Biochemical Pharmacology, Vol. 29, pp. 2225–2233.

0006-2952/80/0815–2225 $02.00/0

THE NATURE OF THE SUBSTRATE-SELECTIVE INTERACTION BETWEEN RAT LIVER MITOCHONDRIAL MONOAMINE OXIDASE AND OXYGEN

CHRISTOPHER J. FOWLER* and LARS ORELAND
Department of Pharmacology, University of Umeå, S-901 87 Umeå, Sweden

(*Received* 7 *February* 1980; *accepted* 17 *March* 1980)

Abstract—The activity of rat liver monoamine oxidase was increased in an uncompetitive manner with increasing concentrations of oxygen. However, the value of the Michaelis constant for oxygen, estimated from determinations of enzyme activity with 6 oxygen concentrations and 5–6 amine substrate concentrations, was dependent upon the amine substrate used to assay for activity. In an attempt to determine the nature of these differences, the value of the Michaelis constants for oxygen have been related to the k_{cat} (rate constant of the limiting step in the overall enzyme-catalysed reaction) values of the enzyme towards the different amine substrates. The results are consistent with the hypothesis that the two forms of monoamine oxidase in rat liver are not independent enzyme forms, but interact one with the other in some way.

Monoamine oxidase (monoamine: O_2 oxidoreductase, MAO, EC 1.4.3.4) is thought to exist as two forms, termed MAO-A and MAO-B, on the basis of the sensitivity to inhibition by substrate-selective inhibitors, such as clorgyline and l-deprenil (see ref. 1). MAO-A is sensitive to inhibition by clorgyline but fairly resistant to inhibition by l-deprenil, whereas the reverse is true for MAO-B [2, 3]. According to this definition, in the rat liver, 5-hydroxytryptamine is metabolized by MAO-A alone, β-phenethylamine and benzylamine by MAO-B alone, and tyramine, dopamine and tryptamine by both forms of the enzyme [4, 5]. Furthermore, clorgyline and l-deprenil are so potent as inhibitors of the two forms of MAO that it has been possible to use them to 'titrate' the concentrations of these two enzyme forms [6–8].

In a variety of tissues, MAO has been shown to follow a ping-pong, or double-displacement reaction pathway [9–12]. As a consequence of this, the activity of MAO is increased in an uncompetitive manner as the concentration of the second substrate, oxygen, is increased. Parallel regression lines on a Lineweaver–Burk plot for enzyme activity assayed under atmospheres of air and oxygen have also been shown for a variety of substrates in rat liver [13], rat heart [14], human platelet [15] and human brain [16], consistent with a ping-pong reaction. However, the degree of increase in activity with increased oxygen concentrations, and hence the value of the apparent Michaelis constant for oxygen (K_0) that can be estimated from these data, depends upon the amine substrate used to assay for MAO activity [13–16]. A similar conclusion can be derived from the data of Von Korff [17] for rabbit brain MAO.

It was suggested previously that these differences in K_0 values could be due to some sort of enzyme heterogeneity unrelated to MAO-A or -B [13]. Thermal inactivation experiments have also suggested heterogeneous forms of MAO-B in mouse tissues and in pig heart [18, 19], and in the rat liver, the deamination of two MAO-B substrates, β-phenethylamine and benzylamine, are affected differentially by tris buffers [20]. The interaction between MAO and oxygen has therefore been studied in greater detail, in an attempt to establish whether or not these differences in K_0 values are due to some novel enzyme multiplicity, or due to the nature of the enzyme reaction pathway. A preliminary report of some of these data has been presented [21].

MATERIALS AND METHODS

Six rats, of body weight 253 ± 1 g (liver weight 12.5 ± 0.3 g), were killed by a blow to the head, and the livers rapidly removed, blotted on filter paper and weighed. The livers were homogenized 1 : 8 (w/v) in 'sucrose buffer' (0.25 M sucrose, 10 mM potassium phosphate, pH 7.8) in an MSE Atomix blender at setting 1 for 10 sec. The homogenates were centrifuged at 600 *g* for 15 min to remove nuclei and cell debris, and the resulting supernatants were then centrifuged at 6500 *g* for 20 min to yield pellets which were washed (by resuspension followed by centrifugation at 6500 *g* for 20 min). The washed pellets were then resuspended in sucrose buffer and stored frozen until used for assay. This procedure has been shown to produce a preparation of mitochondrial membranes with little contamination from mitochondrial matrix, lysosomal or microsomal marker enzymes [14], presumably due to the shearing action of the Atomix homogenizer. This membrane structure was also confirmed by electron microscopy [14]. The properties of the MAO in the membrane fraction were the same as those found for the enzyme in crude homogenates homogenized by either Atomix or in a conical glass homogenizer [14].

Monoamine oxidase activity was assayed radiochemically by the method of Callingham and Laverty [22], as modified by Fowler *et al.* [15]. Briefly, ali-

* To whom all correspondence should be addressed.

quots of the enzyme preparation were incubated in 15 ml glass centrifuge tubes with the radiolabelled substrate (of specific activity 0.5–2.0 μCi/μM, dissolved in sucrose buffer) at 37° in a final volume of 100 μl before inactivation by the addition of 10 μl of 3M hydrochloric acid. Time courses of the reaction were drawn for incubation periods of between 1 and 20 min, and incubation periods throughout were chosen to be on the linear phase of the reaction. The deaminated metabolites were extracted into ethyl acetate–toluene (1 : 1 v/v, saturated with water) and the radioactivity in each sample determined by scintillation spectroscopy. Values from blanks (where distilled water was used in place of the enzyme preparation) were subtracted from the values in the presence of the enzyme preparation to give the net recovered radioactivity, which was converted into nmoles of substrate metabolized. All stages of the assay up to the acid inactivation, with the exception of the incubation periods, were carried out at 0°. Unless otherwise stated, all incubations were carried out under an atmosphere of air.

The efficiency of extraction of the deaminated metabolites into the organic layer was determined in two ways. First, a concentrated preparation of mitochondrial membranes was incubated with substrate (20 μM) for 20 min, and the metabolites were then extracted and counted for radioactivity. At incubation times of longer than 20 min, no further deamination was found, so it could be assumed that all of the available substrate had been metabolized. The efficiency of extraction was then calculated as the net radioactivity recovered/net radioactivity originally added, and expressed as a percentage. This value represents a lower limit to the extraction efficiency, as it does not take into account other time-dependent factors that might be present, such as the stability of the substrate over the long incubation periods, which would prevent the reaction going to completion. Secondly, acid-inactivated reaction mixtures, containing 20 μM substrate before incubation, were extracted four times into the organic layer, the extraction efficiency being calculated as the net recovered radioactivity from the first extraction/the net recovered radioactivity in all four extractions, and expressed as a percentage. This method gives an upper limit to the extraction efficiency as it biases against products that are very poorly extracted. None of the procedures employed in this investigation were found to change the efficiency of extraction.

In experiments where the concentration of oxygen was varied, the assay mixtures were gassed with different mixtures of oxygen and nitrogen (the partial pressure of oxygen ranging from 10 to 100 per cent of the gas mixture) at a flow rate of 55 ml/sec for 3 sec prior to stoppering the tubes with rubber stoppers, and allowing the gas and liquid phases to equilibrate. The oxygen concentrations in the homogenates were taken to be directly proportional to the partial pressure of oxygen in the gas phase, the concentration of oxygen in air saturated water at 37° (0.217 mM) being taken as reference (see refs. 23, 24).

When clorgyline and l-deprenil were used to inhibit the enzyme activity, they were preincubated with the enzyme preparation for 240 min at 37° before addition of substrate. This time allows for the selective inhibition of MAO-A by clorgyline and MAO-B by l-deprenil to reach completion [25]. Preincubation with distilled water for 240 min did produce a small (about 15 per cent) decrease in the observed activity of MAO with respect to samples that were not preincubated. However, this decrease in activity was the same for all substrates used in the investigation. After the preincubation step, no further inhibitor was added to the reaction mixture to reduce the possibility of further enzyme–inhibitor reactions taking place. Thus, the concentrations of clorgyline and l-deprenil given throughout refer to those at preincubation.

Specific activities of MAO, corrected for the mean efficiency of extraction, are expressed as nmoles (of substrate metabolized)/mg protein/min. Protein concentrations of the enzyme preparations were determined by the method of Lowry *et al.* [26], with human serum albumin as standard.

The radioactive substrates for MAO, 5-hydroxytryptamine-[side chain-2-^{14}C]-binoxalate (5-HT), tyramine-[ethyl-1-^{14}C]-hydrochloride, dopamine-[ethyl-1-^{14}C]-hydrobromide, tryptamine-[side chain-2-^{14}C]-bisuccinate and β-phenethylamine-[ethyl-1-^{14}C]-hydrochloride were obtained from New England Nuclear, Boston, MA. Benzylamine-[methylene-1-^{14}C]-hydrochloride was obtained from ICN Pharmaceuticals Inc., Irvine, CA. All non-radioactive substrates, with the exception of benzylamine, were bought as salts, as the purity of the salts over a long period could be guaranteed better than the free bases, and substrate contamination has been shown to produce inaccuracies in kinetic experiments (see ref. 27). Benzylamine was converted into the hydrochloride by dissolution of the free base in ethyl acetate and addition of concentrated hydrochloric acid, the insoluble hydrochloride being collected and recrystallized in ethyl acetate–ethanol.

Clorgyline hydrochloride was a gift from May & Baker Ltd., Dagenham, U.K. l-Deprenil hydrochloride was a gift from Prof. J. Knoll, Department of Pharmacology, Semmelweis University of Medicine, Budapest, Hungary. All other reagents were standard laboratory reagents of analytical grade wherever possible. Male Sprague–Dawley rats were obtained from Anticimex AB, Stockholm, Sweden.

RESULTS

The efficiencies of extraction of the metabolites from all six substrates used in this study, determined by both lower and upper limit methods, are shown in Table 1. All the data included in this paper have been corrected for the appropriate mean extraction efficiencies.

Preincubation of rat liver mitochondrial membranes for 240 min at 37° with 2×10^{-8} M clorgyline was found completely to inhibit the activity of MAO-A with little effect on the activity of MAO-B. The reverse was found when 2×10^{-8} M l-deprenil was used. Thus, for each substrate, the percentage of the total activity metabolized by MAO-A was given by the percentage activity remaining in the presence of 2×10^{-8} M l-deprenil, and that of MAO-B by the percentage activity remaining in the presence of

Table 1. Extraction efficiencies of the deaminated products of six monoamines into the organic layer of the assay medium*

Substrate	Extraction efficiency (%)		
	Lower limit	Upper limit	Mean
5-Hydroxytryptamine	56.5	92.4	74.4
Tyramine	88.9	95.9	92.4
Dopamine	53.6	80.2	66.9
Tryptamine	39.2	97.9	68.6
β-Phenethylamine	85.8	99.2	92.5
Benzylamine	74.3	99.3	86.8

* The lower and upper limit extraction efficiencies of the deaminated metabolites into the ethyl acetate–toluene (1:1 v/v, saturated with water) extraction medium were determined as described in Materials and Methods. Each value represents the mean of four determinations in a pooled preparation of mitochondrial membranes derived from six rats. The substrate concentration was in all cases 20 μM.

2×10^{-8} M clorgyline. The relative activities of the two forms of MAO towards all six substrates are shown in Table 2.

When the membranes were preincubated with different concentrations of clorgyline for 240 min at 37°, the inhibition of the MAO activity towards 5-HT increased in a manner linear with increasing concentrations of clorgyline (Fig. 1A). The inhibition of tryptamine oxidation by clorgyline when preincubated simultaneously with 2×10^{-8} M l-deprenil was also linear (Fig. 1A). Similarly, the inhibition of benzylamine oxidation by l-deprenil was linear, as was the inhibition of tryptamine oxidation by this compound in the presence of 2×10^{-8} M clorgyline (Fig. 1B). Essentially similar results were obtained when tyramine and dopamine were used as substrates for both forms of MAO, and when β-phenethylamine was used as a substrate for MAO-B. From the concentration of each inhibitor required to produce 100 per cent inhibition of enzyme activity, the concentration of each enzyme form could be calculated. These values are shown in Table 2.

When the enzyme preparations were incubated with amine substrates under different oxygen–nitrogen gas mixtures, the activity was in every case increased in an apparent uncompetitive manner with increasing concentrations of oxygen. Similarly, when the data was replotted as $1/v$ against 1/oxygen concentration, increasing amine concentrations caused an uncompetitive increase in activity to be found. As examples, the data with dopamine and 5-HT as substrates are shown in Figs. 2A and B and 3A and B. Secondary plots of the intercepts of these lines on the $1/v$ axis against 1/(the concentration of the substrate that was held constant for each line) were linear in all cases, and were used to calculate the maximum velocity (V_{max}), and the Michaelis constants of the enzyme towards the amine substrate

Table 2. Relative activities and absolute concentrations of MAO-A and MAO-B in rat liver*

Substrate	Relative activity (%)		Enzyme concentration (pmoles/mg protein)	
	MAO-A	MAO-B	MAO-A	MAO-B
5-Hydroxytryptamine	82.7 ± 2.1	13.9 ± 3.0	11.8 ± 0.4	—
Tyramine	54.1 ± 3.9	59.3 ± 1.6	10.1 ± 0.5	11.0 ± 0.2
Dopamine	57.0 ± 3.0	49.2 ± 2.3	10.1 ± 0.4	11.2 ± 0.3
Tryptamine	58.4 ± 4.4	57.6 ± 1.2	10.2 ± 0.5	11.8 ± 0.1
β-Phenethylamine	21.0 ± 0.9	84.5 ± 4.2	—	15.9 ± 0.8
Benzylamine	5.1 ± 1.3	103.1 ± 2.0	—	13.3 ± 0.4

* Relative activities of MAO-A were calculated as the per cent activity remaining in the presence of 2×10^{-8} M l-deprenil. Relative activities of MAO-B were calculated as the per cent activity remaining in the presence of 2×10^{-8} M clorgyline. The MAO-A enzyme concentration with 5-HT as substrate was determined from the amount of clorgyline required to produce 100 per cent inhibition of enzyme activity. MAO-B enzyme concentrations assayed with β-phenethylamine and benzylamine were calculated in the same manner but with l-deprenil as inhibitor. For tyramine, dopamine and tryptamine as substrates, MAO-A concentrations were calculated from the inhibition by six concentrations of clorgyline in the presence of 2×10^{-8} M l-deprenil, and the MAO-B concentrations from the inhibition by seven concentrations of l-deprenil in the presence of 2×10^{-8} M clorgyline. In all cases, the membrane preparations were preincubated with the inhibitor for 240 min before the addition of substrate. The mean protein concentration at preincubation was 0.78 mg/ml. The substrate concentration was in all cases 100 μM. Each value represents the mean (± S.E.M. or S.E.R.) of duplicate determinations in six membrane preparations.

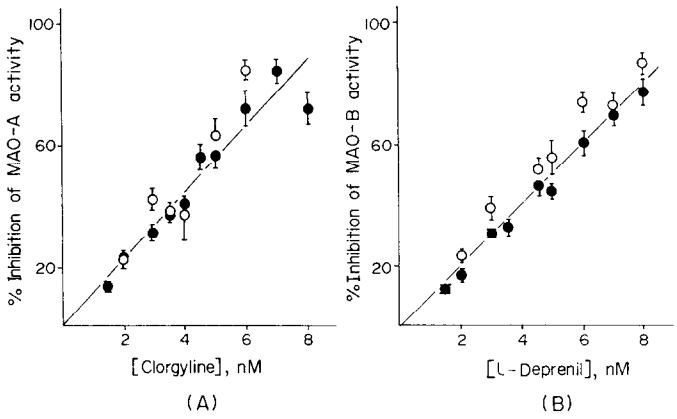

Fig. 1. The effect of clorgyline and l-deprenil upon rat liver mitochondrial MAO. Each point represents the mean (± S.E.R.) of duplicate determinations of the per cent inhibition of the MAO activity in six mitochondrial membrane preparations. The enzyme preparations were preincubated with inhibitor for 240 min prior to addition of substrate. The mean protein concentrations of the fractions at preincubation were 0.78 mg/ml.
Panel A: Clorgyline used as inhibitor. Substrates used were: 100 μM 5-HT (●) and 100 μM tryptamine (○). When tryptamine was used as substrate, the membrane preparations were preincubated in the presence of 2×10^{-8} M l-deprenil. Panel B: l-Deprenil used as inhibitor. Substrates used were: 100 μM benzylamine (●) and 100 μM tryptamine (○). When tryptamine was used as substrate, the membrane preparations were preincubated in the presence of 2×10^{-8} M clorgyline.

and oxygen (K_{am} and K_o, respectively). These kinetic parameters are summarized in Table 3. The values of the Michaelis constants of the enzyme towards oxygen were significantly higher when tyramine, tryptamine and β-phenethylamine were used as substrates than when 5-HT and benzylamine were used. The values for 5-HT and benzylamine were not significantly different one from the other, and nor were the values for tyramine, tryptamine and β-phenethylamine. Only the values for tryptamine were significantly different ($P < 0.005$) from those obtained when dopamine was used.

DISCUSSION

In the assay of MAO activity, a variety of solvents have been used to separate the deaminated products from the amine substrates (for review, see ref. 28). The data shown in Table 1 would suggest that the ethyl acetate–toluene solvent system extracts the metabolites from all the substrates used in this study reasonably well. This conclusion was also reached by Tipton and Youdim [28] for benzene–ethyl acetate, although this solvent system did not extract the metabolites of dopamine particularly well. This dif-

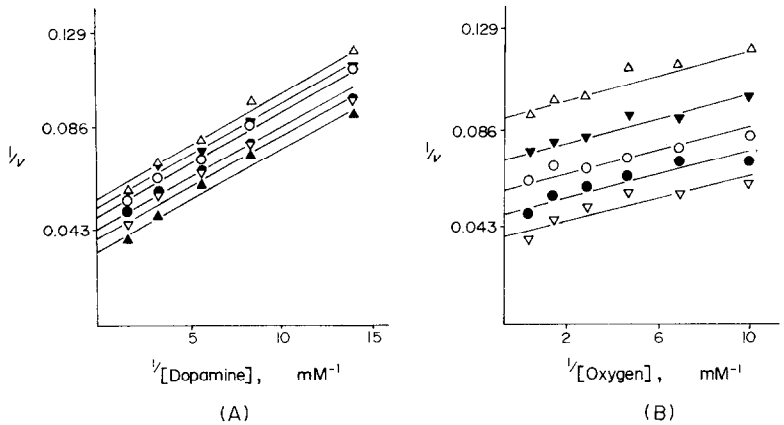

Fig. 2. Double reciprocal plots of the activity of rat liver MAO assayed with various concentrations of oxygen and dopamine. Each point represents the mean of duplicate determinations of activity in six preparations of rat liver mitochondrial membranes.
Panel A: Data plotted as 1/(initial velocity in nmoles/mg protein/min) against 1/(dopamine concentration in mM). Oxygen concentrations were: 108.5 μM (△), 151.9 μM (▲), 217.0 μM (○), 325.5 μM (●), 542.5 μM (▽) and 1085.0 μM (▼). Panel B: Data plotted as 1/(initial velocity in nmoles/mg protein/min) against 1/(oxygen concentration in mM). Dopamine concentrations were: 72 μM (△), 120 μM (▲), 180 μM (○), 300 μM (●) and 600 μM (▽).

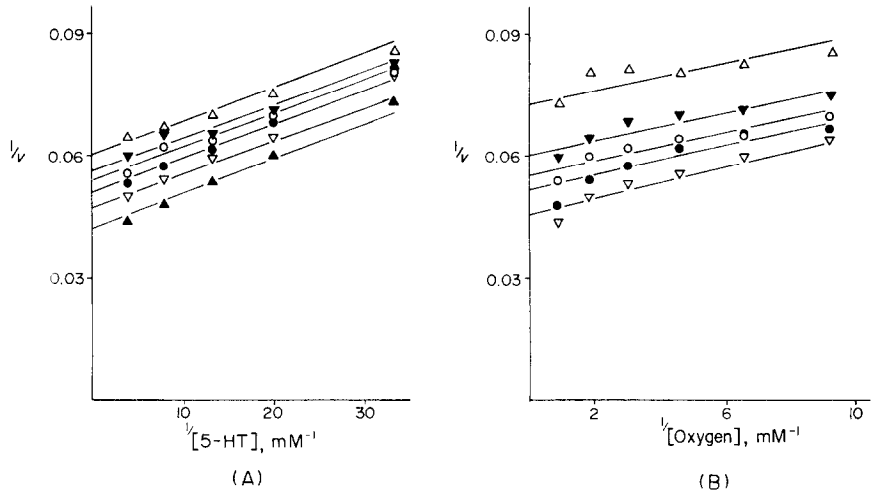

Fig. 3. Double reciprocal plots of the activity of rat liver MAO assayed with various concentrations of oxygen and 5-HT. Each point represents the mean of duplicate determinations of activity in six preparations of rat liver mitochondrial membranes.
Panel A: Data plotted as 1/(initial velocity in nmoles/mg protein/min) against 1/(5-HT concentration in mM). Oxygen concentrations were: 108.5 μM (△), 151.9 μM (▼), 217.0 μM (○), 325.5 μM (●), 542.5 μM (▽) and 1085.0 μM (▲). Panel B: Data plotted as 1/(initial velocity in nmoles/mg protein/min) against 1/(oxygen concentration in mM). 5-HT concentrations were: 30 μM (△), 50 μM (▼), 75 μM (○), 125 μM (●), and 250 μM (▽).

ference is not due to the substitution of the benzene by toluene [14, 28], but could possibly be due to the difference in the water saturation of the two solvent systems. Although the actual extraction efficiencies of tyramine, β-phenethylamine and benzylamine can be estimated with a reasonable degree of accuracy by the use of the lower and upper limit extraction efficiency methods (Table 1), the differences between the two values for 5-HT, dopamine and particularly tryptamine are large. However, similar values were found for this solvent system when human brain rather than rat liver was used as the enzyme source [29]. Another method for the estimation of the upper limit extraction efficiency, involving back-extraction of the deaminated metabolites (see ref. 6), gave extraction efficiencies that were in all cases higher than the upper limit values determined by the repeated extraction method described here. Due to the differences between the upper and lower limit values, the mean extraction efficiencies used here must be regarded as approximate, especially as the ratio of acid to alcohol metab-

Table 3. Kinetic parameters of rat liver monoamine oxidase*

Substrate	V_{max} (nmoles/mg protein/min)	K_{am} (μM)	K_o (μM)
5-Hydroxytryptamine	26.9 ± 2.9	26 ± 3	88 ± 19
Tyramine	110.3 ± 17.9	188 ± 35	303 ± 30†
Dopamine	39.4 ± 8.6	202 ± 31	156 ± 39
Tryptamine	56.0 ± 6.9	35 ± 5	299 ± 25†
β-Phenethylamine	95.2 ± 12.4	17 ± 2	228 ± 24†
Benzylamine	47.1 ± 3.6	97 ± 7	109 ± 14

* K_{am} values were determined by linear regression analysis of the initial velocities at infinite oxygen concentrations for each amine substrate concentration, plotted as S/v against S. K_o values were determined in the same way from plots of initial velocities at infinite amine concentration for each oxygen concentration. V_{max} values obtained from the two methods were in all cases within 8 per cent of each other, and the average value was taken. Initial velocities at the infinite first or second substrate concentrations were determined by linear regression analysis for duplicate determinations of activity at a fixed concentration of one substrate and a varying concentration of the other plotted as S/v against S. In all cases six oxygen concentrations and five to six amine concentrations were used. The values shown in the table represent means ± S.E.M. of determinations in six membrane preparations. Oxygen concentrations varied between 108.5 and 1085.0 μM. Amine concentrations varied between 20 and 250 μM (5-hydroxytryptamine), 60 and 500 μM (tyramine), 72 and 600 μM (dopamine), 12 and 100 μM (tryptamine and β-phenethylamine) and 30 and 250 μM (benzylamine).

† Significantly higher than values for 5-hydroxytryptamine and benzylamine ($P < 0.005$, two tailed paired t-test).

olites has been shown to change as the monoamine substrate concentration is changed (see ref. 30), but the values at least allow some comparison to be made between the different substrates.

The acetylenic inhibitors of MAO are thought to cause inhibition by a reversible association with the active centre of the enzyme followed by a 'suicide reaction' to form a covalent adduct with the flavine prosthetic group (see refs. 31–33). Clorgyline and l-deprenil are so potent as inhibitors of MAO-A and -B, respectively, that the free concentrations of these inhibitors are depleted by the formation of reversible and irreversible enzyme–inhibitor complexes (see refs. 6, 34), and in consequence can be used to titrate the number of enzyme active centres [6–8]. After 240 min of preincubation, when all reactions between enzyme and inhibitor have stopped [25], the inhibition of MAO-A and -B by clorgyline and l-deprenil, respectively, is linear with inhibitor concentration (Figs. 1A and B). The number of enzyme active centres in the membranes can be calculated from the concentration of inhibitor required to produce 100 per cent inhibition (see ref. 6). In this way, the concentration of MAO-A and -B has been determined for each substrate. For substrates metabolized by both forms of MAO, the enzyme concentration of each form was determined in the presence of 2×10^{-8} M inhibitor of the other form. This concentration was found to produce almost complete inhibition of the activity of one form with little effect on the other (Table 2), although some residual oxidation of the MAO-B substrate β-phenethylamine was found. This effect may be due to the relatively high concentration of β-phenethylamine used to assay for activity in these experiments (100 μM), since a significant deamination of this substrate by rat liver MAO-A has been found at high, but not low substrate concentrations [35]. The concentrations of MAO-A determined as described in Table 2 were similar when 5-HT, tyramine, dopamine and tryptamine were used as substrates, which would suggest that these substrates are metabolized by the same enzyme 'pool' in the mitochondrial membranes. The same conclusion appears to be true for MAO-B when tyramine, dopamine, tryptamine, β-phenethylamine and benzylamine were used as alternative amine substrates (Table 2), in agreement with a recent study (with benzylamine and β-phenethylamine as substrates) [36]. This method, however, does not take into account factors such as the non-specific binding of the inhibitor to sites other than the active centre of MAO, and metabolism of the inhibitor, both of which have been demonstrated for clorgyline in rat liver crude homogenates, although much shorter preincubation periods were used and reversible reactions were still present [14, 34]. Non-specific binding has also been demonstrated for l-deprenil in the human brain [29]. These factors have been found to be absent, however, for the inhibition of rat heart and human brain MAO-A by clorgyline [6, 29]. The use of long periods of preincubation (in order to allow the irreversible reaction between enzyme and inhibitor to go to completion, and thus displace both specific and non-specific equilibria), together with the use of a preparation of mitochondrial membranes with little microsomal contamination (to minimize drug metabolism), should reduce these effects considerably.

The activity of MAO with all six substrates was increased in an uncompetitive manner with increasing concentrations of oxygen, consistent with a ping-pong, or double displacement reaction. The Michaelis–Menten equation can be written (see ref. 12):

$$v = \frac{V_{max}}{1 + \frac{K_{am}}{[\text{amine}]} + \frac{K_o}{[\text{oxygen}]}}, \qquad (1)$$

where K_{am} and K_o are the Michaelis constants for the amine substrate and oxygen, respectively. It should be stressed, however, that these Michaelis constants are not dissociation constants, but are groups of rate constants derived from the steady-state equations (see refs. 9, 12). V_{max} is defined as the maximum velocity attainable when the enzyme is saturated with both of its substrates, and K_{am} and K_o are the concentrations of the appropriate substrate (the amine substrate and oxygen, respectively) necessary to produce half-maximum velocity when the enzyme is saturated with the other substrate. The V_{max}, K_{am} and K_o values for the enzyme assayed with six amine substrates are shown in Table 3. The K_o values for tyramine and benzylamine are similar to those found in a previous study where considerably lower concentrations of oxygen were used [12, 37], which would indicate that the differences in K_o values for the different amine substrates reported in this study are not due to substrate-inhibitory effects by high concentrations of oxygen. In agreement with previous estimations of the K_o values from determinations of MAO activity in oxygen and air [13], the value of K_o appears to depend upon the amine substrate used to assay for activity. The values obtained when tyramine, tryptamine and β-phenethylamine were used as substrates were found to be significantly higher than when 5-HT and benzylamine were used (Table 3).

When first reported, these differences in K_o values were thought to be due to some sort of enzyme heterogeneity unrelated to MAO-A or -B, and a new classification of MAO activity was tentatively suggested [13]. However, it was stated at the time that this heterogeneity would need to be proved rigorously before such a classification could be justified.

Equation 1 above shows that a graph of 1/initial velocity against 1/oxygen concentration will have a slope of K_o/V_{max} which will be independent of the concentration of the amine [the amine concentration is incorporated in the intercept, thus leading to the parallel lines found when the data is plotted in this way (see Figs. 2B and 3B)]. For a single enzyme acting on a variety of different amine substrates, the value of K_o/V_{max} would be expected to be constant at a constant enzyme concentration. In the case of monoamine oxidase, however, there are two species of enzyme present with different activities towards each substrate, and this would be expected to lead to a departure from this simple relationship. The effects of differences in the concentrations of the two enzyme forms upon this relationship can, how-

Table 4. k_{cat} values for rat liver MAO-A and -B*

Substrate	k_{cat} (moles/mole MAO/min) MAO-A	MAO-B
5-Hydroxytryptamine	2270 ± 197	—
Tyramine	5221 ± 854	5204 ± 805
Dopamine	2089 ± 453	1632 ± 351
Tryptamine	2720 ± 293	2387 ± 326
β-Phenethylamine	—	5911 ± 526
Benzylamine	—	3561 ± 316

* k_{cat} values for each form of MAO were calculated as (V_{max} × fractional activity due to enzyme form)/(concentration of enzyme form). The values represent means ± S.E.M. for k_{cat} values determined in six rat liver mitochondrial preparations. The data used to calculate the k_{cat} values are summarized in Tables 2 and 3.

ever, be nullified, since V_{max} is defined by the relationship:

$$V_{max} = k_{cat} \cdot e,$$

where e represents the enzyme concentration and k_{cat} is the rate constant for the limiting step in the overall enzyme-catalysed reaction (i.e. the maximum molecular turnover number). The ratio k_{cat}/K_o represents the apparent first-order rate constant for the combination between the reduced enzyme and oxygen and should be independent of the nature of the amine substrate used.

Values of k_{cat} can be calculated for both enzyme forms acting on the different amine substrates from the enzyme concentrations and the relative contributions of the two forms (Table 2) together with the values of V_{max} (Table 3), and are shown for the two forms in Table 4. The values of k_{cat} for the two enzyme forms towards common substrates are rather similar. From these data, assuming that the two enzyme forms are separate entities, k_{cat} values for the enzyme system as a whole can be calculated, and are shown plotted against K_o in Fig. 4A. The expected proportionality between these two constants is not seen, and the correlation coefficient of the linear regression analysis is rather low ($r = 0.497$). Furthermore, the regression line appears to cut the x-axis away from the origin, which would not be expected if the strict proportionality between k_{cat} and K_o were obeyed. A possible explanation for this behaviour would be that the two forms of the enzyme differed in their rates of interaction with oxygen. However, this seems unlikely since there is a very good correlation ($r = 0.860$) when the values of K_o are plotted against k_{cat} with 5-HT (substrate for MAO-A alone), β-phenethylamine and benzylamine (substrates for MAO-B alone) as amine substrates.

The enzyme rhodanese (thiosulphate : cyanide sulphurtransferase, EC 2.8.1.1), which also obeys a ping-pong mechanism, has also been shown not to

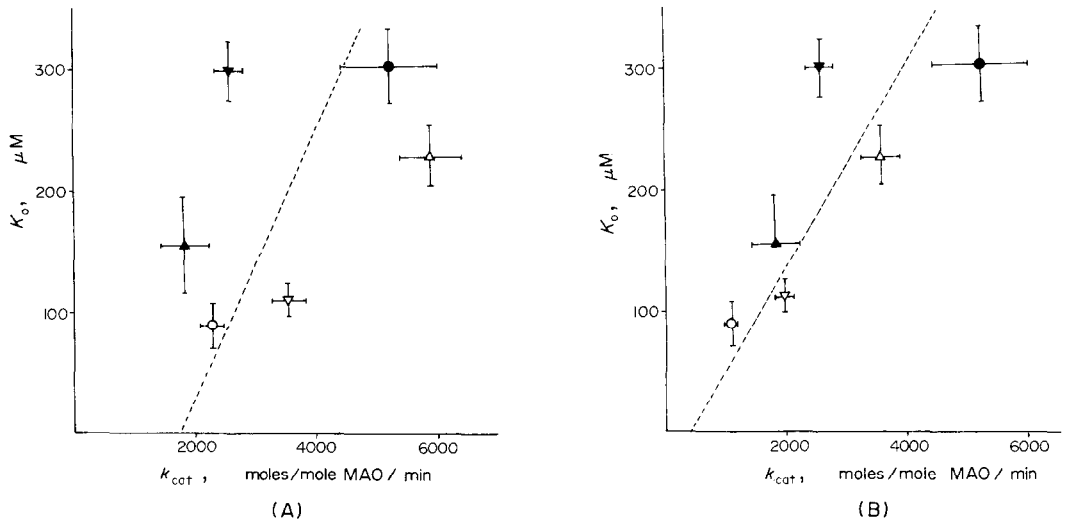

Fig. 4. Panel A: Relationship between the k_{cat} values and the K_o values for rat liver MAO, assuming that MAO-A and -B are entirely separate entities (see Discussion). Ordinate: K_o values in μM; abscissa: k_{cat} values in moles/mole MAO/min. Each point represents the mean ± S.E.M. of determinations in six mitochondrial fractions. The dotted line indicates the regression line (of the type $y = ax + b$, where $y = K_o$ and $x = k_{cat}$) calculated from the 36 individual points. The correlation coefficient of the regression line was 0.497. Substrates used were: ○, 5-HT; ●, tyramine; ▲, dopamine; ▼, tryptamine; △, β-phenethylamine; ▽, benzylamine. The regression line cuts the x-axis at a k_{cat} value of 1804 ± 591 (mean ± S.E.M.) moles/mole MAO/min. Panel B: Relationship between the k_{cat} values and the K_o values for rat liver MAO, assuming that the two forms of the enzyme interact one with the other (see Discussion). Ordinate: K_o values in μM; abscissa: k_{cat} values in moles/mole MAO/min. Each point represents the mean ± S.E.M. of determinations in six mitochondrial fractions. The dotted line indicates the regression line (of the type $y = ax + b$, where $y = K_o$ and $x = k_{cat}$) calculated from the 36 individual points. The correlation coefficient of the regression line was 0.729. Substrates used were: ○, 5-HT; ●, tyramine; ▲, dopamine; ▼, tryptamine; △, β-phenethylamine; ▽, benzylamine. The regression line cuts the x-axis at a k_{cat} value of 423 ± 414 (mean ± S.E.M.) moles/mole MAO/min.

give a constant value of k_{cat}/K_m towards its second substrate (cyanide); the value being dependent upon the nature of the sulphur-donor substrate [38]. In this case, the behaviour has been explained in terms of 'enzyme memory', which suggests that different conformational changes are induced in the enzyme by different donor substrates, and that these persist when the sulphur-substituted enzyme is formed, thus affecting the interaction between this form and cyanide. A similar model might provide an explanation of the behaviour of monoamine oxidase, but in this case, there is also an alternative, simpler, explanation for the results.

The calculations used above have assumed that the two enzyme forms are separate entities, as has indeed been claimed to be the case from the results of mixed-substrate experiments carried out with the enzyme from rat lung [39]. Similar studies with the enzyme from rat liver have, however, suggested that the two forms may not be independent, but interact in some way [5, 40, 41]. If the enzyme forms are dependent one upon the other, the calculations of the k_{cat} values for 5-HT, β-phenethylamine and benzylamine must take into account the contributions from both enzyme forms, even though the activity of one of these forms is zero. In this case, the values of k_{cat} are calculated most simply as $V_{max}/([MAO\text{-}A] + [MAO\text{-}B])$. When the k_{cat} values calculated in this way are plotted against the K_o values, there seemed to be good proportionality between k_{cat} and K_o (Fig. 4B), with a much higher correlation coefficient for the regression line ($r = 0.729$). Furthermore, the regression line appeared to pass effectively through the origin. Thus it would seem that a model for rat liver MAO where the two forms interact one with the other is more compatible with the data presented in this study than a model where the two enzyme forms are wholly independent.

One explanation for this kinetic behaviour is that the substrates for both forms of MAO display 'half-sites reactivity', i.e. although there are two sites for the substrate, only one of the sites is catalytically active at any one time, although, if one of the two sites is inhibited (by clorgyline or l-deprenil), the other can function independently. A variety of enzymes, including ox liver glutamate dehydrogenase and yeast aldehyde dehydrogenase, display half-sites reactivity (for review, see ref. 42). Another possibility is that 5-HT, β-phenethylamine and benzylamine are in fact metabolized by both MAO-A and -B active centres, but these active centres are arranged on the enzyme in such a way that selective inhibition of one of the active sites (by clorgyline or l-deprenil) produces inhibition of the other active site. This seems unlikely, as it would then be expected that, for example, the oxidation of 5-HT would be equally sensitive to inhibition by clorgyline and l-deprenil, which it is not (Table 2).

Although the values for tryptamine appear to differ somewhat from the values for the other five substrates (Fig. 4B), it is unlikely that this is due to enzyme heterogeneity, particularly as the concentrations of the two forms responsible for the metabolism of this substrate are the same as for the other substrates used here (Table 2). It seems more likely that this anomalous result is due to the large error in the estimation of the efficiency of extraction of the deaminated metabolites of tryptamine (Table 1). It should be possible to resolve this difficulty by the use of an assay procedure where no organic step is required, such as the aldehyde dehydrogenase coupled assay of Houslay and Tipton (see ref. 43).

Thus the results would indicate that the differences in the K_o values found when different substrates are used to assay for activity are not in themselves an indication of enzyme heterogeneity. A similar conclusion has been drawn for human brain MAO [16]. Furthermore, the data presented above would also support the notion that, in the rat liver, the two enzyme forms are interactive and may display half-sites reactivity, although, in view of the errors involved in the analysis described above, further work is required to prove this point conclusively. In a variety of tissues, including the rat liver, both purified and 'native' MAO have been shown to exist in subunits [44–49]. Furthermore, immunological studies [50] have indicated that, in the human liver, the MAO-A faces the inner surface of the outer mitochondrial membrane, whereas the -B form faces the outer surface. It is tempting to suggest that the two forms of MAO are located as a complex across the mitochondrial outer membrane. Such a hypothesis would allow an easy explanation for the interaction between the two forms of MAO postulated above.

Acknowledgements—The authors would like to express their gratitude to Prof. K. F. Tipton, for his invaluable advice and help. L. O. would like to thank the Swedish Medical Research Council (Grant No. 04X-4145) for their continued support. During the course of this work, C. J. F. was in receipt of a Royal Society European Postdoctoral Science Research Fellowship.

REFERENCES

1. C. J. Fowler, B.A. Callingham, T. J. Mantle and K. F. Tipton, *Biochem. Pharmac.* **27**, 97 (1978).
2. J. P. Johnston, *Biochem. Pharmac.* **17**, 1285 (1968).
3. J. Knoll and K. Magyar, *Adv. biochem. Psychopharmac.* **5**, 393 (1972).
4. D. W. R. Hall, B. W. Logan and G. H. Parsons, *Biochem. Pharmac.* **18**, 1447 (1969).
5. M. D. Houslay and K. F. Tipton, *Biochem. J.* **139**, 645 (1974).
6. C. J. Fowler and B. A. Callingham, *Molec. Pharmac.* **16**, 546 (1979).
7. T. Egashira, B. Ekstedt, H. Kinemuchi, Å. Wiberg and L. Oreland, *Med. Biol.* **54**, 272 (1976).
8. L. Oreland and C. J. Fowler, in *Monoamine Oxidase; Structure, Function and Altered Functions* (Eds. T. P. Singer, R. W. Von Korff and D. L. Murphy), pp. 389–396. Academic Press, New York (1979).
9. K. F. Tipton, *Eur. J. Biochem.* **5**, 316 (1968).
10. A. G. Fischer, A. R. Schulz and L. Oliner, *Biochim. biophys. Acta* **159**, 460 (1968).
11. S. Oi, K. Shimada, M. Inamasu and K. T. Yasunobu, *Archs Biochem. Biophys.* **139**, 28 (1970).
12. M. D. Houslay and K. F. Tipton, *Biochem, J.* **135**, 735 (1973).
13. C. J. Fowler and B. A. Callingham, *Biochem. Pharmac.* **27**, 1995 (1978).
14. C. J. Fowler, Ph.D. Thesis, University of Cambridge (1978).
15. C. J. Fowler, B. Ekstedt, T. Egashira, H. Kinemuchi and L. Oreland, *Biochem. Pharmac.* **28**, 3063 (1979).

16. J. A. Roth, in *Monoamine Oxidase; Structure, Function and Altered Functions* (Eds. T. P. Singer, R. W. Von Korff and D. L. Murphy), pp. 153–168. Academic Press, New York (1979).
17. R. W. Von Korff, *Biokhimiya* **42**, 396 (1977).
18. R. F. Squires, *Biochem. Pharmac.* **17**, 1401 (1968).
19. G. A. Lyles and J. W. Greenawalt, *Biochem. Pharmac.* **27**, 923 (1978).
20. C. J. Fowler, B. A. Callingham and M. D. Houslay, *J. Pharm. Pharmac.* **29**, 411 (1977).
21. C. J. Fowler and L. Oreland, in *Monoamine Oxidase; Structure, Function and Altered Functions* (Eds. T. P. Singer, R. W. Von Korff and D. L. Murphy), pp. 145–151. Academic Press, New York (1979).
22. B. A. Callingham and R. Laverty, *J. Pharm. Pharmac.* **25**, 940 (1973).
23. M. Dixon and K. Kleppe, *Biochim. biophys. Acta* **96**, 357 (1965).
24. L. Della Corte, Ph.D. Thesis, University of Cambridge (1975).
25. T. Egashira, B. Ekstedt and L. Oreland, *Biochem. Pharmac.* **25**, 2583 (1976).
26. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
27. K. F. Tipton, T. J. Mantle and M. D. Houslay, *Biochem. Pharmac.* **26**, 1525 (1977).
28. K. F. Tipton and M. B. H. Youdim, in *Monoamine Oxidase and its Inhibition. Ciba Foundation Symposium* 39 (Eds. G. E. W. Wolstenholme and J. Knight), pp. 393–403. Elsevier, Exerpta Medica, North Holland, Amsterdam (1976).
29. C. J. Fowler, L. Oreland, J. Marcusson and B. Winblad, *Naunyn-Schmiedeberg's Archs Pharmac.*, **311**, 263 (1980).
30. K. F. Tipton, M. D. Houslay and A. J. Turner, *Essays Neurochem. Neuropharmac.* **1**, 103 (1977).
31. R. R. Rando, *Science N.Y.* **185**, 320 (1974).
32. L. Oreland, H. Kinemuchi and B. Y. Yoo, *Life Sci.* **13**, 1533 (1973).
33. A. L. Maycock, R. H. Abeles, J. I. Salach and T. P. Singer, *Biochemistry* **15**, 114 (1976).
34. C. J. Fowler and B. A. Callingham, *J. Pharm. Pharmac.* **30**, 304 (1978).
35. E. J. Dial and D. E. Clarke, *Pharmac. Res. Commun.* **11**, 491 (1979).
36. C. J. Fowler and L. Oreland, *J. Pharm. Pharmac.*, in press.
37. K. F. Tipton, *Adv. biochem. Psychopharmac.* **5**, 11 (1972).
38. R. Jarabak and J. Westley, *Biochemistry* **13**, 3237 (1974).
39. Y. S. Bakhle and M. B. H. Youdim, *Br. J. Pharmac.* **56**, 125 (1976).
40. B. Ekstedt, *Biochem. Pharmac.* **25**, 1133 (1976).
41. T. J. Mantle, K. Wilson and R. F. Long, *Biochem. Pharmac.* **24**, 203 (1975).
42. F. Seydoux, O. P. Malhotra and S. A. Bernhard, *CRC crit. Rev. Biochem.* **2**, 227 (1974).
43. M. D. Houslay and K. F. Tipton, *Biochem. J.* **135**, 173 (1973).
44. L. Oreland, H. Kinemuchi and T. Stigbrand, *Archs Biochem. Biophys.* **159**, 854 (1973).
45. G. G. S. Collins and M. B. H. Youdim, *Biochem. Pharmac.* **24**, 703 (1975).
46. N. Minamiura and K. T. Yasunobu, *Archs Biochem. Biophys.* **189**, 481 (1978).
47. N. Minamiura and K. T. Yasunobu, *Biochem. Pharmac.* **27**, 2737 (1978).
48. B. A. Callingham and D. Parkinson, in *Monoamine Oxidase; Structure, Function and Altered Functions* (Eds. T. P. Singer, R. W. Von Korff and D. L. Murphy), pp. 81–86. Academic Press, New York (1979).
49. D. J. Edwards and K. Y. Pak, *Biochem. biophys. Res. Commun.* **86**, 350 (1979).
50. S. M. Russell, J. Davey and R. J. Mayer, *Biochem. J.* **181**, 7 (1979).