J. Pharm. Pharmac., 1976, **28**, 478–482 *Received January* 26, 1976

α-Methyl derivatives of biogenic amines as inhibitors of monoamine oxidase

F. INOUE, J. B. ROBINSON* AND SUSAN A. STANSFIELD

Faculty of Pharmacy, University of Toronto, Toronto, Ontario, Canada

The values of Km app and Vmax for three natural substrates of monoamine oxidase have been determined at various stages in the isolation of the enzyme from rat liver tissue. The results are consistent with the presence in the enzyme preparation of at least two distinct molecular forms of the enzyme.

Using the α-methyl derivatives of the natural substrates as inhibitors of the enzyme, the substrate dependence of Ki further substantiates this view. In addition, the kinetics of the inhibition suggest that the value of Km app may not for all substrates, necessarily be a measure of the affinity of the substrate for the enzyme.

Current evidence for the presence of isoenzymic forms of monoamine oxidase (MAO) [monoamine: O_2 oxidoreductase (deaminating) E.C. 1.4.3.4] is derived from two differing approaches. Electrophoresis of solubilized enzyme preparations on polyacrylamide gel has shown the separation of the enzyme into several bands of enzymatic activity although the number of such bands detected is variable and also depends on the tissue and species used (for review see Sandler & Youdim, 1972). More recently however, the specificity of the commonly employed detection method for MAO (nitrobluetetrazolium staining of the gel in the presence of substrate) (Glenner, Burtner & Brown, 1957) has been questioned (Diaz Borges & D'Iorio, 1973; Inoue, Robinson & Dost, 1976) and it has been suggested that some of the bands of activity detected may be artifacts arising from the method of isolation (Houslay & Tipton, 1973).

Alternative evidence, derived from the substrate dependent biphasic nature of the inhibition of MAO by certain inhibitors, e.g. clorgyline and deprenyl, has suggested that at least two different molecular forms of MAO with differing substrate specificities are present, the proportions of which are variable within different tissues (Johnston, 1968; Hall, Logan & Parsons, 1969; Goridis & Neff, 1971; Yang, Goridis & Neff, 1972; Yang & Neff, 1973; Coquil, Goridis & others, 1973; Neff & Yang, 1974; Houslay & Tipton, 1974).

Although within the present work it has not been possible to demonstrate the presence of isoenzymes separable by electrophoresis (Inoue & others, 1976) the possibility of isoenzymes of MAO being present cannot be discounted. Accordingly, MAO isolated from rat liver and from rat brain has been studied at various stages in the isolation procedure using three different substrates and also using the α-methyl derivatives of these substrates as potential substrate-specific or substrate-selective inhibitors as a means of detecting the presence of isoenzymes of MAO not separated by electrophoresis.

* Correspondence.

METHODS

In the isolation of MAO preparations all steps were performed at 0–5° unless otherwise stated.

Preparation of pre-column enzyme sample

Male Sprague-Dawley rats (250–300 g) were killed by cervical dislocation and the liver and brain immediately removed, washed in ice-cold 0·3 M sucrose, blotted, weighed and suspended in 9 volumes of sucrose solution (0·3 M) and homogenized. Liver samples were homogenized in a Waring blender for 30 s then with a Pyrex glass homogenizer. The homogenate was centrifuged (1500 *g*: 15 min), the tissue and cell debris removed and the supernatant solution further centrifuged (22 000 *g*; 20 min), the sedimented mitochondria collected and suspended in phosphate buffer (pH 7·4; 0·05 M).

To this suspension was added slowly benzylamine (final concentration, 1×10^{-3} M) followed by Triton X-100 (final concentration 1·5%v/v) and ammonium sulphate, to 25% saturation. The mixture was gently stirred at 4° for 4 h, centrifuged (22 000 *g*; 20 min) and ammonium sulphate added to the supernatant solution to 55% saturation. After stirring for 15 min the precipitate was sedimented by centrifugation (25 000 *g*; 20 min), collected and redissolved in the minimum quantity of phosphate buffer (pH 7·4; 0·05 M). The solution was dialysed against a total of

500 volumes of the same buffer for 60 h. The protein precipitating during the dialysis was removed by centrifugation (27 000 *g*; 20 min) and the supernatant solution retained. This solution constituted the pre-column enzyme sample.

Preparation of post-column enzyme sample

The pre-column enzyme sample (5–7 ml) was added to a Sephadex G-200 column (98 × 2 cm) which had been previously equilibrated with phosphate buffer (pH 7·4; 0·05 M) and the column eluted with the same buffer at a flow rate of 9 ml h^{-1}, and 8 ml fractions collected. Those fractions showing enzymic activity (radiochemical method) were pooled and the solution concentrated under vacuum without heat to approximately two-thirds volume. This solution constitutes the post-column enzyme sample.

Preparation of post-electrophoresis enzyme sample

A small volume of the post-column enzyme sample was mixed with an equal volume of Sephadex G-200 prepared in tris-HCl buffer (pH 8·60; 0·05 M) and 0·1 ml fractions applied to 5% polyacrylamide gel rods. Electrophoresis was conducted at 4° using a Pharmacia GE-4 gel electrophoresis apparatus and a constant current of 8 mA per tube for 2–3 h.

The gel rods, after removal from the support tube were sectioned into discs (3–5 mm long) and those discs showing enzymic activity (radiochemical method) were pooled, macerated by forcing the gel through a No. 18 hypodermic needle and phosphate buffer (pH 7·4; 0·05 M) added to the macerate. After standing overnight at 4° the mixture was centrifuged (1000 *g*; 10 min) and the supernatant solution separated. This solution constitutes the post-electrophoresis enzyme sample.

Enzyme activity was estimated (37°; pH 7·4) radiochemically using [^{14}C]phenethylamine, [^{14}C] tyramine or [^{14}C]tryptamine (New England Nuclear, Canada) in an incubation mixture containing labelled substrate solution (50 μl) enzyme preparation (25 μl) [50 μl was used in studies employing the post electrophoresis enzyme preparation] and phosphate buffer (0·05 M; pH 7·4) to 300 μl. After incubation with shaking at 37° for 1 h, hydrochloric acid (200 μl; 2N) followed by toluene (6 ml) was added and the mixture extracted by shaking in a Vortex test tube mixer. An aliquot (5 ml) of the toluene was removed and added to a Liquifluor/toluene mixture (New England Nuclear, Canada) (5 ml) and counted in a Packard Tricarb liquid scintillation spectrophotometer. Counting efficiency was determined by the use of an external standard. All determinations and all countings were in duplicate and the mean count, after correction for the blank, was used in calculations of the enzyme catalysed oxidation.

Inhibitor studies were made similarly except that the inhibitor solution (25 or 50 μl) was pre-incubated with the reaction mixture for 15 min before the addition of substrate and the volume of phosphate buffer added (to a total volume of 300 μl) was adjusted for the volume of inhibitor solution.

All kinetic data were derived from double-reciprocal plots (Lineweaver & Burk, 1934) the best line fitting the experimental data points being obtained both visually and by a least squares regression analysis.

Of the inhibitors studied (±)-α-methyltryptamine was prepared by the method of Heinzelman, Anthony & others (1960), the intermediates and final product agreeing in physical characteristics and spectral data (ultraviolet, infrared, nmr) with literature data. (±)-Amphetamine sulphate and (±)-*p*-hydroxyamphetamine hydrobromide were the generous gifts of Smith, Kline and French (Canada) Ltd.

RESULTS

Several authors have previously reported methods for the isolation of MAO from various tissues and the methods we used are derived, with only slight modification, from these (Hawkins, 1952; Youdim & Collins, 1971; Shih & Eiduson, 1973).

The yields at each stage in the isolation procedure together with the activity of the fractions and purification attained are shown in Table 1. Although the purification obtained is relatively low (16 to 18 × relative to the original homogenate) the overall purification is in good agreement with literature data from a variety of isolation procedures (Nara, Gomes & Yasunoba, 1966; Erwin & Hellerman, 1967; Gomes, Igaue & others, 1969; Hollinger & Oreland, 1970; Collins & Sandler, 1971).

Table 1. *Purification of rat liver monoamine oxidase.* (Liver from 5 rats, 69·5 g of liver tissue).

Fraction	Vol. (ml)	Prot. (mg ml^{-1})	Total prot. (mg)	Val. (mol. h^{-1} mg^{-1})	Enzyme units (EU)	Spec act. (EU mg^{-1})	Pur.
I	682	23·2	15 822	$2{\cdot}24\times10^{-11}$	354	0·02	1·0
II	179	28·7	5137	$4{\cdot}48\times10^{-11}$	229	0·04	2·0
III	25	15·8	395	$7{\cdot}86\times10^{-11}$	31·04	0·08	4·0
IV	45	2·5	112·5	$3{\cdot}35\times10^{-10}$	37·73	0·34	17·0

Enzyme unit is that amount of enzyme required to oxidize 1×10^{-9} mol of tryptamine h^{-1} at 37° and at a substrate concentration of 1×10^{-4} M.

I, Homogenate of liver in 0·3 M sucrose. II, Mitochondrial suspension in phosphate buffer (0·05 M; pH 7·4). III, Ammonium sulphate precipitation (25–55% saturation), dialysed 3 days against phosphate buffer (0·05 M; pH 7·4). IV, Gel filtration on Sephadex G-200.

Kinetic data employing the three substrates on the liver MAO at different stages in the purification and on the post-electrophoresis brain MAO preparation are given in Table 2. For the liver enzyme preparation, the Vmax values determined for tryptamine and β-phenethylamine show a marked decrease after gel filtration of the enzyme whereas the Vmax value determined using tyramine as substrate increases at all stages in the purification procedure. In addition, the Km values determined for each substrate fail to show consistency at the various stages of the enzyme purification procedure, a marked decrease in Km being seen on the post-column enzyme preparation when using tryptamine and β-phenethylamine as substrates.

Table 2. *Values of Km* ($\times 10^4$M) *and Vmax* ($\times 10^7$) *of MAO during isolation and purification.*

	Liver enzyme preparation					Brain enzyme Prep.	
	Pre-column		Post-column		Post-electro-phoresis	Post-electro-phoresis	
	Km	Vmax	Km	Vmax	Km	Vmax	Km	Vmax
Tyramine	7·3	1·0	8·2	1·7	3·8	2·9	1·0	0·5
Tryptamine	4·2	2·0	0·1	0·3	0·2	0·9	0·09	0·6
β-Phenethylamine	1·3	0·8	0·2	0·4	0·2	1·4	0·01	0·09

Units of Vmax are mol h^{-1} mg^{-1} $\times 10^7$.

The values of Ki for each of the inhibitor molecules in the presence of each of the substrate molecules are shown in Table 3. Because the kinetics of inhibition are not uniformally competitive it is not possible to compare the Ki values of each inhibitor in the presence of each of the substrates. However, in the few instances where this is possible (e.g. pre-column liver enzyme with tyramine or β-phenethylamine as substrate) it is apparent that the Ki value is substrate-dependent.

DISCUSSION

Although it has not been possible to demonstrate by electrophoretic separatory techniques the presence of isoenzymes of MAO within the liver and brain MAO preparations used (Inoue & others, 1976), the presence of more than one form of the enzyme cannot be discounted. Indeed, a number of authors have suggested that at least two forms of MAO showing differing substrate specificities are present in certain tissues (Houslay & Tipton, 1974; Neff & Yang, 1974 and references cited there).

The kinetic data derived from the three substrates at the various stages in the enzyme isolation (Table 2) would tend to support these suggestions. Indeed, if only one form of the enzyme were present, whether that single form carried only a single active site or

Table 3. *Values of Ki determined when using the α-methyl derivatives of natural substrates as inhibitors of MAO.*

		Liver enzyme preparation						Brain enzyme Preparation	
		Pre-column		Post-column		Post-electro-phoresis		Post-electro-phoresis	
Substrate	Inhibitor	Ki $\times 10^{-5}$	(I)	Ki $\times 10^{-5}$	(I)	Ki $\times 10^{-5}$	(I)	Ki $\times 10^{-5}$	(I)
Tyramine	α-Methyl-tryptamine	5·5	C	7·6	C	10·4	C	0·6	C
		5·7	C	7·6	C	12·1	C	0·6	C
	Amphetamine	6·9	C	7·5	C	20·3	C	1·9	C
		7·2	C	9·5	C	18·9	C	2·2	C
	HO-amphetamine	8·4	C	15·6	C	58·2	C	2·0	C
		9·0	C	18·3	C	47·7	C	2·2	C
Tryptamine	α-Methyl-tryptamine	4·6	NC	3·6	Mix	3·7	C	0·3	C
		4·1	NC	3·4	Mix	3·8	C	0·3	C
	Amphetamine	6·3	NC	8·3	C	25·7	C	2·1	C
		8·9	NC	8·8	C	23·2	C	2·4	C
	HO-amphetamine	6·9	NC	7·4	C	46·2	C	2·1	C
		10·4	NC	8·3	C	41·1	C	2·2	C
β-Phenethyl-amine	α-Methyl-tryptamine	130·0	C	8·0	UC	28·9	UC	12·3	UC
		111·0	C	9·0	UC	31·2	UC	12·7	UC
	Amphetamine	64·3	C	3·3	UC	7·8	UC	10·5	UC
		63·2	C	3·5	UC	7·4	UC	10·5	UC
	HO-amphetamine	318·0	C	22·1	UC	70·4	UC	29·6	UC
		254·0	C	20·0	UC	70·2	UC	25·7	UC

(I) Type of inhibition; C competitive; NC Noncompetitive, UC uncompetitive; Mix mixed inhibition.

more than one active site each of which showed some degree of substrate specificity, the dramatic change in the value of Km app on gel filtration when using tryptamine or β-phenethylamine as substrate would be inexplicable. In addition, one would have expected that the value of Vmax would increase at each stage of the enrichment process. The results obtained are considered therefore to be consistent with the presence of at least two different molecular forms of MAO as originally suggested by Johnston (1968).

From the substrate-dependent biphasic nature of the inhibition of MAO in the presence of a limited number of inhibitors, some substrate specificity or selectivity is inferred for these different molecular forms of MAO (Johnston, 1968; Neff & Yang, 1974). These conclusions are further strengthened by the data derived from the use of the α-methyl derivatives of the substrate molecules as enzyme inhibitors (Table 3). If only a single form of the enzyme were present with a single active site, the values of Ki for the competitively inhibited reactions should be substrate-independent. Thus, although (±)-α-methyltryptamine is consistently the most potent inhibitor when tyramine is a substrate, (±)-amphetamine is the most potent competitive inhibitor of the pre-column liver enzyme when β-phenethylamine is acting as substrate. These results however are consistent with the presence of two forms of the enzyme, form A having a greater affinity for the more polar substrates and inhibitors and form B having a greater affinity for the more non-polar substrates and inhibitors. Such a system is analogous to previous proposals by Neff & Yang (1974) and Houslay & Tipton (1974) although from our current data it is not possible to suggest that the form B enzyme is specific for β-phenethylamine (Neff & Yang, 1974) or for β-phenethylamine and tryptamine (Houslay & Tipton, 1974), rather that each of the substrates has differing affinities for each of the forms of the enzyme.

A further interesting feature of the inhibition studies arises in considering the kinetics of the inhibition, particularly when using β-phenethylamine as substrate. The occurence of uncompetitive kinetics is generally ascribed to the formation of an ESI complex as shown below and is rare in simple enzyme systems. Hence, interpretations based on such kinetics should be made with caution. These apparent uncompetitive kinetics are probably explained

$$E + S \rightleftharpoons ES \xrightarrow{[I]} ESI \xrightarrow{\times} E + I + P$$

as arising from an unusual form of competitive inhibition. Employing the simple equation for an enzyme-catalysed reaction and applying steady-state kinetics:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

$$\text{Km app} = \frac{k_{-1} + k_2}{k_1}$$

The value of Km app approximates to the true Km if $k_{-1} >> k_2$ and in such instances the value of Km app determined experimentally will be a measure of the affinity of the substrate for the enzyme, an apparent assumption made in work with MAO substrates (McEwan & Sober, 1967). However, in the rare cases when $k_2 > k_{-1}$, the Km app will approximate to k_2/k_1 (Dixon & Webb, 1964). In such an instance, an inhibition affecting the enzymatic breakdown of the ES complex to product will produce proportional changes in both Vmax and Km app values of such an enzyme with the appearance of apparent uncompetitive kinetics. Thus, it would appear that, in the case of β-phenethylamine acting as a substrate, we possibly have an example of $k_2 > k_{-1}$, whereas for the other substrates the Km app values are an approximate measure of the affinity of the substrate for the enzyme active site.

Finally, some comment should perhaps be made with regard to the relevance of data generated from purified enzyme preparations in relation to the *in vivo* situation. The enzyme MAO is believed to be closely associated with the outer membrane of the mitochondrial particle and it is thus possible that the use of various disruptive processes (sonication and solubilization in detergents) in the isolation procedure may cause rearrangement of membrane bound material. This possibility coupled with the fact that the partially purified preparations of MAO frequently only contain a fraction of the total MAO activity of the original tissue may mean that one is studying an enzyme preparation different from that present *in vivo*. Therefore, although we are using an enzyme preparation which has been dialysed free from detergent and which on gel filtration appears as an aggregated molecule, the values of Km derived for each of the three substrates on our most highly purified liver enzyme preparation (post-electrophoresis preparation) are similar to those reported for a rat liver mitochondrial outer membrane preparation prepared without the use of detergents. This latter preparation contained 90% of the original

MAO activity and has been shown to contain two kinetically distinct MAO activities (Houslay & Tipton, 1974).

Acknowledgements

The authors acknowledge with thanks the gift of (±)-amphetamine and (±)-*p*-hydroxyamphetamine from Smith, Kline and French (Canada) Ltd, and also the support of this project in the form of a grant (MA-4033) from the Medical Research Council of Canada.

REFERENCES

COLLINS, G. G. S. & SANDLER, M. (1971). *Biochem. Pharmac.*, **20**, 289–296.
COQUIL, J. F., GORIDIS, C., MACK, G. & NEFF, N.H. (1973). *Br. J. Pharmac.*, **48**, 590–599.
DIAZ BORGES, J. M. & D'IORIO, A. (1973). *Can. J. Biochem.*, **51**, 1089–1095.
DIXON, M. & WEBB, E. C. (1964). *Enzymes*, 2nd Edn., p 234, London: Longmans, Green.
ERWIN, G. V. & HELLERMAN, L. (1967). *J. biol. Chem.*, **242**, 4230–4238.
GLENNER, G. C., BURTNER, H. J. & BROWN, G. W. (1957). *J. Histochem. Cytochem.*, **5**, 591–600.
GOMES, G., IGAUE, I., KLOEPFER, H. G. & YASUNOBA, K. T. (1969). *Archs Biochem. Biophys.*, **132**, 16–27.
GORIDIS, C. & NEFF, N.H. (1971). *Br. J. Pharmac.*, **43**, 814–818.
HALL, D. W. R., LOGAN, B. W. & PARSONS, G. H. (1969). *Biochem. Pharmac.*, **18**, 1447–1454.
HAWKINS, J. (1952). *Biochem. J.*, **50**, 577–581.
HEINZELMAN, R. V., ANTHONY, W. C., LYTTLE, D. A. & SZYMUSZKOVICZ, J. (1960). *J. org. Chem.*, **25**, 1548–1558.
HOLLINGER, G. & ORELAND, G. (1970). *Archs Biochem. Biophys.*, **139**, 320–328.
HOUSLAY, M. D. & TIPTON, K. F. (1973). *Biochem. J.*, **135**, 173–186.
HOUSLAY, M. D. & TIPTON, K. F. (1974). *Ibid.*, **139**, 645–652.
INOUE, F., ROBINSON, J. B. & DOST, K. A. (1976). *J. Pharm. Pharmac.*, **28**, 521–522.
JOHNSTON, J. P. (1968). *Biochem. Pharmac.*, **17**, 1285–1297.
LINEWEAVER, H. & BURK, D. (1934). *J. Am. chem. Soc.*, **56**, 658–666.
MCEWEN, C. M. & SOBER, A. J. (1967). *J. biol. Chem.*, **242**, 3068–3078.
NARA, S., GOMES, B. & YASUNOBA, K. T. (1966). *Ibid.*, **241**, 2774–2780.
NEFF, N. H. & YANG, H-Y. T., (1974). *Life Sci.*, **14**, 2061–2074.
SANDLER, M. & YOUDIM, M. B. H. (1972). *Pharmac. Rev.*, **24**, 331–348.
SHIH, J. C. & EIDUSON, S. (1973). *J. Neurochem.*, **21**, 41–49.
YANG, H-Y. T., GORIDIS, C. & NEFF, N. H. (1972). *Ibid.*, **19**, 1241–1250.
YANG, H-Y. T. & NEFF, N. H. (1973). *J. Pharmac. exp. Ther.*, **187**, 365–371.
YOUDIM, M. B. H. & COLLINS, G. S. S. (1971). *Eur. J. Biochem.*, **18**, 73–78.