I \rightleftharpoons E_A \cdot I \rightarrow E_A - I$$

$$E_B + I \rightleftharpoons E_B \cdot I \rightarrow E_B - I.$$

In the first step, there is a non-covalent interaction occurring between enzyme and inhibitor which takes place rapidly, so that one observes an instantaneous inhibition, the extent of which depends on inhibitor concentration. In the second step, a covalent bond between E and I is formed at a much slower rate. The rate of this second reaction would not be the same for both sites.

The mixed inhibitor studies (Tables 2 and 3) also indicated that MAO in beef brain mitochondria might contain different sites which are distinct for the different inhibitors but perhaps are not mutually exclusive for any particular substrate. There appears to be a site which reacts with clorgyline and harmaline and another site for Deprenyl and pargyline. Both sites are involved in the deamination of all the substrates and the effect of using a combination of inhibitor of an A type and a B type is an additive one. Thus, one is able to completely inhibit MAO activity using relatively low concentrations of such a combination whereas if a single inhibitor or even a combination of inhibitors of the same type is used at the same concentrations, one only observes partial inhibition. It is interesting to note the difference in the effects of the combination of clorgyline and harmaline and that of Deprenyl and pargyline, where the A inhibitors show more of an antagonistic effect (Table 3). This probably reflects the dissimilarity between the structural nature of clorgyline and harmaline, or a difference in the type of inhibition produced, being irreversible with clorgyline and reversible with harmaline, as confirmed by the dialysis experiments. Deprenyl and pargyline are both propargylamine derivatives and are irreversible inhibitors.

The deamination of 5-HT deviated slightly from the general pattern of additive inhibition with A–B combinations of inhibitors in that there was only 85 per cent inhibition with the combination of clorgyline and Deprenyl in contrast to the other substrates (Table 2). Recently it was reported that there is a clorgyline-insensitive 5-HT-oxidizing activity in the circumventricular structure of the rat brain [26]. Since it was observed here that total inhibition of 5-HT deamination was not achieved with high concentrations of clorgyline (Fig. 1), it may well be that this remaining 5-HT-oxidizing activity in beef brain is due to a site or form which is distinct from those previously mentioned. Nonetheless, with regard to the other substrates, the present results would seem to be in partial agreement with a recent study *in vivo* by Green and Youdim [27] who gave clorgyline plus Deprenyl simultaneously to rats and found total inhibition of both 5-HT and PEA oxidation with brain tissue. Higher doses of a single inhibitor were able to inhibit by 100 per cent the oxidation of only one amine but not the other. With beef brain MAO, it is possible to completely inhibit PEA oxidation with higher concentrations of clorgyline and 5-HT oxidation with sufficiently high concentrations of Deprenyl. Green and Youdim [27] suggested that, while 5-HT may normally be metabolized by predominantly the A type of MAO, when this form is inhibited, type B may continue to act on the amine and that MAO acts *in vivo* as an integrated enzyme system with properties that differ from the individual forms studied *in vitro*. The present report seems to indicate that intact mitochondria might be regarded as an integrated system *in vitro*.

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REFERENCES

1. E. Usdin (Ed.) *Neuropsychopharmacology of Monoamines and Their Regulatory Enzymes.* Raven Press, New York (1974).
2. M. Sandler and M. B. H. Youdim, *Pharmac. Rev.* **24,** 331 (1972).
3. M. B. H. Youdim, *Adv. Biochem. Psychopharmac.* **5,** 67 (1972).
4. R. W. Fuller and B. W. Roush, *Archs int. Pharmacodyn Thér.* **198,** 270 (1972).
5. J. B. Ragland, *Biochem. biophys. Res. Commun.* **31,** 203 (1968).
6. H.-Y. T. Yang, C. Goridis and N. H. Neff, *J. Neurochem.* **19,** 1241 (1972).
7. R. F. Squires, *J. Neurochem.* **24,** 47 (1975).
8. J. A. Roth and C. N. Gillis, *Molec. Pharmac.* **11,** 28 (1975).
9. J. P. Johnston, *Biochem. Pharmac.* **17,** 1285 (1968).
10. R. F. Squires, *Adv. Biochem. Psychopharmac.* **5,** 355 (1972).
11. N. H. Neff and C. Goridis, *Adv. Biochem. Psychopharmac.* **5,** 307 (1972).
12. C. Braestrup, H. Andersen and A. Randrup, *Eur. J. Pharmac.* **34,** 181 (1975).
13. M. B. H. Youdim and B. Holman, *J. neural Trans.* **37,** 11 (1975).
14. F. M. Achee, G. Togulga and S. Gabay, *J. Neurochem.* **22,** 651 (1974).
15. S. Gabay, F. M. Achee and G. Mentes, *J. Neurochem.* **27,** 415 (1976).
16. F. M. Achee, P. King and S. Gabay, *Fedn Proc.* **34,** 727 (1975).
17. S. Gabay and F. M. Achee, *Psychopharmac. Bull.* **13,** 45 (1977).
18. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).

19. D. W. R. Hall and B. W. Logan, *Biochem. Pharmac.* **18,** 1955 (1969).
20. J. L. Webb, *Enzymes and Metabolic Inhibitors*, Vol. 1, p. 507. Academic Press, New York (1963).
21. H. Yasuhara, *Jap. J. Pharmac.* **24,** 523 (1974).
22. T. J. Mantle, M. D. Houslay, N. J. Garrett and K. F. Tipton, *J. Pharm. Pharmac.* **28,** 667 (1976).
23. B. Ekstedt, *Biochem. Pharmac.* **25,** 1133 (1976).
24. T. J. Mantle, K. Wilson and R. F. Long, *Biochem. Pharmac.* **24,** 2031 (1975).
25. K. F. Tipton, *Biochem. Pharmac.* **22,** 2933 (1973).
26. D. Williams, J. E. Gascoigne and E. D. Williams, *Brain Res.* **100,** 231 (1975).
27. A. R. Green and M. B. H. Youdim, *Br. J. Pharmac.* **35,** 415 (1975).