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SUBSTRATE-SELECTIVE ACTIVATION OF RAT LIVER MITOCHONDRIAL MONOAMINE OXIDASE BY OXYGEN

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Abstract—The activation by oxygen of the activity of monoamine oxidase (MAO) in preparations of rat liver mitochondrial membrane vesicles has been studied. The increase of activity with the substrates 5-hydroxytryptamine, tyramine, β-phenethylamine and benzylamine were in all cases uncompetitive. However, the degree of activation depended upon the particular substrate used. An attempt has been made to explain these results by use of an assumption that MAO activity can be divided into two forms unrelated to MAO-A and MAO-B. These two forms, tentatively called MAO-1 and MAO-2, differ in their Michaelis constants for oxygen.

There is now a considerable weight of evidence to show that the enzyme monoamine oxidase (monoamine: O_2 oxidoreductase, MAO, E.C. 1.4.3.4) appears to exist in more than one catalytically active form (see [1, 2]). Use of the irreversible MAO inhibitor clorgyline and a variety of substrates enabled Johnston [3] to classify the enzyme into two forms, MAO-A and MAO-B, the former being more sensitive to the inhibitor. According to this classification, in the rat liver, 5-hydroxytryptamine (5-HT) is metabolised by MAO-A alone, benzylamine and β-phenethylamine by MAO-B alone, and tyramine by both forms of the enzyme [4, 5].

Although this classification has been widely used, and has proved to be a valuable aid to the study of MAO activity in many animal tissues, there has been a gradual accumulation of experimental observations that cannot fully be explained by this simple binary classification [see 6]. One discrepancy concerns the possible heterogeneity of MAO-B. Squires [7, 8] showed that the response of the MAO-B in rat and mouse liver to thermal denaturation could be resolved into several components when kynuramine was used as substrate. Kinetic studies of the enzyme reaction with several substrates and inhibitors have also indicated that the MAO-B may be heterogeneous in rat liver [9], human blood platelets [10] and pig heart [11]. Tris buffer has also been found to inhibit the metabolism of β-phenethylamine but not benzylamine in rat liver mitochondria [12].

So far, little attention has been given to the influence of changes in the concentration of the second substrate, oxygen, upon the apparently different catalytic forms, although there are several reports that the degree of activation of MAO activity by increasing the oxygen tension depends upon the particular substrate used [13, 14, 15, 16]. In consequence, we have extended these observations on the effect of oxygen on MAO activity and have attempted to relate these findings to the possible heterogeneity of the enzyme.

Although the bulk of the MAO activity in rat liver is found in the outer mitochondrial membrane (see [17]), possible contamination by extramitochondrial sources of MAO could account for some of the apparent heterogeneities observed. In order to avoid such a possibility, the experiments in this study have been performed on a preparation of vesicles of mitochondrial membranes in which contamination from other sources has been kept to a minimum.

METHODS AND MATERIALS

Rats, of body weight 350–375 g were killed by a blow to the head, and the livers rapidly removed, blotted on filter paper and weighed. The livers were pooled and homogenised 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. The duration of homogenisation was 10 sec. The homogenates were centrifuged at 600 *g* for 15 min to remove nuclei and cell debris. The resulting supernatant fractions ('crude homogenates') were centrifuged at 6,500 *g* for 20 min and the supernatants discarded. The pellets were resuspended in 'sucrose buffer' by agitation either with a glass rod or a vortex mixer and centrifuged as before, the pellets ('crude mitochondrial fraction') being retained. A sample from each fraction (0.9–1.0 g) was layered on to an 18 ml linear sucrose gradient (40–70% w/v, buffered with 10 mM potassium phosphate, pH 7.8; made with an MSE gradient former) and centrifuged at 50,000 *g* for 135 min in a swing-out rotor. A tight band of protein formed two-thirds of the way down the gradient ('purified mitochondrial fraction') which was collected, and resuspended in 10 mM potassium phosphate buffer, pH 7.8, in order to reduce the sucrose concentration to 0.25 M without any change in the ionic phosphate concentration. This was centrifuged at 18,000 *g* for 30 min. The pellet was then resuspended in 'sucrose buffer' and set to a protein concentration of 1 $mg.ml^{-1}$. This fraction ('vesicle fraction') was then stored frozen until used for assay.

This method was chosen in preference to the more

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refined method for the production of mitochondrial outer membranes described by Sottocasa *et al.* [18] since it was quicker and the yield of MAO was greater.

Enzyme assays. MAO activity was measured radiochemically by the method of McCaman *et al.* [19] as modified by Callingham and Laverty [20]. [^{3}H] tyramine, [^{3}H] 5-hydroxytryptamine (5-HT), [^{14}C] β-phenethylamine and [^{14}C] benzylamine were used as substrates. Unless otherwise stated, assays were carried out at 37° in an atmosphere of oxygen. Preliminary experiments were undertaken to establish the linearity of the reaction with the four substrates. Progress curves were drawn from incubation periods of 1.5, 3.0, 4.5 and 6.0 min. There was no significant deviation from linearity in any case up to 3.0 min of incubation. In consequence, all incubation periods used subsequently were within this time. Specific activities of MAO are expressed in terms of nmol (of substrate metabolised) (mg protein)$^{-1}$.h^{-1} calculated as mean values ± standard error of the mean. No corrections were made for the efficiencies of extraction of the deaminated metabolites of the different substrates. None of the procedures employed changed the efficiency of extraction. In experiments where clorgyline was used to inhibit the MAO, the homogenates were preincubated for 20 min with the drug to allow the irreversible inhibition of MAO-A [21, 22]. No preincubation was needed when Tris buffer was used to inhibit the enzyme [23].

Succinic dehydrogenase (SDH), fumarase, malate dehydrogenase (MDH) and acid phosphatase activities were assayed by standard spectrophotometric methods [24–27] respectively.

Protein content of the fractions was assayed by the method of Lowry *et al.* [28].

Statistical significance between groups was determined by 95 per cent confidence limits of a ratio [29] where appropriate.

Materials. The radioactive substrates for MAO, tyramine-[G-^{3}H]-hydrochloride and β-phenethylamine-[ethyl-1-^{14}C]-hydrochloride were obtained from New England Nuclear GMBH, Dreieichenchain, Germany; 5-hydroxytryptamine-[G-^{3}H]-creatinine sulphate from the Radiochemical Centre, Amersham, England and benzylamine-[methylene-^{14}C]-hydrochloride from ICN Pharmaceuticals, Hersham England.

Clorgyline hydrochloride was a gift from May & Baker Ltd., Dagenham, England. Tris, (tris-(hydroxymethyl)-methylamine) was obtained from Fisons Scientific Apparatus Ltd., Loughborough England. All other reagents were standard laboratory reagents of analytical grade wherever possible. Male Wistar rats were obtained from A. J. Tuck and Son, Rayleigh, England.

RESULTS

Table 1 shows the yields of the marker enzymes obtained at various steps in the production of the 'vesicle fraction'. The homogenisation period was 10 sec. Longer periods of homogenisation increased the microsomal and lysosomal contamination and decreased the yields of MAO. Shorter periods resulted in incomplete homogenisation. There was a good yield of the mitochondrial membrane enzymes MAO and SDH, but the yields of the matrix enzymes MDH and fumarase were lower. Electron microscopy confirmed that the purified mitochondrial fractions consisted largely of membrane material with only small amounts of matrical contamination.

These vesicles were produced when the crude mitochondrial fraction was resuspended by either gentle agitation with a glass rod, or by vigorous vortex mixing, indicating that the vesicles were mainly formed during homogenisation.

The properties of the MAO in three groups of vesicles, each derived from the livers of two rats, were compared with their respective crude homogenates (Table 2). The yields of MAO towards all four substrates were about the same, and indicated that there was a 9–10 fold purification of the MAO by these procedures. The K_m of the MAO towards each substrate was not substantially changed, and nor was the sensitivity towards inhibition by Tris buffer.

The crude homogenates and vesicles appeared to contain the same ratios of MAO-A: MAO-B, since there was no change in the heights of the plateaux separating the two sigmoid regions of the curves

Table 1. Relative percentage yields of some marker enzyme activities during the preparation of rat liver mitochondrial membrane vesicles

	Relative yields (per cent) of enzyme activities		
	Crude homogenate	Crude mitochondria	Vesicles
Protein	100	10.9 ± 2.4	7.2 ± 1.5
MAO (Tyramine) (outer mitochondrial membrane)	100	42.9 ± 6.6	33.9 ± 6.2
Succinate dehydrogenase (inner mitochondrial membrane)	100	62.0 ± 16.0	51.4 ± 14.1
Fumarase (mitochondrial matrix)	100	7.1 ± 3.0	6.2 ± 2.5
Malate dehydrogenase (mitochondrial matrix)	100	20.0 ± 5.3	7.0 ± 3.0
Acid phosphatase (lysosomes)	100	24.8 ± 3.2	10.9 ± 2.6

Results are shown in terms of means ± S.E.R. for triplicate determinations in three groups, each of six rats.

Table 2. Kinetic parameters of MAO activities in crude homogenates and mitochondrial membrane vesicles from rat liver

Yield (per cent)	5-HT	Tyramine	β-Phenethylamine	Benzylamine
Crude homogenate	100	100	100	100
Vesicles	38.1 ± 6.4	38.1 ± 0.5	38.3 ± 1.6	33.8 ± 3.1
Yield of protein			4.2 ± 0.5	
K_m (μM)				
Crude homogenate	77 ± 12	215 ± 31	28 ± 8	128 ± 28
Vesicles	137 ± 44	277 ± 61	31 ± 3	187 ± 74
V_{max}(nmole (mg protein)$^{-1}$.h^{-1})				
Crude homogenate	131 ± 5	616 ± 3	286 ± 26	185 ± 12
Vesicles	1210 ± 124	5836 ± 479	3099 ± 110	1568 ± 213
Oxygen ratio				
Crude homogenate	1.43 ± 0.03	1.42 ± 0.03	1.98 ± 0.03	1.24 ± 0.02
Vesicles	1.40 ± 0.14	1.40 ± 0.06	2.03 ± 0.14	1.24 ± 0.04
Maximum oxygen ratio				
Vesicles	1.49 ± 0.10	1.93 ± 0.12	2.34 ± 0.12	1.45 ± 0.15
Inhibition by 20 mM Tris-HCl (per cent)				
Crude homogenate	57.0 ± 1.2	48.1 ± 1.0	32.5 ± 1.6	8.2 ± 1.7
Vesicles	59.3 ± 1.4	55.6 ± 2.5	33.7 ± 1.3	15.7 ± 1.3

Results are shown in terms of means ± S.E.M. or S.E.R. where appropriate for triplicate determinations in three groups each of two rats. K_m and V_{max} values were calculated from the initial velocities at six substrate concentrations. Yields were calculated from individual V_{max} values. Oxygen ratios (MAO activity in oxygen/activity in air) and per cent inhibition by 20 mM Tris–HCl buffer were determined at substrate concentrations of: 5-HT, 0.25 mM; tyramine, 0.25 mM β-phenethylamine, 0.05 mM; benzylamine, 0.25 mM. Maximum oxygen ratios were calculated from individual V_{max} values in oxygen and air.

produced when the deamination of tyramine was inhibited by increasing concentrations of clorgyline in the two preparations (Figs. 1A and B). 5-HT was metabolised by MAO-A, and β-phenethylamine and benzylamine by MAO-B in both cases. However the MAO in the vesicle fraction was slightly more sensitive to inhibition by clorgyline.

The ratios of the MAO activities in an atmosphere of oxygen compared with air ('oxygen ratio') with all four substrates are shown in Table 2. The oxygen ratios for each substrate, at a concentration near its respective K_m in oxygen, appeared to be identical in both crude homogenates and vesicle fractions, but differed from substrate to substrate. Further experiments showed that these kinetic parameters did not change if the homogenates were prepared by hand in a conical glass homogeniser.

The kinetic parameters of MAO in the vesicle fractions were determined in atmospheres of both air and oxygen (Fig. 2A, B, C, D). In each case, the increase in oxygen concentration caused an apparent uncompetitive activation of the MAO, but the degree of activation again depended upon the substrate employed. The V_{max} of each fraction was calculated by the method of Wilkinson [30]. These were used to determine the 'maximum oxygen ratios' for these oxygen concentrations. These are shown in Table 2. Benzylamine and 5-HT possessed similar 'maximum oxygen ratios' of about 1.5, which were significantly smaller than those for tyramine and β-phenethylamine, which were about 2.1. The differences between values for either tyramine and β-phenethylamine or benzylamine and 5-HT were not significant. Benzylamine and β-phenethylamine competitively inhibited the deamination of each other in both air and oxygen, with K_i values near those of their K_m values (Table 3).

DISCUSSION

The shearing action of the mechanical blender appears to produce a preparation of mitochondrial membranes with only moderate matrical contamination, similar to that described by Caplan and Greenawalt [31]. However, these authors used osmotic pressure to rupture the mitochondria, whereas the preparation shown here was produced in an isotonic

Table 3. Mutual inhibition of β-phenethylamine and benzylamine deamination in rat liver mitochondrial membrane vesicles

	Oxygen		Air		
	K_m(μM)	K_i(μM)	K_m(μM)	K_i(μM)	Maximum oxygen ratio
β-Phenethylamine	33 ± 4	21	12 ± 2	20	2.44 ± 0.08
Benzylamine	131 ± 9	161	75 ± 28	60	1.30 ± 0.04

30 μM β-phenethylamine and 125 μM benzylamine were used to inhibit the deamination of six concentrations of [^{14}C]benzylamine and [^{14}C]β-phenethylamine respectively. In all cases, the inhibition was competitive. Apparent K_m values were calculated by the method of Wilkinson [30] and K_i values calculated from these figures. The results are expressed in terms of means ± S.E.M. or S.E.R. where appropriate from triplicate determinations in three groups each of two rats. The maximum oxygen ratios for the two substrates in these preparations are also shown.

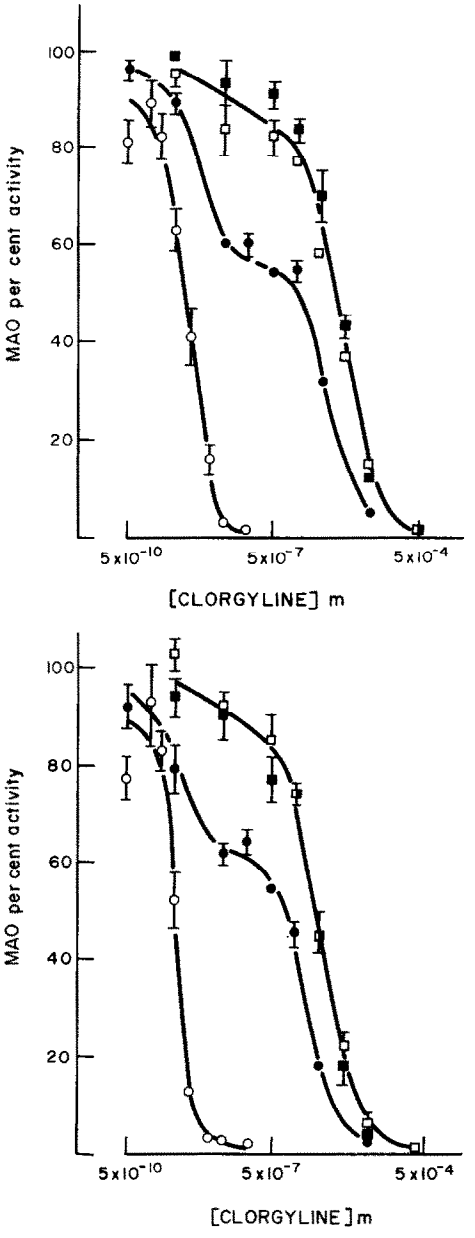

Fig. 1. The effect of clorgyline upon the *in vitro* deamination of (○), 0.25 mM 5-HT; (●), 0.25 mM tyramine; (□), 0.03 mM β-phenethylamine; (■), 0.15 mM benzylamine in A: crude homogenates; B: mitochondrial membrane vesicles from rat liver. Each point represents the mean MAO activity assayed in triplicate in three groups each of two rats ± S.E.R. expressed as a percentage of its respective uninhibited control, plotted against the molar concentration of clorgyline.

medium. The MAO in this preparation appears to have similar properties to those found in the crude homogenates (Table 2). The vesicle enzyme does, however, show a slightly increased sensitivity to inhibition by clorgyline (Fig. 1A and B), although the ratio of MAO-A:MAO-B is unchanged.

Clorgyline is such a potent inhibitor of the deamination of 5-HT by rat liver MAO that the concentration of inhibitor is of the same order as that of the enzyme. Thus any change in either the number of MAO active centres or the number of sites of nonspecific binding would cause a change in the apparent sensitivity of the enzyme preparation to inhibition by clorgyline [see 22]. The homogenates and the vesicles used for the experiments shown in Fig. 1 were set to the same content of MAO. Consequently, the vesicle fraction had a much lower protein content than the crude homogenates, and so presumably displayed less non-specific binding, resulting in a shift to the left of the inhibition curves (Fig. 1).

It has been known for a long time that MAO activity increases as the oxygen tension rises [32, 33]. Comparisons of the activity of the enzyme in air and in oxygen ('oxygen ratio') have shown that this ratio depends upon the particular substrate used [13, 14, 15, 16]. The oxygen ratios for benzylamine and β-phenethylamine, at concentrations near their respective K_m values in oxygen are significantly different (Table 2).

Houslay and Tipton [34] showed that the deamination of benzylamine by the MAO of rat liver followed a ping-pong, or double displacement reaction. A similar situation is thought to occur for the MAO of pig brain [35], ox liver [36] and ox thyroid gland [37].

The kinetics of the reaction with rat liver MAO can be described:

$$v = \frac{V_{max}}{1 + \frac{K_s}{[S]} + \frac{K_o}{[O_2]}} \quad (1)\ [\text{see } 34]$$

where K_s and K_o are the apparent Michaelis constants for the enzyme towards the amine substrate and oxygen respectively. It should be noted that these constants are not dissociation constants, but are derived from the steady state equations (see [1, 34]). Equation (1) states that the activation by oxygen is uncompetitive. This uncompetitive activation, previously shown only when benzylamine was used as substrate [34] has been shown here to hold for all four substrates tested (Fig. 2A, B, C, D), which would suggest that the general mechanism postulated for rat liver MAO [34] applies to all four substrates used.

At different oxygen concentrations, equation 1 can be rewritten:

$$\frac{v_2}{v_1} = 1 + \frac{(O_{II}/O_I - 1)}{(1 + K_s/[S])(O_{II}/K_o) + 1} \quad (2)$$

where v_1 and v_2 are the velocities at oxygen concentrations of O_I and O_{II} respectively.

At infinite amine substrate concentrations, $K_s/[S] = 0$, and equation 2 becomes:

$$\frac{v_2}{v_1} = 1 + \frac{O_{II}/O_I - 1}{O_{II}/K_o + 1} \quad (3)$$

Thus, at infinite substrate concentrations, the higher this oxygen ratio, the higher the K_o. The maximum oxygen ratios can be calculated from the data shown in Fig. 2 by the method of Wilkinson [30], and are shown in Table 2.

From these data, assuming that the oxygen concentrations in the reaction mixtures under air and oxygen are 0.217 and 1.085 mM [see 16, 38], approximate K_o values can be calculated from equation 3. The mean values (± S.E.R.) are respectively: 5-HT, 153 ± 36 μM; tyramine, 335 ± 58 μM; β-phenethylamine, 553 ± 69 μM and benzylamine, 144 ± 52 μM.

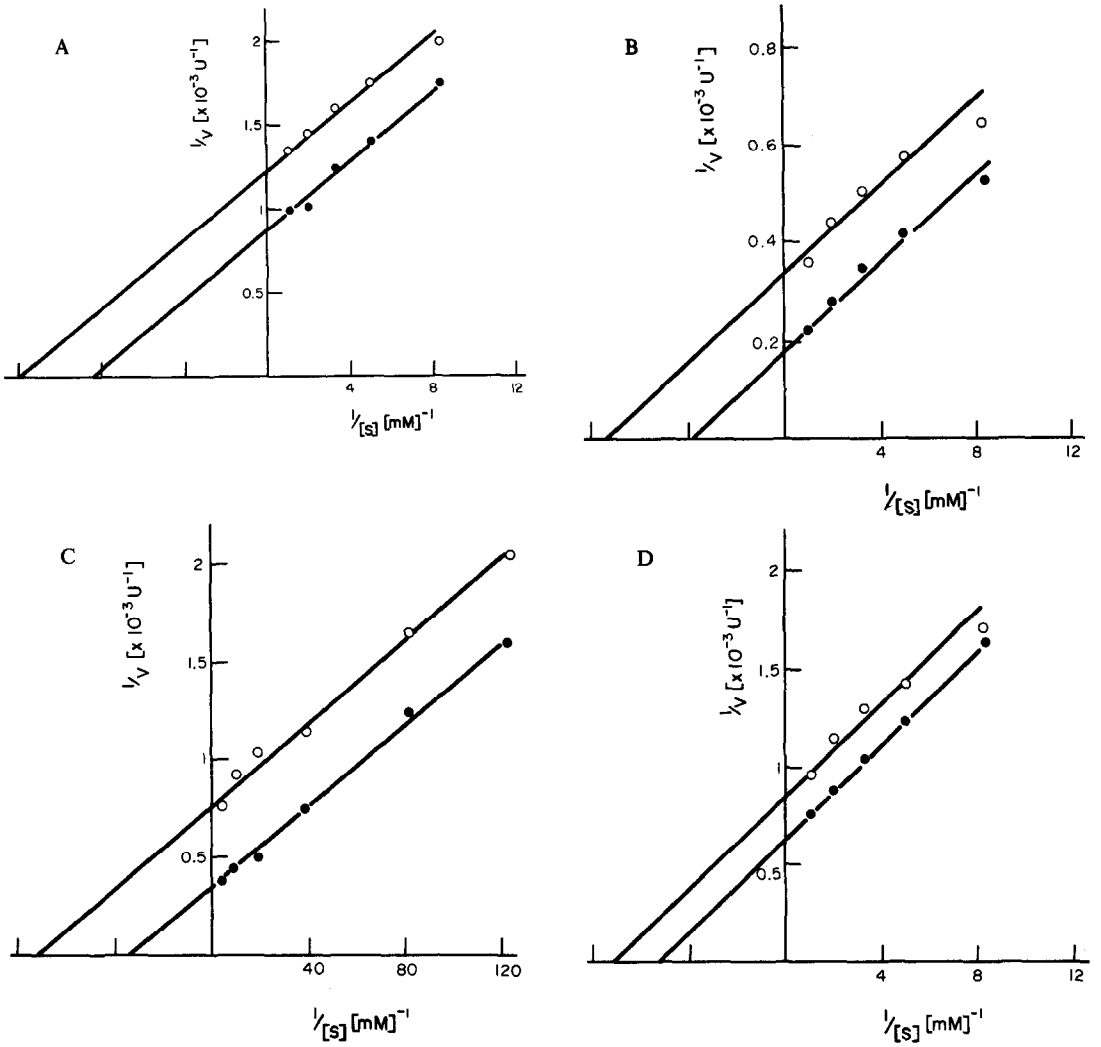

Fig. 2. Double reciprocal plots of the MAO activity of rat liver mitochondrial membrane vesicles assayed in an atmoshpere of air (○) and oxygen (●). The substrates used were: A, 5-HT; B, tyramine; C, β-phenethylamine; D, benzylamine. Each point represents the mean of triplicate determinations in three groups each of two rats. At all incubation periods used, no deviation from linearity could be detected.

Although these values are only approximate, the value for benzylamine compares very well with that of 156 μM obtained by Houslay and Tipton [34].

It is possible that the differences in the apparent K_o values with the different substrates could be due to substrate inhibition by high concentrations of oxygen with some of the amine substrates. However, this is not the case when benzylamine is used as substrate [34]. Moreover, in a double-displacement reaction, substrate inhibition would not be expected to produce parallel regression lines on a Lineweaver–Burk plot (see [39]). In these experiments, no such deviation could be discerned.

The maximum oxygen ratios, and hence the K_o values, appear to divide into at least two groups, the value for tyramine and β-phenethylamine being higher than that for 5-HT and benzylamine. This apparent difference in K_o values does not appear to be affected by changes in ionic strength, or by sonication of the vesicles [40].

The 'maximum oxygen ratios' for 5-HT and tyramine in these experiments are similar to those that can be calculated from the results of Jain, Sands and Von Korff [14] for the deamination of these two substrates by rabbit brain mitochondrial MAO.

Cleland [41] has suggested that, in a double displacement reaction, an inhibitor with an affinity for both oxidised and reduced forms of the enzyme would produce non-competitive kinetics. However, if the concentration of the second substrate is high, or if the apparent Michaelis constant for the second substrate is low, the inhibition will now appear to be competitive in nature. Roth [42] has shown that amitriptyline inhibits the deamination of β-phenethylamine by MAO-B of human brain in a non-competitive manner when the reaction was performed in air, whereas in an atmosphere of oxygen the inhibition was competitive. However, when benzylamine was used as substrate, the inhibition was competitive in both cases. Furthermore, in air, the two substrates

mutually inhibited the deamination of each other in a non-competitive manner which became competitive in oxygen. However, in the experiments with rat liver reported here (Table 3), the inhibition by β-phenethylamine of benzylamine deamination and vice versa was competitive in both air and oxygen. It is tempting to suggest that in the human brain the differences between the kinetics of inhibition of the deamination of β-phenethylamine and benzylamine in air could be due to a lower K_o for the activity against benzylamine. This may also be the explanation of the effects of some tricyclic compounds that inhibit MAO-B in human platelets [10, 43].

These results would reinforce the conclusion made earlier [6] that the simple binary classification of MAO into MAO-A and MAO-B is an oversimplification. It is possible that the multiple forms could be redefined according to their K_o, whereby 'MAO-1' has a K_o value higher than that for 'MAO-2'. If this is the case, the deamination of 5-HT in the rat liver can be said to be brought about by 'MAO-A2', β-phenethylamine by 'MAO-BI', and benzylamine by 'MAO-B2'. Tyramine may be deaminated by both 'MAO-A1' and 'MAO-B1'. The results of the mutual inhibition experiments with β-phenethylamine and benzylamine could be interpreted as evidence for a single amine binding site together with two binding sites for oxygen. The amine binding site would therefore be responsible for the differential sensitivity to inhibition by clorgyline. However, many more experiments with a variety of tissues and substrates are needed before this division of MAO activity can be justified.

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