A Kinetic Evaluation of Monoamine Oxidase Activity in Rat Liver Mitochondrial Outer Membranes

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1. A preparation of mitochondrial outer membranes from rat liver can be shown to contain two kinetically distinct monoamine oxidase activities. These activities are distinguishable by their different sensitivities to the irreversible inhibitor clorgyline, and by the effect of the reversible inhibitors benzyl cyanide and 4-cyanophenol. 2. The substrate specificities of the preparation and the two enzyme species have been elucidated.

A number of lines of evidence have led us to conclude that the multiple forms that can be separated from partly purified preparations of rat liver and human brain monoamine oxidase (EC 1.4.3.4) result from the binding of differing amounts of membrane material to a single enzyme species (Houslay & Tipton, 1973*a*, 1974; Tipton *et al.*, 1973). These results do not, however, exclude the possibility that populations of the enzyme with different properties might exist within an intact cell or organ [see Houslay & Tipton (1973*a*) and Tipton *et al.* (1973) for discussion]. Such a situation could arise from the existence of different portions of the enzyme in different lipid environments, and in this connexion reports that heterogeneous populations of mitochondria, with different monoamine oxidase activities, exist in both rat brain and liver (Kroon & Veldstra, 1972; Neff, 1974) and that pre- and post-synaptic monoamine oxidase activities in the rat vas deferens have different specificities (Jarrott, 1971) may be relevant. An alternative possibility, which cannot be excluded, stems from the fact that the partially purified preparations of monoamine oxidase that were used in these studies contained only about 45% of the activity towards benzylamine as substrate that was present in the original mitochondrial suspension. This procedure may have selectively purified a specific monoamine oxidase type. In addition, the purification procedure used entailed exposing the enzyme to the drastic conditions of prolonged sonication and detergent treatment which could cause rearrangement of the bound membrane material (cf. Houslay & Tipton, 1973*a*), thereby generating an enzyme population very different from that present in the intact tissue.

To investigate the properties and possible existence of multiple species of the enzyme under conditions closer to those in the intact cell, we have studied the properties of the monoamine oxidase in a preparation of rat liver mitochondrial outer membranes that was prepared without the use of detergents and contained

90±5% of the monoamine oxidase activity initially present (Houslay & Tipton, 1973*a,b*).

Methods

Preparation of an outer-membrane fraction

The shrink-swell method of Sottocasa *et al.* (1967) was used as previously described (Houslay & Tipton, 1973*b*). The material chosen for study was that from fraction 2 of the preparation, the combined discontinuous-gradient-centrifugation supernatants after dialysis against 0.01 M-Tris-HCl buffer, pH 7.2. This dialysed material was then either used directly as the enzyme source, or in a three- to four-fold concentrated form. The concentration was achieved by using an Amicon Ultrafiltration apparatus (Amicon Corp., Lexington, Mass., U.S.A.) with a PM-10 membrane.

Enzyme assays

The coupled monoamine oxidase assay with NAD⁺-linked aldehyde dehydrogenase (EC 1.2.1.3) as described by Houslay & Tipton (1973*a*) was used, except that as a routine potassium phosphate buffer (pH 7.2; final concn. 0.12 M) was used and the final concentration of NAD⁺ was 0.3 mg/ml instead of 0.1 mg/ml. The activity of aldehyde dehydrogenase was not rate-limiting under any of the assay conditions.

Direct spectrophotometric assays for monoamine oxidase with benzylamine and its substituted derivatives were carried out as described by Tabor *et al.* (1954).

O₂-electrode assays for monoamine oxidase were carried out as described by Tipton & Spires (1971) with a Clark electrode (Yellow Spring Instrument Co., Yellow Springs, Ohio, U.S.A.).

Other enzyme assays were as described previously (Houslay & Tipton, 1973*a,b*).

All assays for monoamine oxidase were carried out at pH 7.2 in potassium phosphate buffer (final concn. 0.12M) at 30°C unless otherwise indicated.

Spectrophotometric assays were carried out in final volumes of 1 ml in 1 cm light-path silica cuvettes by using a Gilford 240 photomultiplier and Beckman D.U. monochromator.

Assays for monoamine oxidase with radioactively labelled compounds were done by the method of Sierens & D'Iorio (1970) when 5-[1-¹⁴C]hydroxytryptamine creatinine sulphate was the assay substrate, and by the method of Squires (1972) when [1-¹⁴C]tyramine hydrochloride was the assay substrate, except that the assay buffer was potassium phosphate, pH 7.2, to give a final concentration of 0.12M.

Substrates

Those amines purchased as their free bases were converted into their respective amine hydrochlorides and recrystallized before use (see Houslay & Tipton, 1973b).

p-Hydroxybenzylamine hydrochloride and *p*-hydroxy-*m*-methoxy-2-phenylethylamine hydrochloride were synthesized from their respective aldehydes via their oxime derivatives with subsequent hydrogenation. The purity and identity of these recrystallized compounds and intermediate oximes were ascertained by i.r. and n.m.r. analysis and melting-point determinations.

Adrenaline, noradrenaline and their respective *m*-*O*-methyl derivatives were stored *in vacuo*, over silica gel at -12°C when not in use.

All solutions were made up fresh before use.

Other methods

Protein concentrations were determined by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin as standard. The concentration of the mitochondrial outer-membrane preparation is expressed in mg of protein/ml.

Sonication was carried out with a Dawe Soniprobe (Dawe Instruments Ltd., London W.3, U.K.) and pH measurements were made with a Radiometer PHM 22r pH meter (Radiometer A/S, Copenhagen, Denmark).

Glass-distilled water was used in all experiments.

Materials

Clorgyline [*N*-methyl-*N*-propargyl-3-(2,4-dichlorophenoxypropylamine)] was a kind gift from May and Baker Ltd., Dagenham, Essex, U.K. Benzyl cyanide (2-phenylacetone nitrile), 4-cyanophenol and all amine substrates and aldehydes were obtained from Ralph N. Emanuel Ltd., Wembley, Middx., U.K., except

for adrenaline bitartrate which was from BDH Chemicals Ltd., Poole, Dorset, U.K. and noradrenaline bitartrate from Koch-Light Ltd., Colnbrook, Bucks., U.K. 5-[1-¹⁴C]Hydroxytryptamine creatine sulphate and [1-¹⁴C]tyramine hydrochloride were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

NAD⁺ and NADH were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K.

All other chemicals were from BDH Chemicals Ltd. and were of the highest purity available.

Experimental

Enzyme preparation

The specific activities of the preparations used varied between 20 and 30 munits/mg of protein with benzylamine as substrate; however, this activity represented 90±5% of the original mitochondrial activity independently of whether benzylamine, tyramine or 5-hydroxytryptamine were used as assay substrates. Experiments carried out as previously described (Houslay & Tipton, 1973b) indicated that no kinetically significant permeability barriers existed, and that all the monoamine oxidase activity was indeed membrane bound. Similarly, enzyme activity bore a linear relationship to membrane protein concentration over the ranges used in assays. All spectrophotometric assays for monoamine oxidase with this material gave linear progress curves for at least 5 min and all assays involving radioactively labelled substrates gave linear progress curves for at least 10 min (in no case was more than 5% of the substrate utilized over this time-period). The preparation contained no observable NADH oxidase, alcohol dehydrogenase or aldehyde dehydrogenase activity.

Substrate specificity

The substrate specificity of the membrane fraction was determined with a range of substrates (Table 1). It must be noted that over a wide range of substrate concentrations no evidence of non-linearity was observed in double-reciprocal plots (Lineweaver & Burk, 1934). The extinction coefficients $\epsilon^{1\text{cm}}$ (with absorption maxima in parentheses) observed for the corresponding aldehydes of substrates assayed by direct means were: benzaldehyde 13.8×10^3 litre·mol⁻¹·cm⁻¹ (250 nm), *p*-chlorobenzaldehyde 17.4×10^3 litre·mol⁻¹·cm⁻¹ (259.5 nm), *p*-hydroxy-*m*-methoxybenzaldehyde (vanillin) 4.7×10^3 litre·mol⁻¹·cm⁻¹ (309 nm) and *p*-hydroxybenzaldehyde 10.8×10^3 litre·mol⁻¹·cm⁻¹ (287 nm).

pH optimum

A pH optimum was determined in an identical fashion with that described by Houslay & Tipton (1973a), to compare with that exhibited by the soluble

Table 1. *Substrate specificity of the monoamine oxidase activity of mitochondrial outer membranes*

All assays were carried out as described in the Methods section. The correlation coefficients refer to the Lineweaver–Burk plots, each of which had 12 determinations of initial velocities at different substrate concentrations over the range shown.

Substrate	Assay method	K_m (μM)	Relative V_{max}	Substrate concentration range (mM)	Correlation coefficient
Benzylamine	Aldehyde dehydrogenase	245	(100)	7–0.01	0.997
Dopamine	Aldehyde dehydrogenase	405	112	3–0.03	0.998
Tyramine	Aldehyde dehydrogenase	282	200	3–0.05	0.992
4-Hydroxy-3-methoxy-2-phenylethylamine	Aldehyde dehydrogenase	475	133	2.2–0.2	0.998
Tryptamine	Aldehyde dehydrogenase	18.5	81	2–0.018	0.996
2-Phenylethylamine	Aldehyde dehydrogenase	20.8	118	5–0.015	0.999
5-Hydroxytryptamine	Aldehyde dehydrogenase	187	124	4–0.03	0.999
4-Methoxy-2-phenylethylamine	Aldehyde dehydrogenase	23.8	96	1–0.015	0.999
3,4-Dimethoxy-2-phenylethylamine	Aldehyde dehydrogenase	1360	108	2–0.4	0.990
5-Methoxytryptamine	Aldehyde dehydrogenase	15.9	66	1–0.015	0.997
Vanillylamine	Direct	201	68	2–0.1	0.998
<i>p</i> -Chlorobenzylamine	Direct	300	254	1–0.1	0.994
<i>p</i> -Hydroxybenzylamine	Direct	2500	114	5–0.5	0.983
<i>N</i> -Methylbenzylamine	Direct	714	91	3–0.01	0.990
Adrenaline	O ₂ electrode	400	70	7–0.3	0.939
Noradrenaline	O ₂ electrode	416	71	4–0.3	0.981
Octopamine	O ₂ electrode	625	149	4–0.3	0.991
<i>m</i> -O-Methylnoradrenaline	O ₂ electrode	200	54	4–0.1	0.986
<i>m</i> -O-Methyladrenaline	O ₂ electrode	267	65	4–0.1	0.986

enzyme. A distinct pH optimum was exhibited (approx. pH9.5), although the lability of the preparation increased at pH values greater than 10, and irreversible inhibition occurred at values greater than pH11.

Inhibition by benzyl cyanide and 4-cyanophenol

Both benzyl cyanide, an analogue of 2-phenylethylamine, and 4-cyanophenol, an analogue of *p*-hydroxybenzylamine, can be shown to reversibly inhibit in a competitive fashion the oxidative activity towards benzylamine that is exhibited by this preparation. Linear Lineweaver–Burk (1934) plots and linear Dixon (1953) plots were obtained, identical with those shown previously (Houslay & Tipton, 1973*b*), with observed K_i values of 10–20 μM for benzyl cyanide and 20–30 μM for 4-cyanophenol.

However, benzyl cyanide inhibited 5-hydroxytryptamine oxidation reversibly in a mixed fashion (Fig. 1) yielding values of K_i (slope) 240 μM and K_i (intercept) 380 μM . 4-Cyanophenol acted in a similar fashion (Fig. 2) yielding values of K_i (slope) 115 μM and K_i (intercept) 150 μM . Johnston (1968) postulated that the monoamine oxidase activity of rat liver mitochondria could be segregated into two groups which he termed the 'A' enzyme (here designated as 'species A') and the 'B' enzyme (designated 'species B') on the basis of their sensitivity to the

irreversible inhibitor clorgyline. The 'species A' enzyme activity was highly sensitive to clorgyline and 5-hydroxytryptamine was postulated to be a specific substrate for this species. The 'species B' enzyme activity was relatively insensitive to clorgyline inhibition and benzylamine was postulated to be a specific substrate for this species. Further evidence that benzylamine is oxidized by a single component in mitochondrial outer membranes has been obtained by kinetic analysis (Houslay & Tipton, 1973*b*). However, tyramine (*p*-hydroxy-2-phenylethylamine), which appears to be a substrate for both enzyme species on the model of Johnston (1968), should exhibit a non-linear response to inhibition by benzyl cyanide or 4-cyanophenol, as the initial-rate equation, ignoring the effect of the second substrate, O₂, which is kept at constant concentration, will be given by:

$$v = \frac{\bar{v}_{\text{AT}}[T]}{K_m^{\text{AT}}(1 + [\text{BC}]/K_i^{\text{BC}}) + [T](1 + [\text{BC}]/K_i^{\text{BC}})} + \frac{\bar{v}_{\text{BT}}[T]}{K_m^{\text{BT}}(1 + [\text{BC}]/K_i^{\text{BC}}) + [T]}$$

where v is the initial observed velocity of tyramine oxidation, $[T]$ is tyramine concentration, K_i^{BC} , K_i^{BC} , K_i^{BC} are inhibitor constants for benzyl cyanide, $[\text{BC}]$ is benzyl cyanide concentration, $\bar{v}_{\text{AT}}$, $\bar{v}_{\text{BT}}$ are maximum velocities of tyramine oxidation by the

Fig. 1. Inhibition of 5-hydroxytryptamine oxidation by benzyl cyanide

Double-reciprocal plot of the initial velocities of 5-hydroxytryptamine oxidation against 5-hydroxytryptamine concentration at several fixed concentrations of benzyl cyanide. Benzyl cyanide concentrations were 0.2 mM (Δ), 0.4 mM ($\blacksquare$), 0.6 mM ($\bullet$) and zero ($\circ$). Arbitrary units are expressed as a change of 0.001 *E* unit/min at 340 nm. The O_2 concentration was held constant at 0.23 mM throughout these experiments. Unless otherwise stated the coupled assay with aldehyde dehydrogenase was used.

Fig. 2. Inhibition of 5-hydroxytryptamine oxidation by 4-cyanophenol

Double-reciprocal plot of the initial velocities of 5-hydroxytryptamine oxidation against 5-hydroxytryptamine concentration at several fixed concentrations of 4-cyanophenol. The 4-cyanophenol concentrations were 0.05 mM (Δ), 0.1 mM ($\blacksquare$), 0.15 mM ($\bullet$) and zero ($\circ$).

'group A' and 'group B' activity respectively, and K_m^{AT} , K_m^{BT} are the apparent Michaelis constants for tyramine exhibited by the 'group A' and 'group B' activity respectively.

Fig. 3. Inhibition of tyramine oxidation by benzyl cyanide

Dixon plot of the reciprocal initial velocities of tyramine oxidation against benzyl cyanide concentration at several fixed tyramine concentrations. The tyramine concentrations were 0.15 mM ($\bullet$), 0.2 mM ($\blacksquare$), 0.25 mM ($\blacktriangle$), 0.5 mM ($\circ$) and 1 mM ($\square$).

In the presence of benzyl cyanide both double-reciprocal plots and Dixon plots (Fig. 3) are non-linear. Although in some instances the non-linearity of double-reciprocal plots was not greatly marked, Dixon (1953) plots were markedly non-linear (hyperbolic). Obviously the degree of non-linearity will depend on the values of K_i^{BC} , $K_i'^{BC}$ and $K_i''^{BC}$, which would appear to vary slightly from preparation to preparation. Similar results were obtained with 4-cyanophenol (Fig. 4).

Thus it seems that these results are consistent with the model proposed by Johnston (1968).

Substrate specificity of the two enzyme fractions

The above experiment suggests that clorgyline inhibition can be used to ascertain the substrate specificity of these two enzyme species. The response of tyramine, dopamine (3,4-dihydroxyphenethylamine), benzylamine and 5-hydroxytryptamine oxidation to clorgyline inhibition is shown in Fig. 5. The pattern so obtained is similar to that obtained by other workers (Johnston, 1968; Hall *et al.*, 1969; Neff & Goridis, 1972). A number of substrates were used to assay the enzyme activity; these are listed in Table 2 as substrates of 'species A' enzyme activity

Fig. 4. Inhibition of tyramine oxidation by 4-cyanophenol

Dixon plot of the reciprocal of the initial velocities of tyramine oxidation against 4-cyanophenol concentration at several fixed tyramine concentrations. The tyramine concentrations were 0.15 mM (○), 0.2 mM (□), 0.25 mM (Δ), 0.5 mM (●) and 1 mM (■).

when they showed a similar response to 5-hydroxytryptamine as assay substrate, as substrates of 'species B' enzyme activity when they showed a similar response to benzylamine as substrate, or as substrates for both enzymes when they showed a biphasic response (as for tyramine).

A sample of enzyme in 10mM-Tris-HCl buffer, pH7.2, was treated with 1 μM-clorgyline for 45 min at 20°C to abolish 'species A' activity (see Fig. 5). After treatment the apparent K_m values for dopamine and tyramine were found to be similar to those of the original preparation. This is further evidence that the K_m values shown towards tyramine and dopamine by both these fractions were similar.

Mixed-substrate experiments

These were carried out as described by Dixon & Webb (1964) with benzylamine and 5-hydroxytryptamine as substrates. A value of $80 \pm 5\%$ was obtained for the ratio (observed rate of oxidation of substrate mixture $\times 100$) / (sum of observed rates of individual oxidation in the absence of the other substance) (when both substrates are present at their respective K_m concentrations). This ratio should yield a value of 66.7% if only one enzyme is responsible for the oxidation of both substrates or 100% if there are two separate enzyme systems. This intermediate

Fig. 5. Effect of clorgyline on monoamine oxidase activity

Enzyme preparation (4.2mg/ml) suspended in 10mM-Tris-HCl buffer, pH7.2, was treated with the appropriate clorgyline solution. This was incubated at 22°C until no further inhibition occurred (usually about 45 min). The treated enzyme was then assayed with various substrates (at concentrations ten times the K_m value); these were 5-hydroxytryptamine (□), 'species A' activity, benzylamine (○), 'species B' activity, tyramine (Δ) and dopamine (▲).

value suggests that the catalytic activity of each enzyme species is not mutually exclusive to binding of the substrate of the other enzyme activity (see Houslay *et al.*, 1974). Indeed double-reciprocal plots (Lineweaver & Burk, 1934) showed that 5-hydroxytryptamine was a competitive inhibitor of benzylamine oxidation (Fig. 6), results yielding a linear Dixon plot and a K_i value which was similar to the K_m value for 5-hydroxytryptamine oxidation. Dopamine, tyramine and tryptamine could similarly be shown to inhibit benzylamine oxidation competitively with apparent K_i values one to two times the K_m values exhibited. Also benzylamine reversibly

Table 2. Substrate specificity for the monoamine oxidase activity of rat liver mitochondrial outer membranes

Mitochondrial outer membranes were treated with clorgyline as described in the legend to Fig. 4. Assay methods were as stated in Table 1; however, substrate concentrations were always ten times the K_m values, except for 4-hydroxybenzylamine which was five times the K_m value.

Substrates for 'species A' activity only	Substrates for 'species B' activity only	Substrates for both activities
5-Hydroxytryptamine	Benzylamine	Tyramine
Adrenaline	4-Hydroxybenzylamine	Dopamine
3-O-Methyladrenaline	4-Hydroxy-3-methoxybenzylamine (vanillylamine)	4-Hydroxy-3-methoxy-2-phenylethylamine (3-methoxytyramine)
Noradrenaline	2-Phenylethylamine	
3-O-Methylnoradrenaline	3,4-Dimethoxy-2-phenylethylamine	
Octopamine	4-Methoxy-2-phenylethylamine	
	Tryptamine	
	5-Methoxytryptamine

Fig. 6. Inhibition of benzylamine oxidation by 5-hydroxytryptamine

Double-reciprocal plot of initial velocities of benzylamine oxidation against benzylamine concentration at several fixed 5-hydroxytryptamine concentrations. The 5-hydroxytryptamine concentrations were 0.1 mM (Δ), 0.25 mM ($\circ$), 0.4 mM ($\square$) and zero ($\bullet$). The direct assay for benzylamine oxidation was used by following benzaldehyde production at 250nm. An arbitrary unit is defined as an increase in extinction of 0.001/min.

inhibited 5-hydroxytryptamine oxidation in a mixed fashion (Fig. 7), results also yielding linear Dixon plots. The value of K_i (slope) obtained from the data of Fig. 7 was 0.17mM and that of K_i (intercept), 0.3mM.

Fig. 7. Inhibition of 5-hydroxytryptamine oxidation by benzylamine

Double-reciprocal plot of the initial velocities of 5-hydroxytryptamine oxidation against 5-hydroxytryptamine concentration at several fixed benzylamine concentrations. Benzylamine concentrations were 0.1 mM (Δ), 0.2 mM ($\square$), 0.4 mM ($\bullet$) and zero ($\circ$). The radioactive assay with 5-[1- 14 C]hydroxytryptamine was used, and samples were counted for radioactivity for 8 min.

Discussion

The linearity of the Lineweaver-Burk plots for all substrates investigated, and the similarity of the K_m values obtained for dopamine and tyramine after abolition of 'species A' activity and those of the original preparation, indicate that rat liver mitochondrial outer membranes do not contain multiple amine-oxidizing enzymes acting on any particular substrate studied that have very different K_m values. This is in contrast with the results of Sierens & D'Iorio (1970), who used a sodium deoxycholate-solubilized preparation from rat liver mitochondria and found that biphasic Lineweaver-Burk plots were obtained when 5-hydroxytryptamine was the assay substrate. However, it may be that the solubilization process used by Sierens & D'Iorio (1970) modified a fraction of the enzyme, a possibility that has been discussed

previously (Houslay & Tipton, 1973*a*, 1974; Tipton *et al.*, 1973). Such a modification could take the form of either a change in K_m value of some fraction of the total enzyme activity or the appearance of negative co-operativity; however, it must be noted that the contamination of the 5-hydroxytryptamine substrate with a partially competitive inhibitor could give a similar Lineweaver-Burk plot (Cleland *et al.*, 1973) to that reported. It has also been claimed that one of the electrophoretically separable multiple forms from rat liver (and human brain) mitochondrial monoamine oxidase exhibits a very much lower K_m value for dopamine than do the other components and also differs from the other components in being positively charged at pH 9.2 (see, e.g., Youdim, 1972*a,b*). In previous studies with detergent-sonicated-solubilized preparations from rat liver (Houslay & Tipton, 1973*a*) and human brain (Tipton *et al.*, 1973; Houslay *et al.*, 1974), prepared similarly to those used by Youdim (1972*a,b*), we have been unable to obtain evidence for the presence of an enzyme with a low K_m value which metabolizes dopamine, before electrophoresis. Such an enzyme form may be produced as an artifact of the electrophoretic system, since it is unusual for a protein that has been purified by adsorption to DEAE-cellulose at pH 7.2 followed by elution by high salt concentrations (Youdim & Sandler, 1968; Collins *et al.*, 1970) to reveal the presence of a positively charged component on electrophoresis at pH 9.2.

The pH curve for benzylamine oxidation differs somewhat from those shown by the Triton X-100-solubilized and 'perchlorate-treated' preparations from rat liver (Houslay & Tipton, 1973*a*) in exhibiting a distinct pH optimum.

The observation that the reversible inhibitor benzyl cyanide shows different inhibition kinetics towards benzylamine and 5-hydroxytryptamine oxidation by monoamine oxidase is interesting in the light of the large amount of attention the problem of monoamine oxidase multiplicity has attracted. The experiments carried out in the present study would appear to support and elucidate the original model proposed by Johnston (1968), being consistent with the proposal of two enzyme species (or groups of enzyme species; see Houslay *et al.*, 1974) with one fraction of the activity ('species A') being highly sensitive to clorgyline inhibition and the other ('species B') being less sensitive. Our results indicate that neither of the active sites of these two enzymes species is mutually exclusive to compounds that are substrates for the other, but such compounds may bind in a reversible (competitive or mixed-type) inhibitory fashion, with K_i values similar to the K_m values exhibited by the species they bind in a catalytically competent fashion. Such a situation may explain anomalies in the interpretation of mixed-substrate experiments that have been carried out (see, e.g., Blaschko *et al.*, 1937; Houslay & Tipton, 1973*a*; Houslay *et al.*, 1974; Kobayashi

& Schayer, 1955; Kohn, 1937; Oswald & Strittmatter, 1963; Pugh & Quastel, 1937).

The substrates for 'species A' would appear to be substituted β -phenylethylamines, β -phenylethanolamines with a free *p*-hydroxyl group, and 5-hydroxytryptamine. It must be noted that the 5-hydroxyl group of 5-hydroxytryptamine is able to attain an analogous relationship with the amino nitrogen, for which a binding site has been postulated (McEwen *et al.*, 1968, 1969*a,b*), as can the *p*-hydroxyl moiety of the substituted β -phenylethylamines and β -phenylethanolamines. If this group is blocked, by *O*-methylation, then the compound ceases to be a substrate for this fraction, as indicated by 4-methoxy-2-phenylethylamine, 3,4-dimethoxy-2-phenylethylamine and 5-methoxytryptamine, which are substrates of the 'species B' enzyme activity only. However, benzylamine derivatives with a free *p*-hydroxyl group (i.e. vanillylamine, *p*-hydroxybenzylamine) are not substrates for the 'species A' activity. This would also explain the action of 4-cyanophenol, which acts in an analogous fashion to benzyl cyanide.

Substrates for 'species B' enzyme activity include the substituted derivatives of benzylamine and β -phenylethylamine, plus tryptamine and 5-methoxytryptamine. However, substituted β -phenylethanolamine derivatives are not substrates.

Substrates for both enzyme activities are those fulfilling the structural conditions of the two classes set out above; these include dopamine and its *m*-*O*-methyl derivative and tyramine (*p*-hydroxy-2-phenylethylamine).

Presumably the 'species A' enzyme requires a free *p*-hydroxyl group to locate the substrate in a catalytically competent complex, whereas amines not possessing such a group can only bind in an inhibitory fashion. The topography of this site, however, does not account for *p*-hydroxy-substituted benzylamine derivatives, which presumably can only bind in an inhibitory fashion. This may be due to the difference in distance between the *p*-hydroxyl group and the amino nitrogen for these compounds.

All the compounds investigated, with the exception of 5-hydroxytryptamine, were substrates for 'species B' activity unless they possessed a β -hydroxyl group. This remains something of an anomaly unless one invokes the existence of a site of steric hindrance for the indole nucleus, which creates a non-catalytic complex that is only encountered with 5-hydroxytryptamine when the free hydroxyl group also reacts with its binding site. This would imply that such a steric hindrance would not be encountered by tryptamine or 5-methoxytryptamine as they have no hydroxyl group, or by dopamine and tyramine, which although they would presumably interact with the enzyme through their *p*-hydroxy groups would not interact with the group exhibiting steric hindrance for the indole nucleus.

This division of oxidation of amines of biological importance between two species of monoamine oxidase activity may represent some complex system for controlling the rates of oxidation of amines and the free steady-state amounts of aldehyde products. It is obviously important to assess whether both enzymes have similar reaction mechanisms, in order to understand fluxes in such a complex system, for already it has been demonstrated that benzylamine oxidation ('species B' activity) is peculiarly sensitive to aldehyde product inhibition (Houslay & Tipton, 1973b).

The importance of a free *p*-hydroxyl group for the oxidation of adrenaline and noradrenaline must be noted, as these compounds are substrates for 'species A' enzyme only. Thus the fact that catechol *O*-methyltransferase predominantly forms the *m*-*O*-methyl derivative (see, e.g. Sharman, 1972) would be important if in some tissues *O*-methylation precedes oxidation by monoamine oxidase (cf. Tipton, 1972). Presumably if the 3,4-*O*-dimethyl derivatives or the 4-*O*-methyl derivatives were to be formed or introduced they could not be further metabolized by monoamine oxidase and would in fact serve to inhibit the enzyme.

This system of monoamine oxidase species appears complex; however, it is highly probable that it also reflects the situation in human brain (Tipton *et al.*, 1973; Houslay *et al.*, 1974) and human liver (see Squires, 1972). Although they used enzyme from human liver, McEwen *et al.* (1968, 1969a,b) concluded that their excellent results showed no evidence of multiplicity; all their assays used benzylamine or vanillylamine as assay substrates, which one would predict to be substrates of 'species B' activity only, and the lack of anomalies with inhibition experiments only serves to support the model proposed here.

It is unclear whether the enzyme activities that reflect these two species may be physically separable, especially in view of the fact that membrane components can affect monoamine oxidase activity and specificity (Houslay & Tipton, 1973a, 1974). Indeed these species might represent the location of a single enzyme species within different lipid environments in the intact cell. However, Gorkin (1963) has claimed to be able to separate from rat liver a *p*-nitrophenylethylamine-oxidizing component from one that can oxidize both *p*-nitrophenylethylamine and *p*-hydroxy-*m*-nitrobenzylamine and this would be in agreement with the proposed model if the nitro group acted in the same manner as the hydroxyl group.

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