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POSSIBLE HETEROGENEITY OF TYPE B MONOAMINE OXIDASE IN PIG HEART MITOCHONDRIA

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Abstract—The metabolism *in vitro* of 5-hydroxytryptamine (5-HT), tyramine and benzylamine by pig heart mitochondrial monoamine oxidase (MAO) has been studied. Linear Lineweaver–Burk plots yielded estimated K_m values (at pH 7.8) of 475 μM (5-HT) and 292 μM (tyramine). In contrast, linear regions of a downward-curving reciprocal plot revealed the presence of a high- and low-affinity metabolizing site (estimated K_m of 39 and 853 μM respectively) for benzylamine. Studies with the irreversible MAO inhibitor clorgyline indicated that metabolism of the three substrates in this tissue was brought about by type B MAO alone. However, the apparent sensitivity toward clorgyline of each substrate-metabolizing activity was not identical. This was due to different degrees of rapid or possibly instantaneous inhibition of enzyme activity toward each substrate. This rapid inhibition appeared to be both partially reversible and irreversible to a relative degree depending upon the substrate-metabolizing activity studied; additional time-dependent inhibition developing with prolonged preincubation was a first-order process, with a similar half-life, whichever substrate was used to assay MAO activity. Ackermann–Potter and Lineweaver–Burk plots also demonstrated differences in the inhibitory effects of clorgyline upon metabolism of each substrate. The ability of 5-HT, tyramine and benzylamine to inhibit each other's deamination *in vitro* was also investigated. Enzyme activity was measured by radiochemical assay with each labeled substrate in the presence and absence of the other non-labeled amines. Lineweaver–Burk analysis revealed a competitive interaction between tyramine and benzylamine, whereas mixed-type inhibition patterns were obtained for mixtures containing 5-HT/tyramine or 5-HT/benzylamine. In this latter case, the present inhibition data could only be assessed accurately with the low-affinity catalytic site for benzylamine. The kinetics of heat denaturation indicated both a thermolabile and thermostable component of each substrate-metabolizing activity. Some substrate-dependent differences in the relative proportions of these components were found. These experiments are discussed in relation to similar studies by other workers and suggest that pig heart MAO may, in fact, be heterogeneous.

Differences in substrate specificity, inhibitor specificity, resistance to heat denaturation and mobility upon polyacrilamide gels are among the criteria which have been used recently to indicate the possible existence of multiple forms of the enzyme monoamine oxidase (MAO) [1]. Studies with selective irreversible MAO inhibitors such as clorgyline have suggested that MAO from a variety of animal tissues may be classified into two types (A and B) which differ in their sensitivities toward this drug, and also in their specificities toward different substrates [2]. Initially, it was found in most tissues studied that 5-hydroxytryptamine (5-HT) was metabolized solely by type A MAO (the form more sensitive to clorgyline), benzylamine solely by type B MAO (the less sensitive form), whereas tyramine could be metabolized by either MAO type [3]. However, more recently, some exceptions to these early findings have been noted for MAO in rat and bovine heart [4, 5]. These exceptions shown by cardiac MAO from two species have prompted us to study amine metabolism in cardiac tissue from another species, the pig.

Previous studies of inhibition by clorgyline of pig liver and brain MAO have indicated a predominance of type B MAO, which in these tissues is able to metabolize 5-HT, tyramine and benzylamine [6]. In contrast, a more recent study has suggested that these tissues also contain type A MAO [7]. In the present paper, we show that 5-HT, tyramine and benzylamine metabolism in pig heart are brought about by type B MAO activity (defined on the basis of sensitivity toward clorgyline). However, from these results showing substrate-dependent differences in sensitivity toward clorgyline, and from mixed-substrate and thermal denaturation experiments, it appears that type B MAO of pig heart mitochondria may, in fact, be heterogeneous.

MATERIALS AND METHODS

Radioactive substrates used were [methylene-^{14}C]benzylamine hydrochloride (ICN Pharmaceuticals Inc., Irvine, CA) (generally labeled [^{3}H]5-hydroxytryptamine creatinine sulfate (Amersham-Searle, Arlington Heights, IL) and (generally labeled [^{3}H]tyramine hydrochloride (New England Nuclear, Boston, MA).

Unlabeled substrates used in mixed-substrate studies were benzylamine hydrochloride (K & K Lab. Inc., Plainview, NY), tyramine hydrochloride (CalBiochem, San Diego, CA) and 5-hydroxytryptamine creatinine sulfate (Sigma, St. Louis, MO).

Clorgyline hydrochloride [*N*-methyl-*N*-propargyl-3-(2,4-dichlorophenoxy)propylamine, M + B

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9302] was kindly provided by May & Baker Ltd., Dagenham, Essex, England.

Pig hearts were obtained (at the time of killing) from the local slaughterhouse, and transported immediately, packed in ice, to the laboratory. Mitochondrial fractions were prepared from minced ventricular tissue which was washed and then homogenized in a buffer (10 ml/g of tissue) containing 0.25 M sucrose, 0.01 M sodium phosphate, pH 7.8. The homogenate was centrifuged at 800 *g* for 10 min in a Sorvall RC-2 refrigerated centrifuge to remove cell debris, and the supernatant was centrifuged at 12,000 *g* for 20 min to pellet the mitochondria. They were resuspended in the homogenizing buffer at a final protein concentration of around 15 mg/ml, and divided into suitable portions for storage at −20°. The following experiments were performed upon mitochondrial fractions that were used within 2 weeks of preparation, and that had been frozen and thawed a single time. MAO activity toward the three substrates was found to be unchanged by a single freezing and thawing, and also was stable in the frozen state during this storage period.

Assay of monoamine oxidase. MAO was assayed by a previously described radiochemical method [8]. In general, assays contained 25 μl mitochondria, 25 μl of distilled water and 50 μl of radioactive substrate (2 mM in 0.2 M potassium phosphate buffer, pH 7.8). However, aliquots of mitochondria and also substrate concentrations were varied in some experiments as indicated in the text while still retaining a total assay volume of 100 μl. All assay tubes were flushed with O_2 and stoppered before incubation. Metabolite production was linear for the 30-min assay incubation used (at 37°) and also with up to 25 μl mitochondria in the assay. After stopping the assay reaction with 10 μl of 3 N HCl, the deaminated metabolites were extracted into 0.5 ml ethyl acetate–benzene (1:1, v/v). Extraction efficiencies for metabolites of 5-HT, tyramine and benzylamine under these conditions were determined to be 70, 81 and 89 per cent respectively. A 0.4-ml sample of the organic phase was counted for radioactivity in a Beckman LS-230 liquid scintillation spectrometer using 12 ml of 0.4% butyl PBD (2-(4′-*tert*-butylphenyl)-5-(4″-biphenylyl)-1,3,4-oxadiazole) in toluene (w/v) as scintillant. All counts were corrected for quench and converted to d.p.m.

Inhibition studies using clorgyline. For these studies, assay tubes containing appropriate concentrations of clorgyline in distilled water were preincubated at 37° with mitochondria in a total preincubation volume of 50 μl. After preincubation, all assay tubes were rapidly ice-cooled before the addition of 50 μl of radioactive substrate. Remaining enzyme activity was then measured as described above.

RESULTS

MAO activity of pig heart mitochondria. The specific activities of pig heart mitochondrial MAO toward the three substrates 5-HT, tyramine and benzylamine were determined at a final substrate concentration of 1 mM. The values obtained are shown in Table 1. It can be seen that pig heart mitochondrial MAO is able to deaminate all three substrates, although 5-HT is the least actively metabolized under these particular conditions.

Table 1. MAO activity of pig heart mitochondria*

Substrate	MAO activity (nmoles/hr/mg protein)
5-HT	37 ± 5
Tyramine	129 ± 8
Benzylamine	89 ± 7

* Each value is the mean ± S. E. of eight different preparations and was measured at 1 mM final substrate concentrations.

K_m values for substrates. K_m values for the three substrates were determined by Lineweaver–Burk analysis of initial reaction velocities obtained over a large range of different substrate concentrations. The particular concentrations studied were 5 μM–4 mM (5-HT) and 5 μM–5 mM (tyramine or benzylamine). At higher concentrations, substrate inhibition became apparent.

Lineweaver–Burk plots for 5-HT and tyramine as substrates were completely linear. However, the plot for benzylamine was linear at higher (1–5 mM) and lower (5–25 μM) concentrations, with a downward-curving region joining these linear portions at the intermediate substrate concentrations. K_m values for a low- and high-affinity site for benzylamine metabolism were estimated from the corresponding linear portions of the plot. Estimated K_m values for all three substrates are shown in Table 2.

Representative Lineweaver–Burk plots obtained with these substrates can be seen in the inhibition studies with clorgyline (see Figs 5 and 6) and also in the mixed-substrate studies (see Figs 7–10) described later in this paper.

Inhibition of pig heart mitochondrial MAO by clorgyline. Identical mitochondrial samples were preincubated for 20 min at 37° as described in Materials and Methods, with various graded doses of clorgyline (final preincubation concentrations of 2×10^{-8} to 2×10^{-4} M). Remaining enzyme activity was then assayed with 5-HT, tyramine or benzylamine as substrates at final concentrations of 1 mM. Figure 1 shows that the enzyme activity toward each substrate (expressed as a percentage of uninhibited control values), when plotted against increasing concentrations of clorgyline (on a logarithmic scale),

Table 2. Michaelis constants (K_m) for pig heart mitochondrial MAO*

Substrate	K_m (μM)	Range (μM)
5-HT	475 ± 50 (6)	290–667
Tyramine	292 ± 18 (5)	270–333
Benzylamine (i)	39 ± 8 (3)	29–56
, (ii)	853 ± 12 (3)	830–870

* Each value was estimated from reciprocal plot analysis as described in the text, and is reported as mean ± S. E. Figures in parentheses represent number of different experiments. See Figs 5–10 for representative plots.

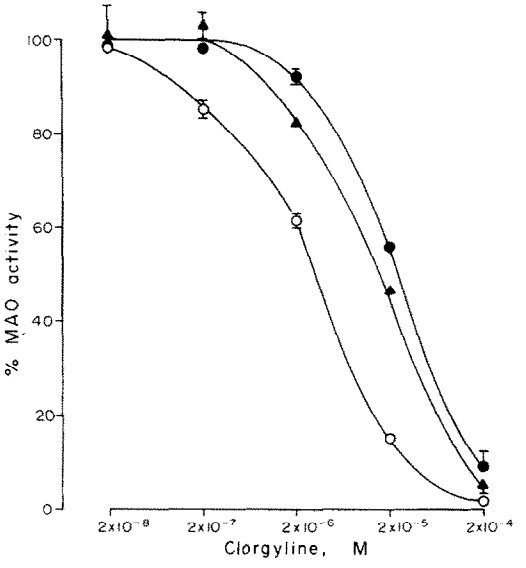

Fig. 1. Inhibition of pig heart MAO by different clorgyline concentrations. Remaining MAO activity in mitochondrial samples was assayed at final substrate concentrations of 1 mM after a 20-min preincubation with clorgyline. Key: ○——○, 5-HT; ▲——▲, tyramine; and ●——●, benzylamine, as substrate. All points represent the mean of triplicate determinations (standard errors shown when they exceed symbol size).

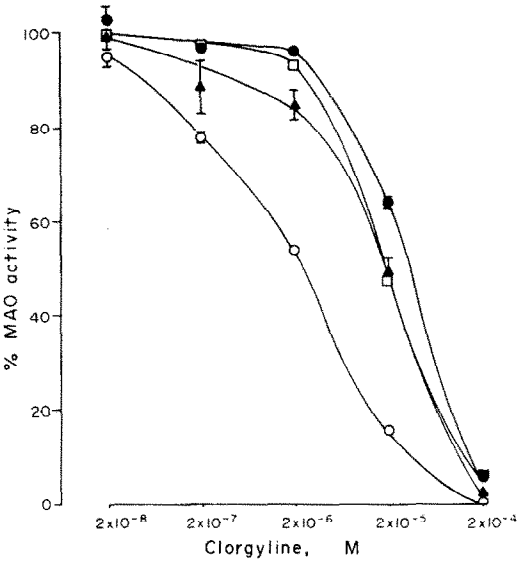

Fig. 2. Inhibition of pig heart MAO by different clorgyline concentrations. MAO activity in mitochondrial samples was assayed at final substrate concentrations of twice estimated K_m values after a 20-min preincubation with clorgyline. Key: ○——○, 5-HT (950 μM); ▲——▲, tyramine (600 μM), □——□, benzylamine (75 μM); and ●——●, benzylamine (1700 μM). All points represent the mean of triplicate determinations (standard errors shown when they exceed symbol size).

gave rise to single-sigmoid inactivation curves. It has been suggested previously that single-sigmoid curves of this nature represent the inhibition of a single enzyme form by clorgyline. Furthermore, the particular range of clorgyline concentrations over which inhibition occurs (2×10^{-7} to 2×10^{-4} M) indicates that pig heart MAO shows a similar sensitivity toward clorgyline as the type B MAO component which has been described in many other animal tissues [e.g. Refs. 2–6]. However, it is clear that these inhibition curves for pig heart MAO show a significantly differing apparent sensitivity toward clorgyline, depending upon the substrate employed to assay remaining enzyme activity. Under the identical conditions of amount of protein, preincubation time, and length of assay period employed here, the estimated ID_{50} values for clorgyline against each substrate-metabolizing activity were 4.0×10^{-6} M (5-HT), 1.5×10^{-5} M (tyramine) and 2.5×10^{-5} M (benzylamine).

Clorgyline plots obtained with MAO substrate concentrations at $2 \times K_m$. It has previously been reported by others that irreversible inhibition of MAO, brought about by inhibitors such as pargyline which interact covalently with the flavin cofactor, may be preceded by an initial reversible binding of inhibitor, in the manner of a substrate [9]. It was important to eliminate the possibility that the different clorgyline plots of Fig. 1 were a result of the addition to the preincubated enzyme–inhibitor mixtures of competing substrates at different relative values of their respective K_m concentrations. We therefore repeated the inhibition studies shown in Fig. 1, with the difference that enzyme activity was measured at substrate concentrations of twice estimated K_m values. For 5-HT and tyramine, the concentrations studied were 950 and 600 μM respectively. For benzylamine, which may be metabolized by two kinetically different components (Table 2), we used 75 and also 1700 μM. The plots obtained are shown in Fig. 2.

It can be seen that, under these conditions, there was still a significant difference between the plots for 5-HT, tyramine and benzylamine (at 1700 μM). However, it is interesting that the activity toward benzylamine at 75 μM is more sensitive toward clorgyline, particularly at an inhibitor concentration of 2×10^{-5} M, than the corresponding activity at 1700 μM. This difference was significant and could be obtained reproducibly in other experiments.

Time-dependent inhibition of MAO by clorgyline. The inhibition plots shown in Fig. 1 indicated that a clorgyline concentration of 2×10^{-5} M produced a moderate degree of MAO inhibition, the extent of which varied with the substrate used to assay MAO. This clorgyline concentration was therefore chosen to study the possibility of time-dependent MAO inhibition during different preincubation periods at 37° of inhibitor with the enzyme. Semilog plots of the data obtained (Fig. 3) showed a linear relationship between decreasing enzyme activity and increasing preincubation time. The slopes of these plots were very similar when each of the three substrates were used at 1 mM final concentrations.

For a first-order process (indicated by these linear plots), the slope has a value of $-K/2.3$, where K is the first-order rate constant. Half-lives ($T_{\frac{1}{2}}$) for each of these plots were calculated from the relationship $T_{\frac{1}{2}} = 0.693/K$ and yielded values of 40.2 min (5-HT), 36.3 min (tyramine) and 38.6 min (benzylamine).

Despite the close agreement between $T_{\frac{1}{2}}$ values

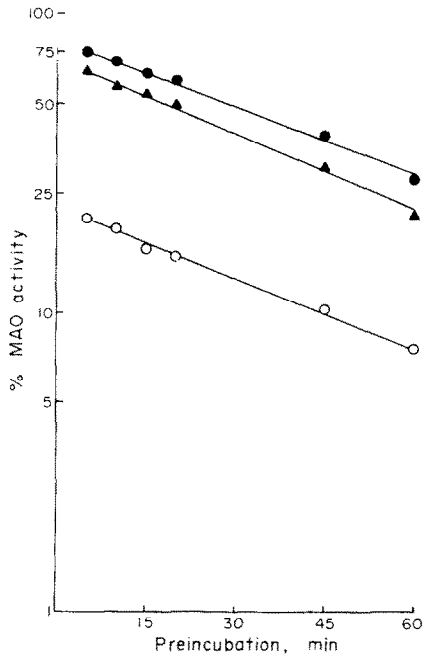

Fig. 3. Time-dependent inhibition of pig heart MAO. MAO activity in mitochondrial samples was assayed at final substrate concentrations of 1 mM after preincubation for various periods with clorgyline (2×10^{-5} M). Key: ○——○, 5-HT; ▲——▲, tyramine; and ●——●, benzylamine, as substrate. All points are the mean of duplicate determinations.

obtained with each substrate, backward extrapolation of these plots to zero time suggested an inhibition of MAO activity which occurred in the absence of preincubation, and also which represented different proportions of the total activity toward the three substrates used.

This possibility was confirmed directly in a subsequent experiment. Clorgyline (final conc. 1×10^{-5} M) and then substrate (final conc. 1 mM) was added to ice-cooled assay tubes containing mitochondrial samples. Enzyme activity was then assayed as usual by incubation for 30 min at 37°. Under these conditions, where no preincubation (at 37°) of enzyme and clorgyline in the absence of substrate had taken place, enzyme activity was still inhibited to different degrees depending upon the substrate used (75.3 per cent inhibition with 5-HT, 25.3 per cent with tyramine and 14.2 per cent with benzylamine).

Inhibition of pig heart MAO without preincubation at 37°. Several experiments were performed in an attempt to investigate the nature of the inhibition caused by clorgyline (2×10^{-5} M) upon pig heart MAO in the absence of the usual preincubation at 37° of drug with the enzyme. There appeared to be two other possible steps in our experiments, besides that of preincubation, during which inhibition of pig heart MAO by clorgyline may develop: (1) the period prior to preincubation, when inhibitor–enzyme mixtures are set up in ice-cooled reaction tubes, and (2) during the assay incubation period at 37° after the MAO substrate has been added to the inhibitor–enzyme mixture.

It was found that as long as mitochondrial samples were kept on ice in the presence of clorgyline, there was no evidence for the development of any significant degree of irreversible inhibition at this temperature for two reasons. First, the substrate-dependent differences in inhibition of MAO subsequently determined were not affected in magnitude, even if enzyme and inhibitor remained in contact on ice for periods from 15 min to 4 hr, thus ruling out a time-dependent inhibition mechanism occurring at this temperature. Second, dilution of similar inhibitor–enzyme mixtures (on ice) with ice-cold buffer resulted in a considerably reduced degree of inhibition of each substrate-metabolizing activity, as shown in Table 3. In fact, 10-fold dilution resulted in virtually no inhibition of enzyme activity. From these results it appears that any initial association of clorgyline (2×10^{-5} M) with pig heart MAO is freely reversible if kept at the temperature of the ice bath.

Another possibility to be considered was that the substrate-dependent differences in inhibition observed in the absence of preincubation may, in fact, represent inhibition that develops during the assay incubation period, in which both inhibitor and substrate are present. However, the different degrees of enzyme inhibition seen with the three substrates were still obtained and remained essentially unaltered, whether determined after assay times of 10, 20 or 30 min. It did not prove possible to assess these inhibitory effects at shorter assay times since the production of radioactive metabolites (particularly from 5-HT) became too low to make accurate estimations. Thus, it appears from these results that, during the periods we were able to study, a time-dependent inactivation of enzyme activity in the presence of substrate does not occur to any significant extent.

However, an indication that clorgyline may be able to combine very rapidly (perhaps instantaneous-

Table 3. Effects of dilution of ice-cold drug–enzyme mixtures upon inhibition by clorgyline of pig heart MAO*

Substrate	Dilution	MAO activity (% of uninhibited controls)
5-HT	None	31 ± 1
	1:5	66 ± 6
	1:10	89 ± 6
Tyramine	None	66 ± 5
	1:5	80 ± 2
	1:10	93 ± 2
Benzylamine	None	91 ± 1
	1:5	94 ± 1
	1:10	101 ± 3

* Reaction mixtures kept ice-cold contained 5.2 mg of mitochondrial protein, clorgyline (final conc. 2×10^{-5} M) in a total volume of 1 ml buffer (0.25 M sucrose, 0.01 M sodium phosphate, pH 7.8). Some mixtures were diluted 5- or 10-fold with ice-cold buffer. Samples (50 μl) were then taken for estimation of MAO activity as described in Materials and Methods at substrate concentrations of 1 mM. Activities are expressed as percentages of corresponding control samples containing no clorgyline. All values are the mean ± standard error of three determinations.

Table 4. Effects of dilution and washing of drug–enzyme mixtures after preincubation for 5 min at 37° upon inhibition by clorgyline of pig heart MAO*

Substrate	Type of sample	MAO activity (% of uninhibited controls)
5-HT	Undiluted	31 ± 1
	Diluted	47 ± 3
Tyramine	Undiluted	67 ± 2
	Diluted	88 ± 9
Benzylamine	Undiluted	89 ± 2
	Diluted	99 ± 7

* Reaction mixtures contained 10.4 mg of mitochondrial protein and clorgyline (final conc. 2×10^{-5} M) in a total volume of 2 ml buffer (0.25 M sucrose, 0.01 M sodium phosphate, pH 7.8). All mixtures were preincubated for 5 min at 37° and then rapidly ice-cooled. Some samples were diluted to a total volume of 20 ml with ice-cold buffer and then centrifuged at 30,000 *g* for 30 min (at 0°). The mitochondrial pellets were resuspended in 20 ml of ice-cold buffer, re-centrifuged at 30,000 *g* for 1 hr, and the resulting pellets were each resuspended in 1 ml buffer. Remaining MAO activity was measured at 1 mM substrate concentrations for samples obtained with and without the dilution and washing treatment. In each case, MAO activity is expressed as a percentage of the appropriate uninhibited control activities which contained no clorgyline. Each value is the mean ± standard error of three determinations.

ly) and to some degree irreversibly at 37° with pig heart MAO was obtained in a subsequent experiment. Mitochondrial samples were preincubated for a short period (5 min) with clorgyline (2×10^{-5} M) to ensure that the inhibitor–enzyme mixture reached the preincubation temperature of 37°. The mixtures were then cooled rapidly, diluted 10-fold with ice-cold buffer and the inhibited mitochondria were pelleted by centrifugation, washed, spun down again, and then resuspended as described in Table 4. All centrifugations were performed at 0° to prevent the possibility of any additional irreversible inhibition by clorgyline developing during these procedures. Remaining enzyme activity in inhibited samples was compared with the activity in control samples containing no clorgyline which had been subjected to the same experimental protocol. Also shown in Table 4 are the activities of inhibited samples (compared with their corresponding controls), which were not subjected to the dilution, centrifugation and washing procedures after the preincubation period, but instead were kept ice-cooled until all samples were ready for assay of MAO at the same time.

It can be seen that inhibited mitochondrial samples which did not receive the dilution and washing treatment again demonstrated the typical substrate-dependent differences in inhibition of MAO shown earlier. On the other hand, 10-fold dilution and washing of mitochondrial samples resulted in the partial reversal of this inhibition. However, this reversal was not as great as might have been expected, especially with 5-HT as substrate. Earlier in this section we have demonstrated that 10-fold dilution of similar mitochondrial samples that had been kept ice-cold with no preincubation at 37° in the presence of clorgyline resulted in almost complete reversal of the inhibition of each substrate-metabolizing activity. However, in the present experiment, 10-fold dilution *and washing* of samples that had been in contact with clorgyline at 37° for a short period of time resulted in virtually complete reversal of inhibition only with benzylamine as substrate. With tyramine and more particularly 5-HT, dilution and washing were less effective. From these results, it seems that besides a slower first-order time-dependent inhibition of pig heart MAO by clorgyline indicated in Fig. 3, a rapid, possibly instantaneous association of clorgyline with the enzyme takes place. At 0° this association appears to be reversible, whereas at 37°, some degree of irreversibility occurs; this latter mechanism is most pronounced with 5-HT, less so with tyramine and almost negligible with benzylamine as substrate. The proportion of enzyme activity which is rapidly inhibited by the combination of these mechanisms varies depending upon the substrate subsequently used to measure remaining enzyme activity. It seems that these differences are largely responsible for the differing clorgyline plots shown

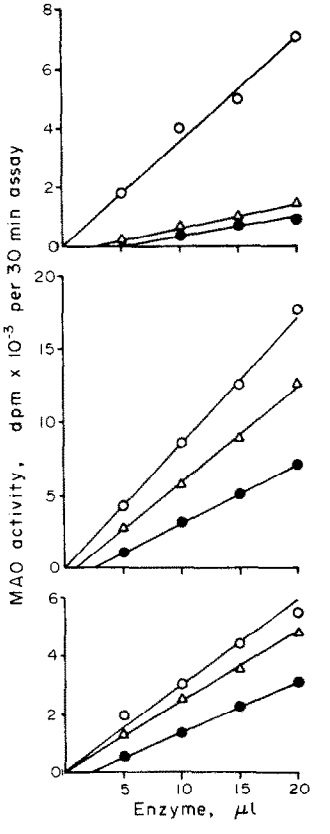

Fig. 4. Ackermann–Potter plots for pig heart MAO. Enzyme concentration was varied by altering amount (μl) of mitochondrial fraction in assay. Upper panel, 5-HT; middle panel, tyramine; and lower panel, benzylamine as substrate, all used at 1 mM final concentration. Key: O——O, no inhibitor; △——△, + clorgyline (2×10^{-5} M) without preincubation; and ●——●, + clorgyline. (2×10^{-5} M) with a 20-min preincubation. Each point is the mean of duplicate determinations.

in Fig. 1 since the slower pseudo first-order time-dependent inactivation of remaining enzyme activity shown in Fig. 3 proceeds with a half-life which is almost identical whichever substrate is used.

Effect of enzyme concentration on inhibition by clorgyline. Ackermann and Potter [10] have previously proposed a plot of reaction velocity against enzyme concentration in the presence and absence of inhibitor as a test for irreversible inhibition. In the absence of inhibition, or in the presence of a reversible inhibitor, these plots should pass through the origin, with a reduced slope for the plot obtained with inhibitor. Irreversible inhibition ideally results in a plot which intersects the enzyme concentration axis and is parallel to the control.

In the present experiments, the relationship between velocity and enzyme concentration for pig heart MAO was plotted by the method of Ackermann–Potter for three different conditions: (1) no inhibitor, (2) clorgyline (2×10^{-5} M) present without preincubation, and (3) clorgyline (2×10^{-5} M) present with preincubation. The results obtained are shown in Fig. 4.

With each of the substrates used at 1 mM final concentration, plots obtained after a 20-min preincubation of inhibitor with enzyme were not parallel to control, but also did not pass through the origin. It has been proposed that plots of this nature represent pseudo-irreversible inhibition of an intermediate type (see Discussion). The intercept on the enzyme concentration axis was greatest with 5-HT and least with benzylamine as substrate. In the absence of preincubation, smaller intercepts were obtained for 5-HT and tyramine, and a "reversible" plot passing through the origin was obtained for benzylamine.

Reciprocal plots in the presence of clorgyline. Reaction velocities were measured at a variety of substrate concentrations for pig heart MAO that had been preincubated for 20 min in the presence and absence of clorgyline (2×10^{-5} M). Reciprocal plots for these results are shown in Figs 5 and 6.

Inhibition by clorgyline of tyramine and benzylamine metabolism (both high- and low-affinity components) appeared to be non-competitive. However, the inhibition of 5-HT metabolism was of a "mixed-type."

Although the reciprocal plots are not shown, these conclusions were identical when similar inhibition experiments were performed on pig heart mitochondria that were not preincubated at 37° with clorgyline before the addition of substrate for the assay.

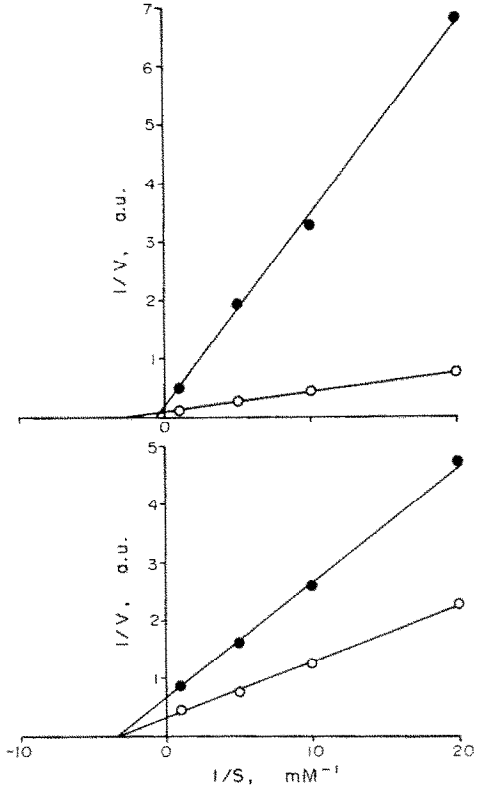

Fig. 5. Reciprocal plots of initial reaction velocities (V_m in arbitrary units) at different substrate concentrations (S) for pig heart MAO. Upper panel, 5-HT; and lower panel, tyramine as substrate. MAO activity in mitochondrial samples was assayed by addition of substrate after a 20-min preincubation. Key: O——O, no inhibitor; and ●——●, clorgyline (2×10^{-5} M) present in preincubation period. Each point is the mean of duplicate determinations.

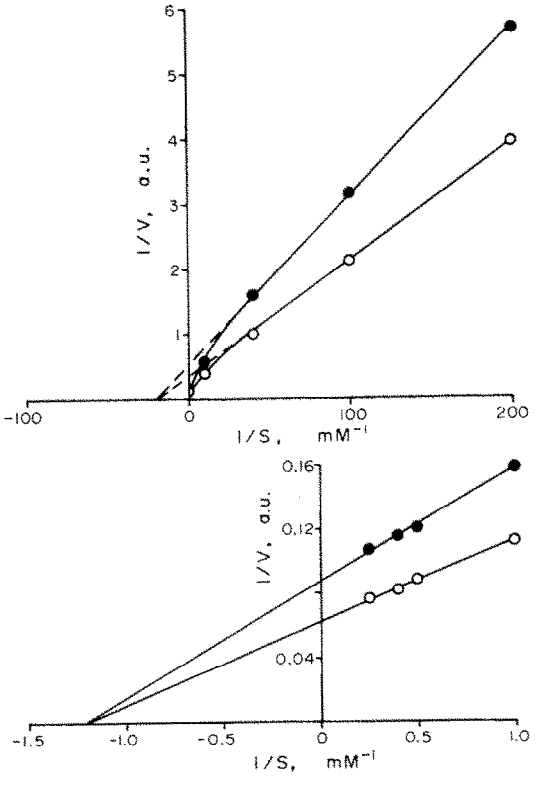

Fig. 6. Reciprocal plots of initial reaction velocities (V, in arbitrary units) at different substrate concentrations (S) for pig heart MAO, using benzylamine at lower concentrations (5 μM–1 mM, upper panel) and higher concentrations (1–4 mM, lower panel). MAO activity in mitochondrial samples was assayed by addition of substrate after a 20-min preincubation. Key: O——O, no inhibitor; and ●——●, clorgyline (2×10^{-5} M) present in preincubation period. Each point is the mean of duplicate determinations.

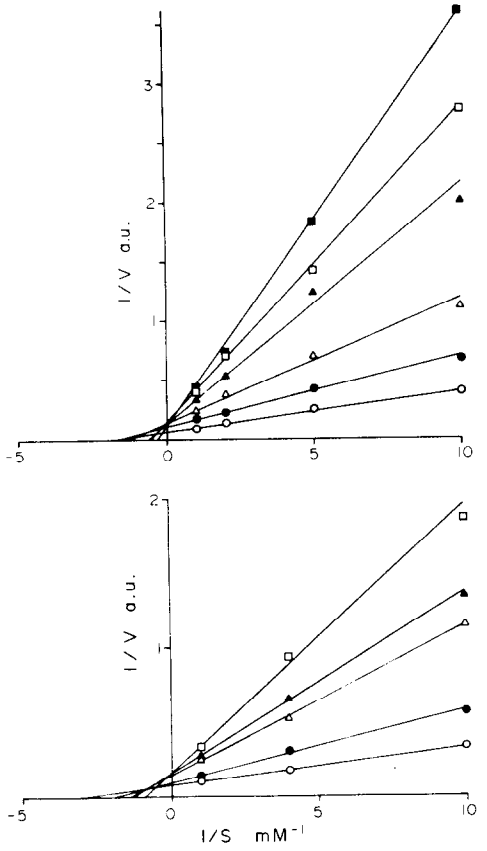

Fig. 7. Effects of unlabeled benzylamine (upper panel) or tyramine (lower panel) upon Lineweaver–Burk plots for [^{3}H]5-HT metabolism by pig heart mitochondrial MAO. Initial reaction velocities (V in arbitrary units) were determined at various 5-HT concentrations (S) in the absence and presence of different concentrations of benzylamine or tyramine. Benzylamine concentrations: ○——○, none; ●——●, 50 μM; △——△, 200 μM; ▲——▲, 500 μM; □——□, 750 μM; and ■——■, 1 mM. Tyramine concentrations: ○——○, none; ●——●, 200 μM; △——△, 500 μM; ▲——▲, 750 μM; and □——□, 1 mM. Each point is the mean of duplicate determinations.

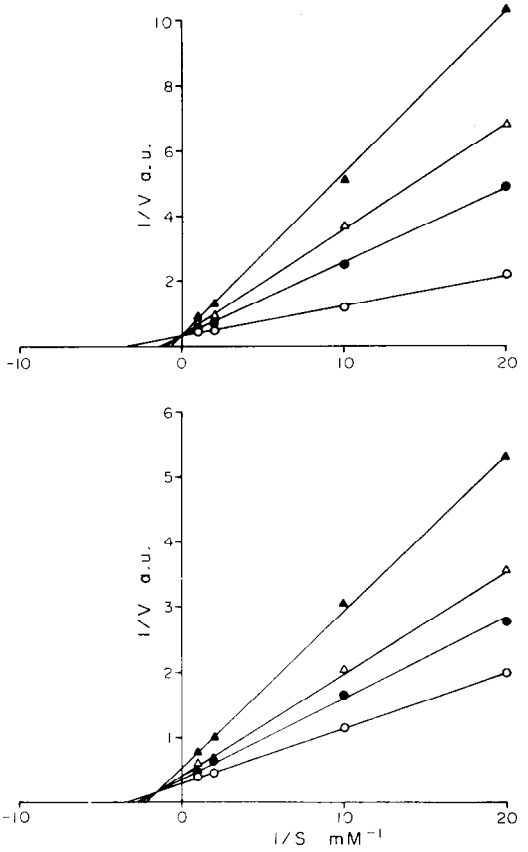

Fig. 8. Effects of unlabeled benzylamine (upper panel) or 5-HT (lower panel) upon Lineweaver–Burk plots for [^{3}H]tyramine metabolism by pig heart mitochondrial MAO. Initial reaction velocities (V in arbitrary units) were determined at various tyramine concentrations (S) in the absence and presence of different concentrations of benzylamine or 5-HT. Benzylamine concentrations: ○——○, none; ●——●, 250 μM; △——△, 500 μM; and ▲——▲, 1 mM. 5-HT concentrations: ○——○, none; ●——●, 1 mM; △——△, 2 mM; and ▲——▲, 4 mM. Each point is the mean of duplicate determinations.

Mixed-substrate studies. The metabolism by pig heart mitochondrial MAO of each radioactively labeled substrate ([^{3}H]5-HT, [^{3}H]tyramine and [^{14}C]benzylamine) was studied in the absence and presence of different concentrations of the other unlabeled substrates. For these studies, initial reaction velocities were measured at varying concentrations of the radiolabeled substrate, and double-reciprocal plot analysis was used to determine the inhibition caused by the various substrates upon the metabolism of each other.

Inhibition of [^{3}H]5-*HT metabolism.* Figure 7 (upper panel) shows the effects of unlabeled benzylamine (50, 200, 500 and 750 μM, and 1 mM) upon [^{3}H]5-HT metabolism. From these double-reciprocal plots it appears that benzylamine is a mixed-type inhibitor of 5-HT oxidation, although the interaction may be complex since there was no common intersection point for these plots. In fact, with 50 μM benzylamine, a non-competitive inhibition mechanism was indicated.

The lower panel of Fig. 7 shows the effects of unlabeled tyramine (200, 500 and 750 μM, and 1 mM) upon 5-HT metabolism. Again, a mixed-type inhibition was indicated.

Inhibition of [^{3}H]*tyramine metabolism.* Tyramine metabolism was inhibited in a competitive fashion by the different concentrations of unlabeled benzylamine used (250 and 500 μM, and 1 mM). These results are shown in Fig. 8 (upper panel). However, 5-HT at concentrations of 1, 2 and 4 mM was a mixed-type inhibitor of tyramine metabolism (lower panel).

Inhibition of [^{14}C]*benzylamine metabolism.* Earlier data in this paper have shown that benzylamine appears to be metabolized by both a low- and high-affinity catalytic site by pig heart MAO (see Fig. 6). Figure 9 shows that, in the presence of unlabeled 5-HT (upper panel) or tyramine (lower panel), the non-linearity of the corresponding reciprocal plots for metabolism of low benzylamine concentrations included those regions of the plots which had previously been used in the absence of other substrates to estimate the K_m of the high-affinity component.

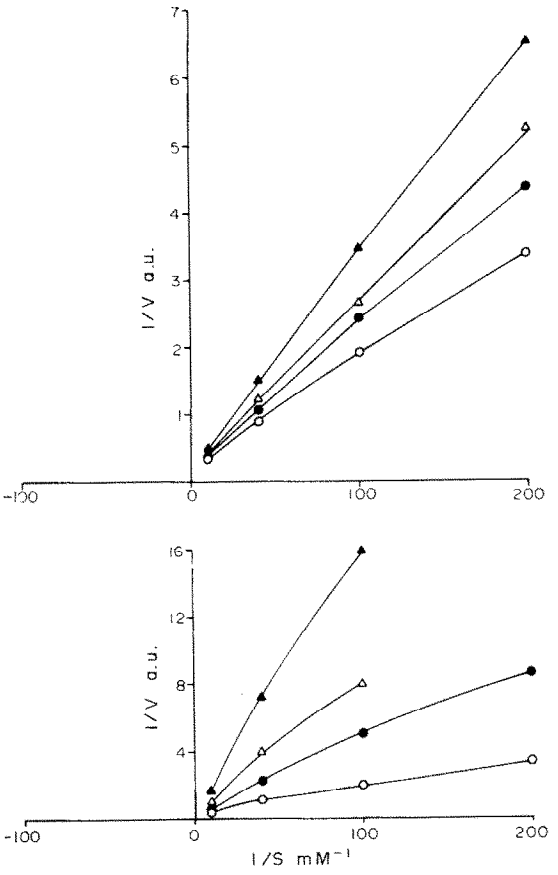

Fig. 9. Effects of unlabeled 5-HT (upper panel) and tyramine (lower panel) upon Lineweaver–Burk plots for metabolism of lower concentrations of [^{14}C]benzylamine by pig heart mitochondrial MAO. Initial reaction velocities (V in arbitrary units) were determined at various benzylamine concentrations (S) in the absence and presence of different concentrations of 5-HT or tyramine. 5-HT concentrations: ○——○, none; ●——●, 1 mM; △——△, 2 mM; and ▲——▲, 4 mM. Tyramine concentrations: ○——○, none; ●——●, 500 μM; △——△, 1 mM; and ▲——▲, 2 mM. Each point is the mean of duplicate determinations.

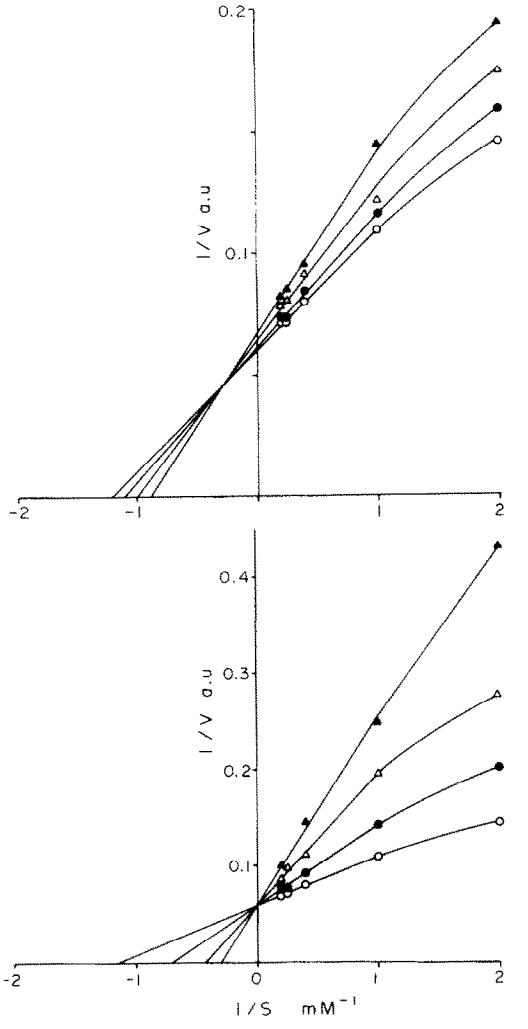

Fig. 10. Effects of unlabeled 5-HT (upper panel) and tyramine (lower panel) upon Lineweaver–Burk plots for metabolism of higher concentrations of [^{14}C]benzylamine by pig heart mitochondrial MAO. Initial reaction velocities (V in arbitrary units) were determined at various benzylamine concentrations in the absence and presence of different concentrations of 5-HT or tyramine. See Fig. 9 for corresponding key to symbols. Each point is the mean of duplicate determinations.

For this reason, it proved impossible to determine with any confidence the nature of the inhibition caused by tyramine or 5-HT on the high-affinity benzylamine-metabolizing component.

In contrast, at the higher benzylamine concentrations which have been used to distinguish a low-affinity site for benzylamine, the reciprocal plots in the presence and absence of unlabeled substrates were linear, and these results (Fig. 10) indicated that this component for benzylamine is inhibited in a mixed fashion by 5-HT (upper panel) whereas a competitive inhibition by tyramine was indicated (lower panel).

Thermal denaturation studies. Remaining MAO activity was assayed in mitochondrial samples that had been heated for different periods of time at 58° in a water bath. The three substrates 5-HT, tyramine and benzylamine were used (at final assay concentrations of 1 mM) to investigate possible differences in the rate of disappearance of enzyme activity toward each of these substrates. Data for the inactivation of MAO activity as a function of heating time were plotted semi-logarithmically as a percentage of unheated control activity as shown in Fig. 11 for 5-HT (upper panel), tyramine (middle panel) and benzylamine (lower panel).

The inactivation curves (denoted by solid symbols) were analyzed into two linear components by the "backward projection" method used by Jarrott [11] in similar experiments. A slowly inactivated linear component could be defined in each curve beyond a heating time of 15–20 min. Backward projection and subtraction of this component from the initial region of the curve yielded data points (open symbols) which indicated a linear, rapidly inactivated enzyme component for each substrate. Estimated proportions of the two components and half-lives for their inactivation are shown in Table 5 for each of the three substrates used to obtain these results.

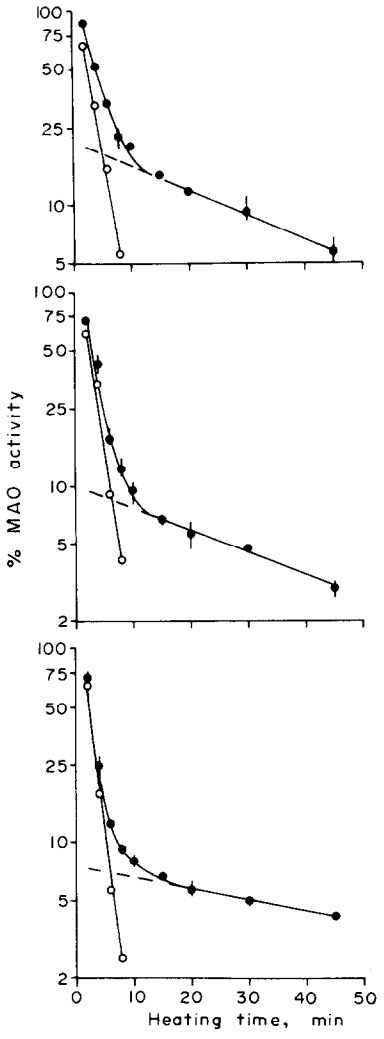

Fig. 11. Time course of heat inactivation at 58° of pig heart mitochondrial MAO. Upper panel, 5-HT; middle panel, tyramine; and lower panel, benzylamine as substrate at 1 mM final concentration. Solid symbols (●) represent experimental data points, shown as means and ranges (where they exceed symbol size) for triplicate determinations. Open symbols (○) represent the rapidly inactivated enzyme component resolved from the experimental curves as described in the text.

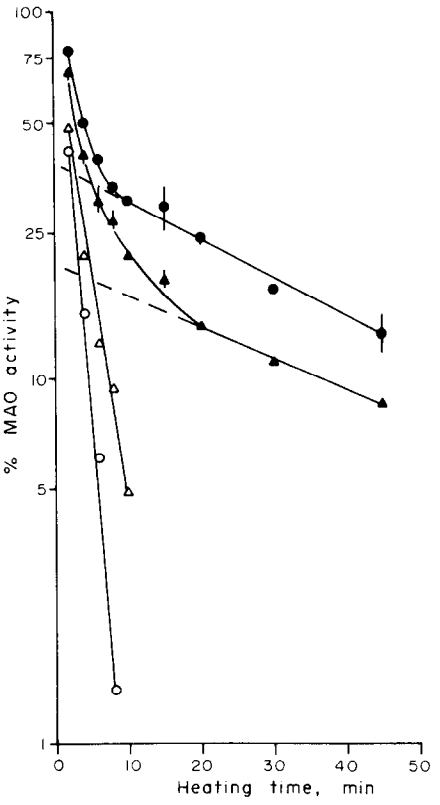

Fig. 12. Time course of heat inactivation at 58° of pig heart mitochondrial MAO activity toward benzylamine. Experimental data points determined at benzylamine concentrations of 25 μM (●) and 1 mM (▲). Each point is the mean and range (where it exceeds symbol size) for triplicate determinations. Rapidly inactivated enzyme components (○, 25 μM; and △, 1 mM benzylamine) were resolved from the corresponding experimental curves as described in the text.

These heat denaturation studies for the three substrates were performed in parallel within the same experiment, using a single mitochondrial fraction from pig heart that had been frozen for storage, and thawed once. When other pig heart mitochondrial fractions were used in this way, it was found that the relative proportions and half-lives of the fast and slow components for each substrate did vary somewhat from preparation to preparation. It is not clear whether these quantitative variations were due to differences in the structural integrity of the mitochondrial membranes or other unknown factors, despite our attempts to ensure that fractions were prepared and stored under identical conditions. In this context, it should be pointed out that the two-component nature of these inactivation

Table 5. Relative proportions and half-lives ($T_{\frac{1}{2}}$) for inactivation of linear components resolved from heat denaturation curves shown in Fig. 11*

	Thermolabile component		Thermostable component	
Substrate	Proportion (%)	$T_{\frac{1}{2}}$ (min)	Proportion (%)	$T_{\frac{1}{2}}$ (min)
5-HT	78	1.6	22	25
Tyramine	90	1.4	10	26
Benzylamine	93	1.2	7	55

* Proportions of each component are expressed as a percentage of the total activity toward the corresponding substrate.

Table 6. Relative proportions and half-lives ($T_{\frac{1}{2}}$) for inactivation of linear components resolved from heat denaturation curves shown in Fig. 12*

Benzylamine concn (μM)	Thermolabile component		Thermostable component	
	Proportion (%)	$T_{\frac{1}{2}}$ (min)	Proportion (%)	$T_{\frac{1}{2}}$ (min)
25	61	1.5	39	31
1000	79	2.3	21	35

* Proportions of each component are expressed as a percentage of the total activity toward benzylamine measured at the corresponding concentration.

curves was not due to the freezing–thawing process, since similar curves resulted from an unfrozen mitochondrial fraction used shortly after preparation.

In the light of these findings, certain general conclusions from these studies could be reached. First, each substrate-metabolizing activity comprises, in major part, a rapidly inactivated enzyme component ($T_{\frac{1}{2}}$ of 1–2 min), along with a smaller, slowly inactivated component ($T_{\frac{1}{2}} > 25$ min). Second, there was consistently a greater proportion of the slowly inactivated component for 5-HT than for tyramine or benzylamine metabolism. The relative proportions of the two components for tyramine and benzylamine were very similar.

Our previous kinetic studies in this paper have indicated that benzylamine may be metabolized by a high- and low-affinity catalytic activity in pig heart mitochondria. It was therefore of interest to investigate whether or not heat inactivation curves are identical when determined at relatively low or high benzylamine concentrations. Figure 12 shows the results of an experiment in which the corresponding curves were obtained using final benzylamine concentrations of 25 μM and 1 mM in the assay. At these concentrations, one is presumably measuring to a large degree (though not exclusively) the properties of the high- and low-affinity component, respectively, for benzylamine. As before, each resultant heat inactivation curve could be analyzed as two linear components representing fast and slow inactivations of enzyme activity. The corresponding proportions and half-lives for these components are shown in Table 6. From these results, it appears that some differences do exist for the heat sensitivity of MAO activity when measured at different benzylamine concentrations. In particular, there was a relatively greater proportion of the slowly inactivated component when 25 μM rather than 1 mM was the final MAO assay concentration employed.

DISCUSSION

At least two functional forms of the mitochondrial enzyme MAO are believed to exist in differing proportions in a variety of animal tissues. These forms, designated type A and type B MAO, were originally defined on the basis of their differing relative sensitivities to the irreversible MAO inhibitor clorgyline [2]. In a number of tissues so far studied, 5-HT and benzylamine are substrates only for type A and type B MAO, respectively, whereas tyramine can be metabolized by either enzyme form [3]. However, recent inhibition studies on cardiac tissue from the rat [4] or ox [5] have indicated that 5-HT and benzylamine can be substrates for both types A and B.

Previous reports in the literature have suggested that the enzyme contained in pig tissues (liver or brain) may be homogeneous with respect to irreversible inhibition by 2-bromo-2-phenylacetaldehyde [12] or clorgyline [6]. These studies with clorgyline indicated the predominance of type B MAO, although a more recent paper has suggested that both type A and type B MAO are present in similar proportions in these tissues [7]. We chose to examine substrate metabolism and its sensitivity toward clorgyline in another pig tissue, the heart.

Pig heart MAO was able to metabolize all three substrates 5-HT, tyramine and benzylamine. K_m determinations by reciprocal plot analysis yielded linear plots for 5-HT and tyramine, but a curved plot with two linear regions for benzylamine, from which a high- and low-affinity K_m value was estimated. It is not clear whether this curved plot results from the phenomenon of negative cooperativity, or whether it represents two distinct sites (or enzymes?) which metabolize benzylamine in this tissue. These results are similar to curved plots obtained for benzylamine metabolism in rat heart mitochondria [4]. However, in contrast to the rat, neither component of benzylamine oxidation (nor also 5-HT or tyramine metabolism) was inhibited after preincubation for 20 min with semicarbazide (2 mM) or potassium cyanide (2 mM). Thus, we have excluded the possibility that the benzylamine metabolism which we have measured includes a significant contribution by either connective tissue or plasma-type enzymes.

Inhibition studies obtained after preincubation of pig heart mitochondrial MAO with a range of clorgyline concentrations yielded single-sigmoid inhibition curves, with apparent sensitivities toward clorgyline which suggested that type B MAO alone was responsible for the metabolism of each substrate used. However, there were slight but significant differences in the apparent sensitivities of the substrate-metabolizing activities indicated by these curves. We have shown previously that variation of such factors as amount of protein in the assay and preincubation time can affect the apparent sensitivity of a given MAO form toward clorgyline [8]. For this reason, the present inhibition curves were obtained together under identical experimental conditions for each substrate. Thus, variations in these factors can be ruled out as a cause of the present results.

Irreversible inhibition of MAO by propargyl-

amine derivatives such as clorgyline or pargyline is believed to involve the covalent and stoichiometric interaction of the drug with the flavin adenine dinucleotide cofactor at the enzyme-active site. It has been proposed that the initial enzyme–inhibitor binding is reversible [9]. A fixed inhibitor concentration can lead to different degrees of reversible competitive inhibition of enzyme activity toward different substrates unless those substrates are used at concentrations which are at a constant ratio to their respective K_m concentrations. This result can sometimes erroneously be interpreted as indicating multiple enzyme forms. Consequently, the log dose inhibition curves were repeated at substrate concentrations which were twice their estimated K_m values. The resultant plots were still significantly different, although the difference between the plot for tyramine and the high-affinity benzylamine-metabolizing component (assayed at 75 μM) had almost disappeared.

We investigated other possible reasons for the differences observed in the inhibition curves. Inhibition of MAO activity toward each substrate was time dependent and (pseudo) first order, presumably indicating that the inhibitor (at 2×10^{-5} M) was considerably in excess of the amount of enzyme present. First-order rate constants (and thus half-lives) for this process were virtually identical for all three substrates used to assay MAO. However, the substrate-dependent differences in sensitivity toward this concentration of clorgyline appear to be due to differences in the proportions of enzyme activity which are rapidly or possibly instantaneously inactivated by clorgyline. Experiments designed to investigate the nature of this initial rapid inhibition of pig heart MAO by clorgyline revealed that any association at ice temperature of the drug with enzyme appears to be almost completely reversible. However, contact for a short period of time at 37° caused, in addition, a degree of irreversible inhibition of enzyme activity which was greater with 5-HT than tyramine, and almost negligible with benzylamine as substrate.

By the criteria of Ackermann and Potter [10] our plots indicated that after preincubation, clorgyline was an irreversible inhibitor of MAO activity toward 5-HT, tyramine and benzylamine, although from the intercepts on the enzyme concentration axis, it appears that the corresponding amounts of enzyme inactivated (in terms of μl of mitochondrial fraction added) are different depending upon the substrate used to assay activity. Both 5-HT and to a lesser degree tyramine metabolism were irreversibly inactivated even in the absence of preincubation with clorgyline, whereas the plot for benzylamine indicated reversible inhibition. Thus, these results provide additional support for similar conclusions discussed earlier in relation to the effects of dilution and washing of mitochondria on the reversibility of the inhibition of these activities.

Mantle *et al.* [13] have pointed out that genuine irreversible inactivation may in some cases lead to "reversible" Ackermann–Potter plots. They showed that irreversible inhibition of the type

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_2} EI'$$

will yield a reversible plot when formation of the irreversibly inactivated enzyme (EI') is occurring relatively slowly (if $K_i = k_{-1}/k_1$ is large and/or k_2 is small) and has not gone to completion. It is only when inhibitor binding is very tight, or inactivation of a fraction of enzyme activity is essentially instantaneous, that the "irreversible" plot is obtained.

Our Ackermann–Potter plots for inhibited pig heart MAO (i.e. neither parallel to control nor passing through the origin) are similar to plots that these workers obtained for inhibition by 5-phenyl-3-(*N*-cyclopropyl)ethylamine-1,2,4-oxadiazole (PCO) of 5-HT metabolism in rat liver mitochondria, a result which they ascribed to a high affinity between PCO and the enzyme, and which they termed "pseudo-irreversible" inhibition of an intermediate type. It therefore seems likely that our plots also represent this "intermediate" type of inhibition, although from our results it appears that relative differences in the degrees of rapid reversible and irreversible inhibition of the substrate-metabolizing activities give rise to the differences observed in the plots of Fig. 4.

Lineweaver–Burk plots for inhibition of tyramine and benzylamine metabolism (both components) by clorgyline indicated a non-competitive inhibition mechanism whether or not preincubation at 37° was employed. Clearly, analysis of this kind does not distinguish between true reversible non-competitive inhibition and the case where irreversible inactivation has removed a proportion of the total enzyme activity [14]. However, previous experiments in this paper have shown clearly that, under these conditions, some degree of reversible inhibition (depending upon the substrate employed) should play a part in determining the nature of the inhibition observed. Thus, it is interesting that clorgyline appears to be a reversible non-competitive inhibitor of tyramine and benzylamine metabolism by pig heart MAO, particularly since previous reports have suggested that other irreversible propargylamine MAO inhibitors are competitive with MAO substrates in their reversible phase [15, 16]. On the other hand, it is not clear why the corresponding plot for inhibition of 5-HT metabolism showing mixed-type inhibition should be different from those using tyramine or benzylamine. This plot could arise from either a "mixed" or "competitive" type of reversible inhibition plot being superimposed upon the "non-competitive" plot expected for the accompanying irreversible inhibition of activity toward 5-HT. Metabolism of amine substrates by MAO is believed to occur by a Ping-Pong mechanism [17] and resulting kinetic studies could be influenced by inhibitors binding reversibly to either the oxidized or reduced (or both) forms of the enzyme. At the present time we are unable to distinguish whether different kinetic mechanisms may exist for the reversible inhibition by clorgyline of 5-HT metabolism compared with tyramine and benzylamine.

In mixed-substrate experiments, the effects of unlabeled substrates upon the metabolism of each radioactively labeled substrate were examined. From the corresponding Lineweaver–Burk plots, tyramine and benzylamine proved to be competitive inhibitors of each other's metabolism. However, in the case of benzylamine oxidation, this conclusion

could only be made confidently for inhibition by tyramine of the low-affinity component of enzyme activity toward benzylamine.

On the other hand, corresponding experiments using substrate pairs involving 5-HT indicated predominantly a mixed type of inhibition. This occurred when [^{3}H]5-HT metabolism was measured in the presence of unlabeled tyramine or benzylamine, or when the converse experiments were performed in which unlabeled 5-HT inhibited the metabolism of [^{3}H]tyramine or [^{14}C]benzylamine (low-affinity component).

Previous data showing mixed or non-competitive inhibition kinetics between substrates for MAO from human platelets, another tissue which from studies with clorgyline contains only the B type enzyme [18, 19], were considered as showing that separate interacting catalytic sites exist for MAO in this tissue [20]. However, similar results from kinetic studies with substrates for type B MAO of human brain have been interpreted as indicating that the substrates used shared a common catalytic binding-site, as well as an inhibitory site on the reduced form of the enzyme, thus rendering it unnecessary to invoke multiple forms of the enzyme as an explanation for the kinetics [21]. In these latter experiments, it was found that non-competitive kinetics from reciprocal plots for the effects of phenylethylamine and benzylamine upon each other's deamination became competitive if enzyme activities were measured at oxygen concentrations which were probably sufficient to saturate the reduced form of the enzyme. For the present results, all assays were performed in reaction tubes that had been flushed with oxygen, although it cannot be assumed that the pig heart oxidase was saturated in our experiments. If 5-HT, tyramine and benzylamine were able to bind to both oxidized and reduced forms of only a single enzyme form in pig heart, mixed-type inhibition kinetics at unsaturating, or competitive kinetics at saturating, oxygen concentrations would be expected. However, under the given conditions employed for our experiments, the results do not seem to be consistent with this interpretation since, for instance, tyramine was a mixed-type inhibitor of 5-HT metabolism, but at similar inhibitory concentrations was a competitive inhibitor upon benzylamine metabolism. Thus, the nature of the inhibition patterns was dependent upon the mixtures of substrates employed, and may indicate that the catalytic binding sites for these substrates are not identical. In addition, Roth [21] found no differences in sensitivity toward clorgyline (or pargyline) of phenylethylamine and benzylamine catalytic sites of human brain MAO, although we have shown in this paper that substrate-dependent differences do exist for pig heart MAO, due mainly to different degrees of rapid irreversible inhibition, thus providing further support for the possible heterogeneity of the pig heart enzyme.

Heat inactivation studies with MAO from various tissues of different animal species have also been cited as evidence for the heterogeneity of this enzyme. For instance, non-linear semi-log plots of loss of enzyme activity as a function of heating time have indicated different inactivation kinetics for multiple components of total enzyme activity within particular tissues [11, 22]. In the present experiments, a thermolabile ($T_{\frac{1}{2}} \sim 1$–2 min) component and a thermostable ($T_{\frac{1}{2}} > 25$ min) component of pig heart MAO were detected with each of the substrates used. The thermolabile component was responsible for the major proportion of total enzyme activity. However, there was an indication that the relative proportions of the two components were different for each substrate-metabolizing activity. In comparisons using any given mitochondrial preparation, 5-HT metabolism was relatively more resistant than tyramine or benzylamine metabolism to the effects of heat treatment, due to a greater proportion of the thermostable component. In these studies, enzyme activities were assayed at final substrate concentrations of 1 mM. Separate experiments indicated a relatively greater proportion of the thermostable component when benzylamine metabolism was measured at 25 μM rather than 1 mM final concentrations. In the light of our evidence that benzylamine may be metabolized at two kinetically distinct sites in pig heart mitochondria, it is interesting that these heat denaturation studies may reflect further differences in the nature of these sites.

It is difficult at this stage to reconcile the double-component heat inactivation curves for MAO in the present results with the inhibition studies obtained with clorgyline earlier. Single-sigmoid inactivation curves showing different sensitivities toward clorgyline of the different substrate-metabolizing activities seem to indicate single non-identical enzyme activities responsible for the metabolism of different amines. Squires [22] has provided additional examples of other animal tissues which from inhibition criteria appear to contain a single MAO form acting upon kynuramine, but possess multiple-enzyme components with respect to heat denaturation. The nature of these different components is unclear but may reflect varying degrees of protection from thermal inactivation of substrate-binding regions by mitochondrial lipids. In this context, Oreland and Ekstedt [23] have demonstrated that binding of lipid to purified pig liver MAO results in a considerable increase in thermal stability.

Taken as a whole, and using criteria which have been employed in the past by others to investigate the possibility that multiple forms of MAO exist, the experiments described here suggest that type B MAO of pig heart mitochondria may, in fact, be heterogeneous. At the present time, it is unclear whether multiple MAO forms represent different protein molecules, or a single protein whose inhibitor and substrate specificities may be regulated by the outer mitochondrial membrane microenvironment of the enzyme [24]. Recent immunological studies on rat liver MAO have supported the latter hypothesis [25]. At present, we cannot distinguish from these studies whether the various substrate-metabolizing activities in pig heart are distributed differently throughout the outer membrane, or alternatively if these results are due to multiple binding-sites located together around a single type of catalytic center. Severina [26] has provided evidence for this latter possibility in studies with pig liver MAO. Whatever the explanation for our results

it appears that any interpretation that type A or B MAO (defined on the basis of sensitivity toward clorgyline) represents single enzyme species in animal tissues may turn out to be an oversimplification of the true situation.

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