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MONOAMINE OXIDASE—I

SPECIFICITY OF SOME SUBSTRATES AND INHIBITORS

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Abstract—A number of synthetic substrates and inhibitors of monoamine oxidase have been studied, using the enzyme from porcine brain. K_m and V_{max} values have been calculated for substrates using Lineweaver–Burk plots. In many cases large variations in K_m and V_{max} were observed for relatively small changes in the structure of substrates. Similar observations were made concerning K_i values for some competitive inhibitors. The effects of hydrogen-ion concentration on enzyme activity are consistent with the view that unprotonated amines are the species which bind to the enzyme. This finding, together with the observation that tertiary amines can act as substrates has led to formulation of a proposed mechanism of dehydrogenation which does not depend upon intermediate formation of Schiff base from the substrate amine.

MONOAMINE oxidase, MAO (monoamine O_2 oxido-reductase (deaminating), EC 1.4.3.4) catalyses the oxidation of a large number of alkylamines and aralkylamines, the latter group including its physiological substrates. Though a number of previous investigations have considered the problem of specificity of MAO,[1–4] the present investigation was prompted by two factors. Firstly, there is increasing evidence that MAO in some tissues may be a mixture of iso-enzymes,[5–7] hence specificity studies using the enzyme from such sources may reveal no more than the overall specificity of the iso-enzyme complex. Secondly, many investigations of MAO have been confined to comparisons of oxidation rates at single concentrations of substrate.

The present communication reports on the specificity of a partially purified preparation of MAO from porcine brain, which is thought to contain only a single MAO enzyme.[8,9] The effects of structural variations in a number of synthetic substrates on K_m values and on the maximum velocity of the enzyme reaction have been investigated. Structural variations have been confined to changes in chain length of phenylalkylamines and to alterations of substituents in the aromatic ring. Effects of pH change on Michaelis–Menten kinetics of the enzyme have also been examined. The findings with these compounds prompted investigation of the specificity of a small number of inhibitors which had structures analogous to the substrates. The effects of structural variations and of pH on the K_i values of these inhibitors was found to be consistent with the conclusions for substrates. Namely, that minor changes, particularly in the relative orientations of amine function and aromatic ring, are important determinants of activity. Suggestions concerning the mode of interaction of enzyme and substrate, and the way in which substrates may be dehydrogenated by MAO, have been made on the basis of the findings.

MATERIALS AND METHODS

Substrates. Benzylamine, *N*-methylbenzylamine, phenylethylamine, γ-phenyl-*n*-propylamine and δ-phenyl-*n*-butylamine (Ralph N. Emanuel, Ltd.) were converted to their HCl salts by dissolving in dry ether and adding a solution of HCl gas in dry ether. 3,4-dimethoxyphenylethylamine and *p*-methoxyphenylethylamine (Koch–Light Laboratories Ltd.) were similarly treated. All these salts were crystallized to constant melting point. Tyramine hydrochloride (Koch-Light Laboratories Ltd.) was crystallized twice from ethanol. *N*,*N*-dimethyltyramine (hordenine) sulphate was from Ralph N. Emanuel Ltd.

3-Phenylpiperidine was prepared from methyl-4-cyano-4-phenylbutyrate[10] as described by El'tsov *et al.*[11] 4-phenylpiperidine was prepared by reduction of 4-phenylpyridine as described by Bally.[12] Amphetamine hydrochloride was a gift from Mr G. Welshman, Biochemistry Department, Belfast City Hospital.

The isomeric fluorobenzylamines were prepared from the corresponding fluorobenzaldehydes, as exemplified by the para compound. To *p*-fluorobenzaldehyde (2·48 g) in ethanol (15 ml) was added hydroxylamine hydrochloride (1·5 g) in water (5 ml), followed by 2N NaOH (10 ml). This mixture was refluxed for 15 min. The resulting solution was reduced to half-bulk by evaporation *in vacuo* and its pH was adjusted to approximately 6 by addition of dilute HCl. Water (30 ml) was added, the precipitate was collected by filtration and dried over P_2O_5. Yield, 2 g, m.p. 113°.

The oxime (1g) in dry ether (50 ml) was added dropwise to $LiAlH_4$ (1 g) stirred in dry ether (100 ml). The vigorous reaction was controlled by the rate of addition. After the addition was complete the mixture was refluxed for a further 30 min, cooled to room temperature and the excess hydride was decomposed by careful addition of water. The suspension was filtered and the residue stirred with ether (50 ml). This extract and the filtrate were combined and dried over $MgSO_4$. The ether was reduced to a volume of *ca.* 50 ml and a solution of HCl gas in ether was added which yielded a white precipitate of the amine salt.

Trans-2-phenylcyclohexylamine was prepared in an analogous manner from 2-phenylcyclohexanone. In this case the period of refluxing at the oxime stage was 30 min, and sufficient ethanol was added to maintain a solution at reflux. The fluorophenylethylamines were prepared by condensation of the appropriate fluorobenzaldehyde with nitro methane to yield the corresponding nitro styrene, as described for ω-nitrostyrene itself.[13] These were reduced with $LiAlH_4$ to the amines as described above.

2-Phenylpiperidine was prepared as follows. 4-benzoyl butyric acid was converted to its oxime as described for 2-phenylcyclohexanone. This oxime was hydrogenated by dissolving in methanol and shaking with hydrogen at room temperature and pressure in presence of 10% palladium-on-carbon catalyst. Sufficient 2N HCl was then added to dissolve the resulting precipitate, and after filtering off the catalyst the solution was concentrated to small bulk *in vacuo*. It was then neutralized (pH 7) with saturated Na_2CO_3 and after keeping the suspension in ice for 1 hr the precipitate of 5-amino-5-phenyl valeric acid was collected. The dried material was heated in an oil bath until it began to melt (170°). The temperature was quickly raised to 200° and held there until effervescence had largely ceased (5 min). The product was crystallized from aqueous methanol to yield 72% of 6-phenyl-2-piperidone. 1·2 g was added in portions to a stirred solution of $LiAlH_4$ (1 g) in dry ether (100 ml). The pro-

duct was isolated as described for *p*-fluorobenzylamine, yielding the HCl salt as a sticky solid. Trituration with acetone (5 ml) removed this stickiness, leaving 0·7 g of 2-phenyl piperidine hydrochloride.

β-Methoxy-*β*-phenylethylamine was prepared thus. α-bromo phenylacetic-acid (8·6 g) was added to a solution of sodium methoxide, prepared by dissolving Na metal (2 g) in methanol (50 ml). The solution was refluxed for 2 hr, after which the solvent was removed *in vacuo*. The residue was treated with water, brought to pH 4 and extracted into ether. The dried ether solution was evaporated to yield an oil which was largely methyl-α-methoxy-phenylacetate. This material was heated under reflux in methanol (50 ml) containing concn. H_2SO_4 (3 ml) to convert any free acid to the methyl ester. After 1·5 hr the methanol was evaporated *in vacuo* and the residue, in ether, was washed successively with 50 ml portions of 10% Na_2CO_3 and water. The ether was removed *in vacuo* and the residue was shaken at room temperature with aqueous ammonia (S.G. 0·88, 30 ml) until crystals began to appear (20 min). The suspension was stored overnight at 4°. The α-methoxy-α-phenyl acetamide (3·3 g) was collected by filtration. Reduction with $LiAlH_4$, as described for 6-phenyl-2-piperidone, gave the desired amine, which was converted to its HCl salt.

All the compounds described were crystallized to constant melting point and analytical purity, usually from *n*-butanol or iso-propanol, with or without the addition of petroleum ether.

Monoamine oxidase. All operations were carried out at 2–4° unless otherwise stated. Protein was estimated by the method of Lowry *et al.*[14] A mitochondrial fraction was isolated from porcine brain as described by Tipton,[15] except that the final preparation was suspended in ice-cold water and stored at −20° for 2 days. The frozen suspension was allowed to thaw at room temperature and was then diluted with water so that the protein concn. was *ca.* 10 mg/ml. To this was added sufficient benzylamine base to bring the pH to 8·2. This suspension, in a stainless steel vessel, was sonicated for 40 min using a MSE 100 watt sonicator with 0·5 in. probe. The temperature was maintained below 8° by external ice-cooling. The resulting preparation was centrifuged at 6000 *g* for 20 min and the sedimented material was discarded. Centrifuging of the supernatant at 100,000 *g* for 2 hr gave a yellow–brown pellet which contained most of the MAO activity. This was resuspended by hand homogenization in sufficient 0·01 M phosphate buffer, pH 7·4, to give a protein concentration of 5 mg/ml. The pH was adjusted as before and the opalescent preparation was treated with a 20% solution of Triton X-100 to a final concentration of 1 per cent. This was gently stirred for 30 min and brought to 15 per cent saturation with solid ammonium sulphate. After a further 10 min the suspension was centrifuged at 20,000 *g* for 20 min and the resulting pellet was discarded. The supernatant was treated with a further portion of ammonium sulphate to 35 per cent saturation, it was stirred for 10 min and centrifuged as before, when the protein was seen to be floating on the surface. The infranant solution was carefully removed and the pale yellow residue was dispersed in 0·05 M phosphate buffer, pH 7·4 (1 ml for each 5 mg of protein in the original sample, before Triton treatment). The suspension was centrifuged at 20,000 *g* for 20 min and the pellet was discarded. The yellow supernatant was treated with ammonium sulphate (40 per cent saturation), and centrifuged as before. The residue was dissolved in a small volume of 0·05 M phosphate buffer, pH 7·4, containing 0·1% of Triton X-100 and dialysed against three changes of the

same buffer for 4 hr. The preparation was diluted so that each 1 ml contained 0·025 units of enzyme activity (see below). This procedure produced about a 20-fold purification of the enzyme. Electrophoresis in 5% acrylamide gel gave a single band when stained with nitrotetrazolium blue, which is specific for MAO.[16]

Measurement of MAO activity. All measurements of MAO activity were made at 30° by following oxygen uptake in an oxygen electrode (Rank Bros., Bottisham, Cambridge) at a polarizing voltage of − 0·7 V, using air as the gaseous phase. For those experiments within the buffering range of phosphate, 0·05 M phosphate buffer was used. Outside this range either 0·1 M acetate or 0·1 M borate buffer was employed. The assay medium was as follows: enzyme preparation, 0·5 ml; buffer, 1·35 ml; 0·02 M KCN in buffer, 0·1 ml. After equilibrating this mixture by stirring in the open electrode compartment the system was sealed and the reaction started by addition of 0·05 ml of a solution of substrate in buffer. The reaction was followed on a Honeywell "Electronik 15" potentiometric recorder.

A unit of enzyme activity was defined as the amount of enzyme catalysing the uptake of 1 μmole of oxygen at 30° and pH 7·4 using tyramine hydrochloride (final concentration 1·5 mM) as substrate.

pK_a *values.* pK_a values of substrates were measured at 30° by potentiometric titration of 0·01 M solutions of the hydrochlorides using 0·1 M NaOH as titrant. The method and apparatus used were as described by Albert and Serjeant.[17]

RESULTS

Rates of oxidation at 30° and pH 7·4 were measured over a range of concentrations for each substrate and the data obtained were plotted by the method of Lineweaver and Burk,[18] to give values for K_m and V_{max}. Results appear in Table 1. The most

TABLE 1. MICHAELIS PARAMETERS FOR SUBSTRATES OF MAO MEASURED AT PH 7·4 AND 30°

Substrate	K_m (μM)	V_{max} (nmoles O_2/min)
N-Methylbenzylamine	377	8·4
p-Fluorobenzylamine	2170	19·6
m-Fluorobenzylamine	500	7·7
Benzylamine	625	18·2
β-Phenylethylamine	11·0	9·2
γ-Phenyl-*n*-propylamine	70·4	13·3
δ-Phenyl-*n*-butylamine	11·0	9·6
o-Fluorophenylethylamine	19·3	8·1
m-Fluorophenylethylamine	18·1	10·2
p-Fluorophenylethylamine	12·9	10·5
p-Hydroxyphenylethylamine	110	14·8
p-Methoxyphenylethylamine	15·1	9·1
3,4-Dimethoxyphenylethylamine	278	6·6
N,*N*-Dimethyltyramine (hordenine)	2330	6·6

obvious feature of the results obtained for substrates is that in general K_m and V_{max} values for benzylamines are considerably higher than for phenylethylamines. Ring substituents are variable in their effects, though it is of interest that *O*-methylation of tyramine causes a large decrease in K_m. The fluorinated compounds are good

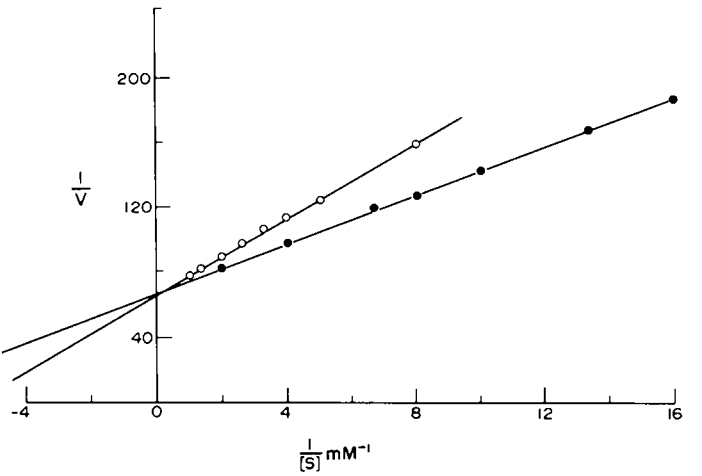

FIG. 1. Lineweaver–Burk plots showing competitive inhibition of tyramine oxidation by *o*-fluorobenzylamine (5×10^{-5} M) pH 7·4, 30°.

substrates, but *o*-fluorobenzylamine was not attacked, in marked contrast to *o*-fluorophenylethylamine. The latter compound was shown to be a competitive inhibitor of the oxidation of tyramine with a K_i value of $8{\cdot}9 \times 10^{-5}$ M (Fig. 1). Some of the phenylethyl compounds showed evidence of inhibition at high concentrations, but this was not apparent in the benzylamine series (Fig. 2). The tertiary amine hordenine is seen to be a substrate for the enzyme albeit a rather poor one.

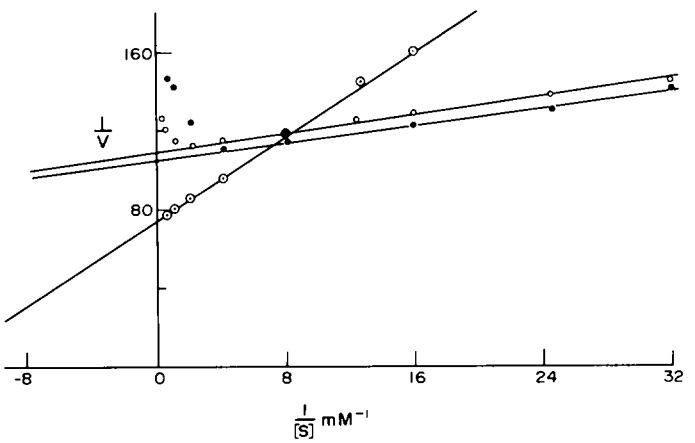

FIG. 2. Lineweaver–Burk plots for oxidation of phenylpropylamine (⊙), phenylethylamine (○) and phenylbutylamine (●) pH 7·4, 30°. The two latter compounds show substrate inhibition at high concentrations, a fact also noted for some phenylethylamines.

pK_a values for substrates appear in Table 2. Those for inhibitors, at 30°, were as follows: 2-phenylpiperidine, 9·53 ± 0·03; 3-phenylpiperidine, 10·06 ± 0·03; 4-phenylpiperidine, 10·35 ± 0·04; *trans*-2-phenylcyclohexylamine, 8·75 ± 0·02. The pK_a value for amphetamine at 30° was calculated as 9·77, from a value at 25° of 9·92,[19] using the formula derived by Perrin.[20]

TABLE 2. K'_m VALUES FOR SOME SUBSTRATES OF MAO AT 30° OBTAINED BY RECALCULATION OF K_m VALUES, ASSUMING THAT THE ACTIVE SPECIES OF AMINE IS THE NEUTRAL MOLECULE. THE QUOTED pK_a VALUES WERE MEASURED AT THE SAME TEMPERATURE

Substrate	pK_a	K'_m (M)
Benzylamine	9·22 ± 0·02	$9{\cdot}3 \times 10^{-6}$*
β-Phenylethylamine	9·64 ± 0·02	$6{\cdot}3 \times 10^{-8}$†
γ-Phenylpropylamine	10·03 ± 0·02	$1{\cdot}3 \times 10^{-7}$*
δ-Phenyl-*n*-butylamine	10·18 ± 0·03	$1{\cdot}8 \times 10^{-8}$†
p-Methoxyphenylethylamine	9·90 ± 0·04	$4{\cdot}8 \times 10^{-8}$†
p-Fluorobenzylamine	9·20 ± 0·02	$3{\cdot}2 \times 10^{-5}$*
o-Fluorophenylethylamine	9·65 ± 0·02	$1{\cdot}1 \times 10^{-7}$†

* Calculated from experimental data at various pH values.
† Derived from K_m value at pH 7·4 only.

Figure 3 shows the effects of pH on the rate of oxidation for four amines. All measurements were made at 1 mM concentrations of substrates. This examination was extended by measuring oxidation rates at varying concentrations of four different substrates over a pH range of about 7–9. Figure 4 is a series of Lineweaver–Burk

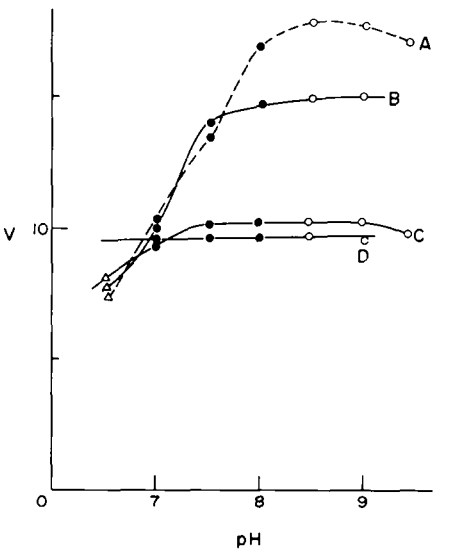

FIG. 3. pH vs velocity curves for oxidation of benzylamine (A), tyramine (B), *p*-fluorophenylethylamine (C) and *p*-methoxyphenylethylamine (D). 30°. 1 mM concentration. Buffers: 0·1 M acetate (▲), 0·1 M borate (○), 0·05 M phosphate (●).

plots obtained from such measurements for benzylamine and indicates that little change in V_{max} occurs over a pH range in which there is a large fall in K_m values. Similar results were obtained for *p*-fluorobenzylamine and phenylpropylamine. At pH values higher than those indicated in Fig. 4, effects upon V_{max} were apparent, but oxygen uptake became non-linear and in some cases terminated quickly so that reliable data were not readily obtained.

These pH effects were found to be similar to those described by McEwen and his co-workers for MAO from human liver.[21,22] These workers showed that K_m values

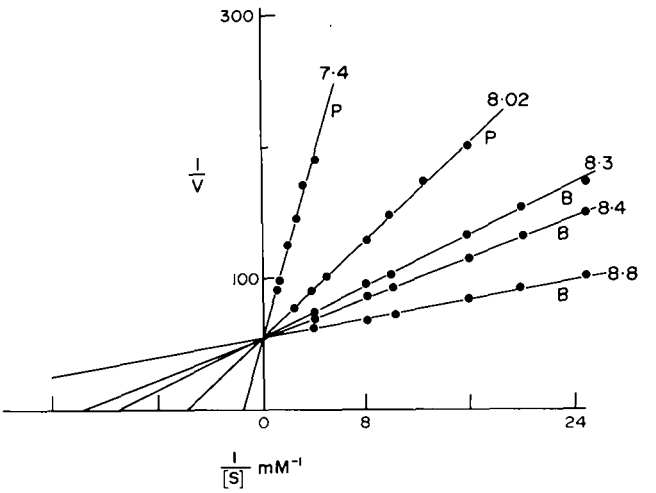

FIG. 4. Lineweaver Burk plots for oxidation of benzylamine at indicated pH values and 30° in phosphate (P) of borate (B) buffer.

for substrates of MAO were related to their pK_a values in a modified form of the Henderson–Hasselbach equation.

$$K_m = K'_m[1 + \text{antilog}\,(pK_a - pH)]$$

where K'_m is the Michaelis constant expressed in terms of the concentration of unionized substrate. This value was found to be constant for a given substrate at all pH values, indicating that the unprotonated amine is the species which interacts with the enzyme. Table 2 gives such values calculated from the present data and it can be seen that K'_m values show little change with pH.

In inhibition studies, initial velocities were determined for a range of substrate concentrations in the presence of a fixed concentration of inhibitor. For studies at pH 7·4 tyramine hydrochloride was used as substrate. Data obtained were plotted by the Lineweaver–Burk method[18] to yield apparent Michaelis constants K_p which were used to calculate K_i values thus:

$$K_i = \frac{i}{K_p/K_m - 1}$$

where i is the concentration of inhibitor. Table 3 lists K_i values obtained at 30°. Figures 5 and 6 show Lineweaver–Burk plots in presence of inhibitors at pH 7·4. The effects of pH on inhibition of MAO were studied in a similar way; phenylpropylamine replacing tyramine as substrate to avoid possible influences of the second ionizable grouping in the latter compound. In keeping with the finding for substrates, inhibitor constants were dependent upon pH in a way which suggested that the unprotonated amine interacts with the enzyme. Figure 7 shows the effects of pH on the inhibition of phenylpropylamine oxidation by 2-phenylpiperidine. Similar results were obtained using 4-phenylpiperidine and amphetamine as inhibitors. K_i values calculated from the graphical data appear in Table 3.

TABLE 3. INHIBITOR CONSTANTS FOR SOME COMPETITIVE INHIBITORS OF MAO AT 30°. THE MIDDLE COLUMN GIVES K_i VALUES MEASURED AT pH 7·4. THE LAST COLUMN SHOWS K'_i VALUES; THE INHIBITOR CONSTANTS EXPRESSED AS THE CONCENTRATION OF UNIONIZED AMINE, WHICH IS INDEPENDENT OF pH

Amine	K_i(M)	K'_i(M)
Amphetamine	$2{\cdot}34 \times 10^{-4}$	$9{\cdot}8 \times 10^{-7}$*
2-Phenylpiperidine	$1{\cdot}35 \times 10^{-4}$	$8{\cdot}9 \times 10^{-7}$*
3-Phenylpiperidine	$4{\cdot}4 \times 10^{-5}$	$9{\cdot}6 \times 10^{-8}$†
4-Phenylpiperidine	$5{\cdot}4 \times 10^{-4}$	$6{\cdot}1 \times 10^{-7}$*
trans-2-Phenylcyclohexylamine	$9{\cdot}6 \times 10^{-4}$	$5{\cdot}5 \times 10^{-6}$†
β-Methoxy-β-phenylethylamine	$2{\cdot}9 \times 10^{-5}$	$1{\cdot}2 \times 10^{-6}$†

* Calculated from experimental data at various pH values.
† Derived from K_i value at pH 7·4 only.

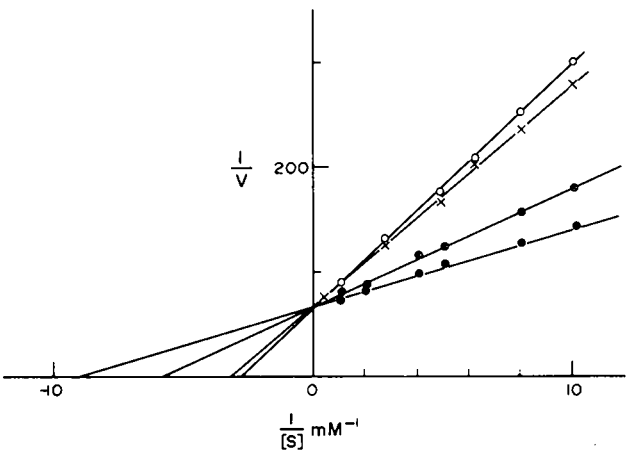

FIG. 5. Lineweaver–Burk plots showing inhibition of tyramine oxidation (●), by 2-phenylpiperidine, 10^{-4} M (⊗), 3-phenylpiperidine, 10^{-4} M (○) and 4-phenylpiperidine, 10^{-3} M (×). pH 7·4, 30°.

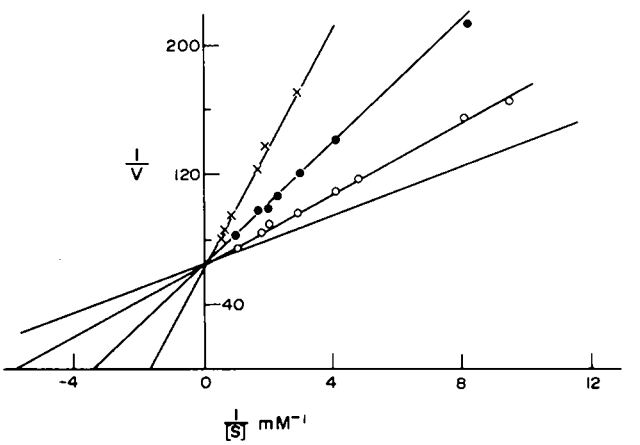

FIG. 6. As Fig. 1, using as inhibitors 2-phenylcyclohexylamine, 5×10^{-4} M (○) and amphetamine, 5×10^{-4} M (●), or 10^{-3} M (×).

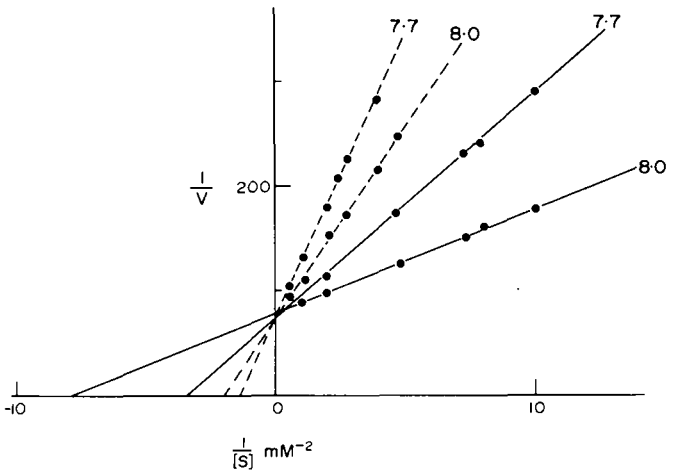

FIG. 7. Lineweaver–Burk plots for phenylpropylamine oxidation (●——●), showing inhibition by 2-phenylpiperidine, 10^{-4} M, (●- - -●) at indicated pH values and 30°.

DISCUSSION

The foregoing results indicate that MAO from porcine brain, in common with other amine oxidases, has a broad substrate specificity, but it appears that small structural changes can lead to large changes in activity. The enzyme attacks primary, secondary and tertiary amines though evidence on the latter is limited to hordenine. Oxidation of tertiary amines by MAO has previously been reported by others. For example, Richter[23] showed that hordenine was slowly attacked by the enzyme from rat liver, and Smith *et al.*[24] have produced clear evidence that *N,N*-dimethyl tryptamine is oxidized by a mitochondrial MAO from liver of the guinea-pig. Although tertiary amines seem to be rather poor substrates for the enzyme, the fact that they are attacked raises questions as to the mechanism of dehydrogenation of amines by MAO. This point is considered later in the discussion.

Alteration of the chain length of phenylalkylamines brings about substantial changes in the activity of MAO. If it is assumed that abstraction of an α-hydrogen as a proton is the rate determining step in oxidation of amines by MAO[25,26] then the high V_{max} value for benzylamine relative to its three homologues (Table 1) is explicable in terms of the electronic characteristics of the methylene group in this compound. Proton abstraction will be facilitated by the electrophilic character of the α-carbon atom. Hellerman and Erwin[26] observed that introduction of electronegative substituents into the para-position of benzylamine increased their rate of oxidation by MAO from bovine kidney. This would be expected if α-proton abstraction was occurring.

Such an explanation does not however account for the higher oxidation rate for phenylpropylamine than for its two congeners, phenylethylamine and phenylbutylamine. It is also noteworthy that K_m values for the latter two amines are very much lower than for their odd-numbered homologues.

A possible explanation for these differences might lie in the varying degrees of flexibility of the molecules. Benzylamine, with only a single carbon atom in its side-chain, may experience severe constraints in binding to the enzyme. It can be envisaged that

to accommodate this substrate, the enzyme may have to undergo a considerable configurational change. Looked at another way, phenylbutylamine, having a more flexible side-chain might more easily adapt itself to the stereochemistry of the active site of MAO. On this basis, it can be predicted that before being able to bind benzylamine, the enzyme adopts a configuration which chances to facilitate abstraction of an α-proton, thus leading to a high rate of oxidation.

The large difference in K_m values of benzylamine and phenylethylamine is paralleled throughout the series of substrates tested. As can be seen from Table 1, amines with a one-carbon side chain have much larger K_m values than their two-carbon congeners, though in both series the effects of ring-substituents are superimposed upon this phenomenon. A consideration of the three C-phenylpiperidines reveals that their K_i values at pH 7·4 vary with the number of carbon atoms between aryl ring and nitrogen function in the same manner as K_m values for substrates. Since K_i values reflect the affinity of an inhibitor for an enzyme, it is possible in the present case that K_m values for amine substrates may to some degree be a reflection of their affinity for MAO. In support of this McEwen *et al.*[22] have shown that amines acting as competitive inhibitors of MAO have K_i values which are virtually the same as their K_m values when acting as substrates.

On this basis, some indications of the determinants of binding of amines to MAO can be discerned. Phenylethyl- and phenylbutylamine can be so arranged that their amino-groups and α-carbon atoms are directly superimposed, with their aromatic rings lying in the same plane, but occupying adjacent positions in an area having a "naphthalene" shape. Neither of the odd-numbered homologues fits easily into such a space; either the aromatic ring resides outside the proposed area, or the $-CH_2NH_2$ group is a much poorer fit, though the greater flexibility of phenylpropylamine compared with benzylamine should enhance its chances of binding to the enzyme. A similar argument can be applied to the C-phenylpiperidines since the best inhibitor of the three is 3-phenylpiperidine which is the only one able to adopt a configuration about the Ar–C–C–N portion of the molecule identical to that of phenylethyl amine.

Support for this idea comes from work on MAO from bovine kidney by Hellerman and Erwin[26] and on the enzyme from human liver by McEwen *et al.*[22] Both these groups have shown that naphthols act as competitive inhibitors of the enzyme, implying that an extensive hydrophobic binding area is present near the active site of MAO, in keeping with the suggestions made above. That this area is not merely a non-specific hydrophobic site is borne out by the disparate behaviour of the phenylalkylamines with the enzyme.

This selectivity of action, and the implication that the relative positions of aromatic ring and $-CH_2NH_2$ group are important determinants of affinity of amines for MAO, are further borne out by a comparison of amphetamine and trans-2-phenylcyclohexylamine. The latter compound bears a formal similarity to amphetamine in that it possesses a Ph–C–C–NH_2 structure substituted on the carbon atom carrying the amino group. Being part of a cyclic structure it has a much greater rigidity than the side chain of amphetamine itself. It can be assumed that, with the cyclohexyl moiety in the chair form, it is energetically favourable for the aromatic ring to adopt a position roughly at right-angles to the plane of the homocyclic ring. In such a configuration it is at a maximum distance from the otherwise hindering amino

hydrogens. K_i values for these two compounds show that trans-2-phenylcyclohexylamine is a poorer inhibitor than amphetamine. It seems unlikely that this is due to the cyclohexyl ring *per se*, in view of the inhibitory potency of 3-phenylpiperidine, for example. More likely is that the molecule is unable to adopt a configuration favourable for binding to the enzyme since the C–C–NH_2 structure has little flexibility independently of the aromatic ring.

It was surprising to find that DL-β-methoxy-β-phenylethylamine was not oxidized under the conditions used here. That this was not due to a lack of affinity for MAO is indicated by its K_i value ($2{\cdot}9 \times 10^{-5}$ M) as a competitive inhibitor. Phenylethanolamines are oxidized by MAO, though it has recently been suggested that the highly polar –OH function reduces their efficiency.[9] By methylating this functional group it was hoped to produce a better substrate for the enzyme. It seems that the presence of the bulky methoxy group close to the α-carbon atom precludes productive binding.

K_m values for substituted phenylethylamines are consistent with the proposed hydrophobic binding site for the aromatic ring. The high K_m value observed for tyramine (110 μM) is markedly reduced by *O*-methylation of the highly polar –OH group (15·1 μM). Differences between mono- and dimethoxyphenylethylamines are less readily explained. This is particularly true of the fall in oxidation rate, but the difference in K_m values may be rationalized on steric grounds since introduction of a third methoxy group leads to a compound (mescaline) which is reported not to be oxidized by MAO.[27]

The contrast between the oxidation of fluorobenzylamines and the fluorophenylethylamines highlights the difference already noted between 1 and 2 carbon sidechains. K_m values for the latter (13, 18 and 13 μM) are close to that of phenylethylamine (11 μM). Similar remarks apply to V_{max} values for para-derivatives (Table 1). *m*-Fluorobenzylamine is oxidized more slowly than might be anticipated, and the ortho derivative is not a substrate, in marked contrast to *o*-fluorophenylethylamine. These findings do not wholly accord with those of Zeller[27] for the enzyme from bovine liver. He showed that meta-substituted benzylamines were always better substrates, as judged by oxidation rates, than were para-derivatives. This finding was independent of the nature of the substituent, and hence of electronic effects. Zeller did however show that ortho-substituted benzylamines were at best very slowly oxidized, which is in keeping with findings made here for *o*-fluorobenzylamine. Since this compound is a good competitive inhibitor it might be anticipated that ortho-substituents in the benzylamine series are not preventing binding to MAO, but may be interfering with α-proton abstraction by hindering approach towards the α-carbon atom of a nucleophilic site on the enzyme. Consideration of the stereochemistry of ortho-substituted benzylamines shows close proximity of the substituent and the α-hydrogens of the side chain. On the other hand, in fluorophenylethylamine, which is a good substrate, the fluorine atom can attain a much greater distance from both the amino group and the α-hydrogens.

The effects of hydrogen ion concentration on the activity of substrates and inhibitors indicate that the unprotonated species of amines bind to the enzyme. Published data on the effect of pH on MAO activity are variable. As already indicated, the findings of McEwen *et al.*[21,22] are similar to the present results. Erwin and Hellerman[28] showed, using the enzyme from bovine kidney, that pH optima for benzylamine and

tyramine were 8·5 and 7·4 respectively. This may indicate the need to consider ionizable groupings in substrates, other than the amino function. For example, McEwen *et al.*[21] report an optimum of pH 9·5 for the oxidation of benzylamine by MAO from human liver. On the other hand, Youdim *et al.*[5] report that isoenzymes from this tissue have pH optima of 7·0, 7·5, 8·0 and 8·7 using kynuramine, which has two ionizable $-NH_2$ groups. The state of purity of the enzyme may also influence the effects of pH, as a highly purified MAO from porcine brain is reported to have an optimum pH of 7·2.[15] However, the K_m for tyramine with this enzyme is apparently little influenced by purity.[8,15] Changes in buffer salts are also reported to influence pH optima for MAO.[29] Huszti[30] has reported that true K_m values for substrates of MAO from rat brain are independent of pH over the approximate pH range 6·5–9, though he has produced evidence for an ionizable grouping in the enzyme which affects apparent K_m values below about pH 7. In studies of MAO from bovine lever Oi *et al.*[31] have shown that the pK_m values for the RNH_2 species of benzylamine, and the V_{max} for the enzymic reaction, were constant over a range of pH from about 7·2–10. They report the existence of two ionizable groupings in the enzyme with pK_a values of 7·0 and 10·3 approximately. These findings are not inconsistent with the present ones which are limited to a somewhat narrower range of pH.

In spite of these reservations, the findings now reported support the widely held view that unprotonated amine substrates interact with MAO.[21,22,24,25,32]

It has long been held that oxidation of amines by MAO occurs via a dehydrogenation step to yield a Schiff base thus:

$$RCH_2NH_2 \rightarrow RCH{=}NH + 2H$$

This idea is based on the very firm evidence that aldehyde and ammonia (in the case of a primary amine) are products of the enzymic reaction. However, if tertiary amines are substrates for MAO, and assuming that they interact with the enzyme in the unprotonated form, then formation of a Schiff base seems unlikely, if not impossible, since such amines do not possess a hydrogen atom attached to the nitrogen atom.

Previous attempts to explain this anomaly seem unsatisfactory, either invoking a protonated substrate[23] or postulating hydride ion abstraction from the α-carbon atom.[24]

It has been suggested[26] that MAO, like the related flavo-enzyme amino acid oxidase, may have a lysine residue at its active centre. If so, a plausible dehydrogenation mechanism, applicable to primary, secondary and tertiary amines can be advanced. Figure 8 outlines such a mechanism in which after initial abstraction of a proton from the α-carbon atom, the ε-amino group of an adjacent lysine residue displaces the amino function of the substrate. Oxidation of the resulting *N*-alkyl lysine to an internal Schiff base provides the second proton for complete reduction of the enzyme. Subsequent hydrolysis of the Schiff base of the enzyme produces aldehyde, and regenerates the lysine residue. Such a mechanism is consistent with many reported features of amine oxidation by MAO, though it appears at first sight to contradict the finding that inhibition of MAO by phenylethylhydrazine occurs by enzymic dehydrogenation to a hydrazone,[33,34] as first suggested by Davidson.[35] Hydrazone formation would not be predicted from the mechanism proposed. However, Tipton's finding[33] of a time-lag between onset of oxidation of phenylethylhydrazine and the

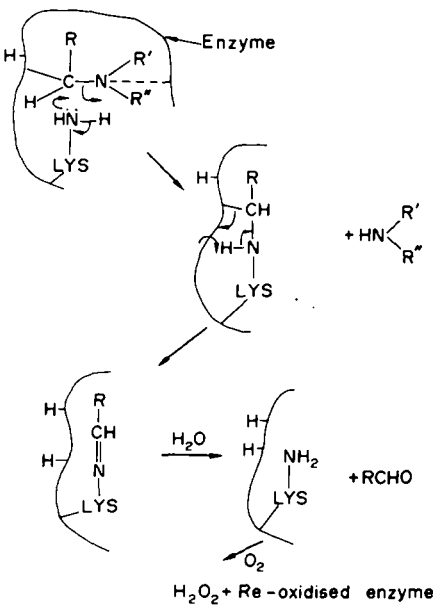

FIG. 8. Suggested scheme for dehydrogenation of amines by MAO to account for oxidation of primary, secondary and tertiary amines. For further details, see text.

development of inhibition, and his observation that addition of phenylacetaldehyde plus hydrazine to MAO causes irreversible inhibition[33] can be interpreted in support of the proposed dehydrogenation mechanism. Oxidation of the hydrazine derivative as suggested by Fig. 8 would lead to formation of hydrazine and phenylacetaldehyde, without intermediate hydrazone formation. Recombination of these products would produce the active inhibitor. The time-lag observed by Tipton would then be consistent with an initially low rate of recombination, due to the low concentrations of reactants in the early stages of oxidation.

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