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SUBSTRATE SPECIFICITY OF THE DIFFERENT FORMS OF MONOAMINE OXIDASE IN RAT LIVER MITOCHONDRIA

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Abstract—Rat liver mitochondrial monoamine oxidase was inhibited by deprenil (selective inhibitor for the 'B form' of monoamine oxidase) to study the 'A form' of the enzyme separately. The activity towards serotonin (usually classified as a substrate for the 'A form') was estimated in the presence of additional monoamine oxidase substrates. All of the additional substrates investigated inhibited the activity towards serotonin competitively. In the deprenil inhibited preparation all of the residual activity towards β-phenylethylamine (usually classified as a substrate for the 'B form') was shown to be sensitive to the 'A form' inhibitor clorgyline, indicating that the 'A form' was also able to oxidize this substrate. The K_m values of the 'A form' for serotonin, tyramine and β-phenylethylamine did not differ significantly.

When the 'B form' of monoamine oxidase was studied after inhibition of the 'A form' by clorgyline, all additional substrates investigated were able to inhibit the activity towards β-phenylethylamine in a competitive fashion. All of the remaining activity towards serotonin in the clorgyline inhibited preparation was sensitive to deprenil. Thus the 'B form' also appears to be able to oxidize this substrate. The K_m values for the 'B form' differed considerably: 4 μM for β-phenylethylamine, 102 μM for tyramine, and 2.5 mM for serotonin.

There is much evidence for the existence of two types of functional forms of monoamine oxidase (monoamine: O_2 oxidoreductase [deaminating] EC 1.4.3.4) in many different tissues from several species [1]. The two forms, called 'A' and 'B' according to their preferential sensitivity to the inhibitors clorgyline [2] and deprenil [3], respectively, have also been shown to have different substrate specificities [1]. Serotonin and norepinephrine are oxidized mainly by the 'A form' in, for example, rat and human liver, while the 'B form' oxidizes preferentially benzylamine and β-phenylethylamine. Some substrates (e.g. tyramine, tryptamine and dopamine) appear to be substrates for both the 'A' and the 'B form'. However, it cannot be excluded that the 'A form' of the enzyme may also oxidize the substrates of the 'B form' to some extent and that the 'B form' of the enzyme may also oxidize those of the 'A form'.

By using mixed substrate experiments Houslay and Tipton [4, 5] obtained results which indicate that the active sites of the two forms can also bind compounds that are substrates for the other form. In the present study the 'A form' of monoamine oxidase in rat liver mitochondria was selectively inhibited by clorgyline and the 'B form' by deprenil in order to investigate this phenomenon further. The inhibitory effect of various monoamines on the 'A form' and on the 'B form' was then investigated separately and the catalytic properties of the two forms towards different monoamines were compared.

MATERIALS AND METHODS

Chemicals

[^{14}C]Serotonin, [^{14}C]tyramine, and β-[^{14}C]-phenylethylamine were obtained from New England Nuclear, Boston, Mass., and the corresponding unlabelled substrates from Sigma Chemical Co., St. Louis, Mo.

Deprenil (phenylisopropylmethylpropionylamine hydrochloride, E-250) was kindly provided by Dr. Magyar, Budapest, Hungary, through Dr. Kinemuchi, Tokyo, and clorgyline [*N*-methyl-*N*-propargyl-3 (2,4-dichlorophenoxy)-propylamine hydrochloride, M & B 9302] from May & Baker Ltd., Dagenham, England (Dr. R. A. Robinson).

Methods

Preparation of mitochondria. Rat liver mitochondria were prepared as described by Hollunger and Orreland [6] for pig liver mitochondria.

Assay of monoamine oxidase. Monoamine oxidase activity was estimated essentially according to Jain *et al.* [7]. KCl was, however, omitted. The incubation medium contained, when not otherwise stated, [^{14}C]serotonin (0.5 mM), [^{14}C]tyramine (0.5 mM) or β-[^{14}C]phenylethylamine (0.05 mM) in a total volume of 275 μl of potassium phosphate (0.01 M, pH 7.4). In some experiments additional unlabelled substrate was present. Usually the reaction was started by the addition of 25 μl of an appropriate dilution of the enzyme preparation. The incubation was carried out for 20 min at 37° and the reaction was stopped by

the addition of 0.2 ml of 2 M hydrochloric acid. The enzyme reaction was found to be linear during the incubation time. The medium was extracted by ether in the case of serotonin and tyramine and with toluene in the case of β-phenylethylamine. Samples of the extracts were then taken for determination of radioactivity in a Packard Tri-Carb Liquid Scintillation Spectrometer with Aquasol (New England Nuclear, Boston, Mass.) as scintillation liquid.

Protein. Protein was estimated according to Lowry *et al.* [8] with human serum albumin as a standard.

RESULTS

Inhibition of the serotonin oxidizing activity by other monoamine oxidase substrates. To investigate whether or not the 'A form' of monoamine oxidase in rat liver mitochondria could be inhibited by other monoamine oxidase substrates, the activity towards [^{14}C]serotonin was studied in the presence of increasing concentrations of other monoamines (Fig. 1). In a control experiment increasing concentrations of unlabelled serotonin was added. As can be seen in Fig. 1 all of the unlabelled substrates added inhibited the activity towards serotonin approximately to the same extent as in the control experiment. At a concentration of the additional monoamine equal to the concentration of the [^{14}C]serotonin (0.5 mM) the activity towards [^{14}C]serotonin was reduced to about 50 per cent. When the concentration of the unlabelled amine was twenty times higher than that of the [^{14}C]serotonin (i.e. 10 mM) almost all of the activity towards the [^{14}C]serotonin was inhibited, irrespective of which additional amine was added.

Inhibition of the β-phenylethylamine oxidizing activity by other monoamine oxidase substrates. β-[^{14}C]-Phenylethylamine, at a concentration of 0.05 mM was used as a substrate for the 'B form' of monoamine oxidase in the mitochondrial preparation. In the control experiment, in which increasing concentrations of unlabelled β-phenylethylamine were added, the

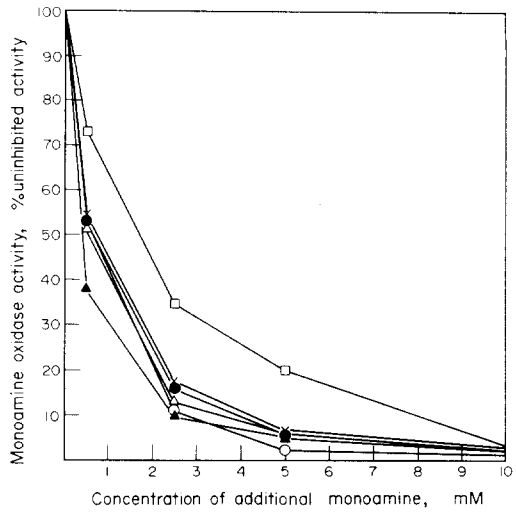

Fig. 1. Inhibition by additional monoamines of rat liver mitochondrial monoamine oxidase activity towards serotonin. The monoamine oxidase activity towards [^{14}C]serotonin at a final concentration of 0.5 mM was estimated in the presence of increasing concentrations of additional unlabelled monoamines. Additional monoamines: × × ×: serotonin; □ □ □: norepinephrine; △ △ △: tyramine; ▲ ▲ ▲: tryptamine; ● ● ●: benzylamine; ○ ○ ○: β-phenylethylamine.

activity towards β-[^{14}C]phenylethylamine was inhibited to about 50 per cent when 0.05 mM unlabelled β-phenylethylamine was present (Fig. 2). At higher concentrations of β-phenylethylamine, substrate inhibition occurred. It is also evident from Fig. 2 that all of the additional monoamines used were able to reduce the activity towards β-[^{14}C]phenylethylamine, but to a lesser extent than in the control experiment. Even when the concentration of unlabelled norepinephrine or serotonin was two hundred times higher than that of the β-[^{14}C]phenylethylamine (i.e.

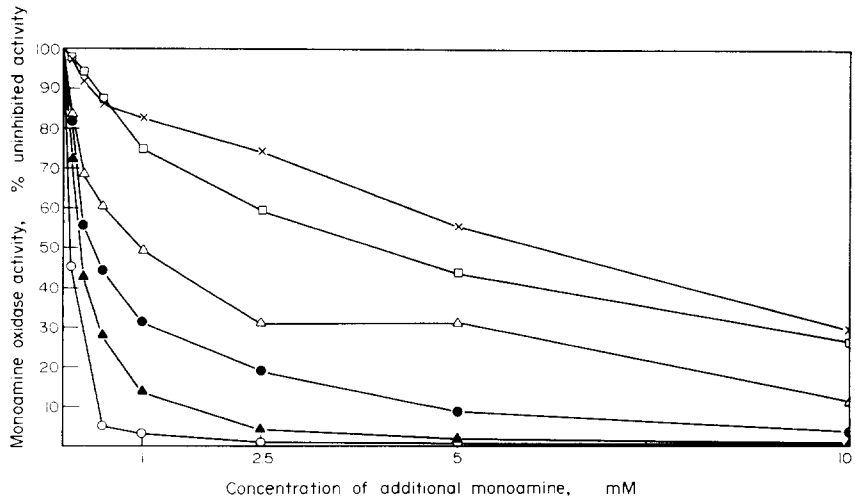

Fig. 2. Inhibition by additional monoamines of rat liver mitochondrial monoamine oxidase activity towards β-phenylethylamine. The activity of monoamine oxidase towards β-[^{14}C]phenylethylamine at a final concentration of 0.05 mM was estimated in the presence of increasing concentrations of additional unlabelled monoamines. The symbols represent the same additional monoamines as indicated in the legend to Fig. 1.

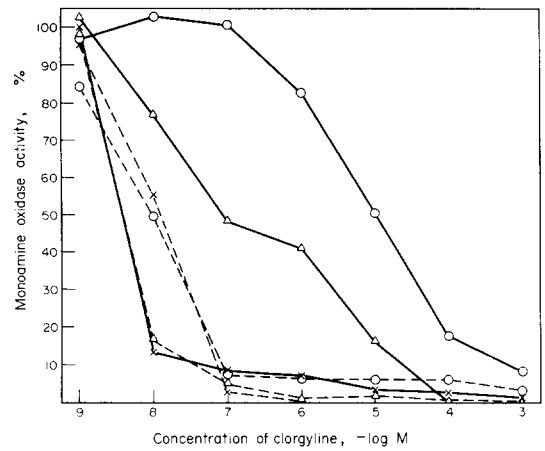

Fig. 3. Clorgyline inhibition of monoamine oxidase activity in uninhibited and deprenil inhibited rat liver mitochondria. The deprenil inhibited mitochondria were obtained from the experiment described in Table 1. Samples of the uninhibited and deprenil inhibited rat liver mitochondria were then incubated in the presence of various concentrations of clorgyline at 25° for 20 min prior to the estimation of monoamine oxidase activity. The activity is expressed as per cent of the activity in the absence of clorgyline. ×—×—×: uninhibited mitochondria with serotonin as substrate; ×- - - -×- - - -×: deprenil inhibited mitochondria with serotonin; △—△—△: uninhibited mitochondria with tyramine; △- - - -△- - - -△: deprenil inhibited mitochondria with tyramine; ○ ○ ○: uninhibited mitochondria with β-phenylethylamine; ○ - - -○- - - -○: deprenil inhibited mitochondria with β-phenylethylamine.

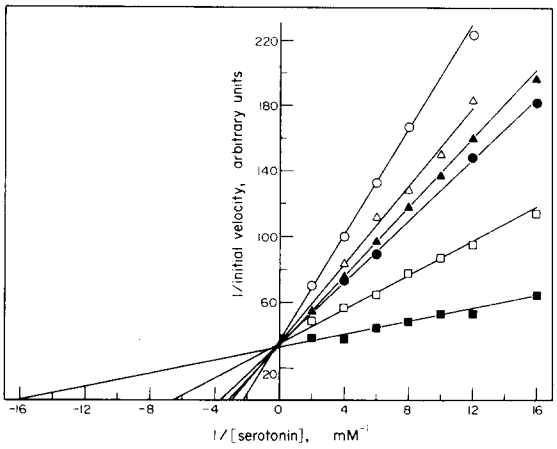

Fig. 4. Inhibition by additional monoamines of serotonin oxidation of deprenil inhibited rat liver monoamine oxidase. Lineweaver–Burk plot of initial velocity of serotonin oxidation against serotonin concentration in the presence of fixed concentrations of additional unlabelled monoamines. Rat liver mitochondria were preincubated in the presence of deprenil as described in Table 1. The monoamine oxidase activity in samples of the deprenil inhibited mitochondrial preparation was estimated with various concentrations of [^{14}C]serotonin as substrate in the presence of additional unlabelled monoamines. Additional monoamines: □—□—□: 75 nmoles of norepinephrine; △—△—△: 75 nmoles of tyramine; ▲ ▲ ▲: 25 nmoles of tryptamine; ○—○—○: 75 nmoles of β-phenylethylamine; ●—●—●: 75 nmoles of benzylamine; ■—■—■: no additional monoamine.

10 mM) 30–35 per cent of the activity towards the β-[^{14}C]phenylethylamine remained.

Inhibition of monoamine oxidase activity by clorgyline and deprenil. As can be seen in Figs. 3 and 5 the activity towards serotonin in the mitochondrial preparation was inhibited by low concentrations of clorgyline and only by high concentrations of deprenil, while the opposite result was obtained with β-phenylethylamine as substrate. With tyramine as substrate about half of the activity was sensitive to clorgyline and half to deprenil. Those results are in full agreement with previous findings [2, 3, 9].

Substrate specificity of the 'A form'. To study the substrate specificity of the 'A form' separately, the 'B form' of monoamine oxidase was selectively inhibited by deprenil leaving 56 per cent of the activity towards serotonin, 20 per cent towards tyramine and 4 per cent towards β-phenylethylamine (Table 1). When the remaining activity was subsequently inhibited by increasing concentrations of the 'A form' inhibitor clorgyline, almost all of the activity towards serotonin, tyramine and β-phenylethylamine was found to be inhibited by low concentrations of clorgyline (Fig. 3), indicating that all these substrates now had been oxidized only by the 'A form' of the enzyme.

The deprenil inhibited preparation was also used to study the type of inhibition of the 'A form' of monoamine oxidase by additional unlabelled monoamines with [^{14}C]serotonin as substrate (Fig. 4). The K_m value was found to be higher in the presence of every one of the additional monoamines, while no significant change in the maximum velocity was obtained, indicating a competitive type of inhibition by both the substrates classified for the 'A' and the 'B form'.

Substrate specificity of the 'B form'. When the 'B form' of monoamine oxidase in the mitochondrial preparation was inhibited by clorgyline to study 'A form' activity selectively, 94 per cent of the activity towards β-phenylethylamine, 55 per cent towards tyramine and 2 per cent of the activity towards serotonin remained (Table 1). The enzyme preparation thus inhibited by clorgyline was then inhibited by increasing concentrations of deprenil. As shown in Fig. 5 almost all of the activity in this preparation was highly sensitive to deprenil, irrespective of the substrate used, in-

Table 1. Per cent of monoamine oxidase activity remaining after inhibition of rat liver mitochondria by clorgyline and deprenil

Substrate	Deprenil inhibited mitochondria*	Clorgyline inhibited mitochondria*
Serotonin	56	2
Tyramine	20	55
β-phenylethylamine	4	94

* Rat liver mitochondria (53 mg protein) were preincubated in the presence of 5 nmoles of deprenil and 5 nmoles of clorgyline, separately, in a total volume of 1.0 ml at 25° for 20 min. After preincubation the monoamine oxidase activity was estimated in aliquots. The rest of the samples were then chilled and used in the experiment described in Figs. 3 and 5 within 10 min. At this time no further reduction of the activity was found.

dicating oxidation of 'A form' substrates by the 'B form' of the enzyme.

The type of inhibition by additional monoamines was also studied in the clorgyline inhibited mitochondrial preparation with β-[^{14}C]phenylethylamine as substrate (Fig. 6). None of the additional monoamines changed the maximum velocity, whereas the K_m value increased. Thus, the 'B form' activity was also inhibited in a competitive fashion.

Determination of K_m *values.* The K_m values for uninhibited ('A' and 'B form'), clorgyline-inhibited ('B form') and deprenil-inhibited ('A form') mitochondrial monoamine oxidase were calculated from Lineweaver Burk plots and are shown in Table 2. Compared to the 'B form' of the enzyme, the 'A form' had a much lower K_m for serotonin, considerably higher K_m for β-phenylethylamine and about half the K_m value for tyramine. In the uninhibited preparation comparatively low K_m values were obtained both for serotonin and β-phenylethylamine, whereas with tyramine the K_m value was found to be in betweeen those of the 'A' and the 'B form'.

DISCUSSION

Evidence has recently been presented that the two forms of monoamine oxidase are also able to bind monoamines which are substrates for the other form of the enzyme [4, 5]. To compare the affinities of the two forms towards different substrates, the monoamine oxidase activity in rat liver mitochondria towards serotonin and β-phenylethylamine was estimated in the presence of various concentrations of other monoamines. As can be seen in Fig. 1 all substrates investigated (i.e. substrates for both the 'A' and the 'B form' of monoamine oxidase) were able to inhibit the activity towards serotonin (the 'A form' activity) to about the same extent. Thus, the 'A form' does not appear to have a high preference for the substrates which are mainly oxidized by this form (serotonin and norepinephrine).

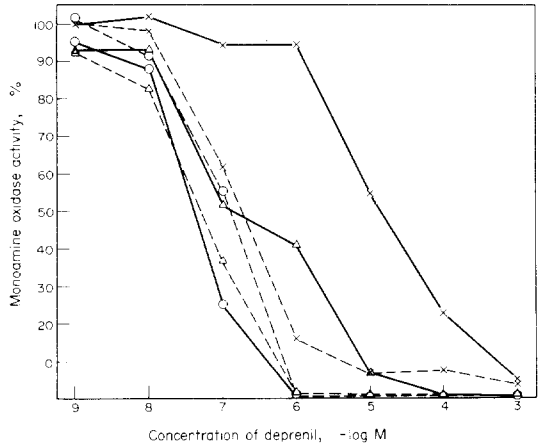

Fig. 5. Deprenil inhibition of monoamine oxidase activity in uninhibited and clorgyline inhibited rat liver mitochondria. The clorgyline inhibited mitochondria were obtained from the experiment described in Table 1. Samples of the uninhibited and deprenil inhibited rat liver mitochondria were then incubated in the presence of various concentrations of deprenil at 25° for 20 min prior to the estimation of monoamine oxidase activity. The activity is expressed as per cent of the activity in the absence of deprenil. × × ×: uninhibited mitochondria with serotonin as substrate; · · ·: clorgyline inhibited mitochondria with serotonin; △ △ △: uninhibited mitochondria with tyramine; △ △ △: clorgyline inhibited mitochondria with tyramine; ○ ○ ○: uninhibited mitochondria with β-phenylethylamine; ○ ○ ○: clorgyline inhibited mitochondria with β-phenylethylamine.

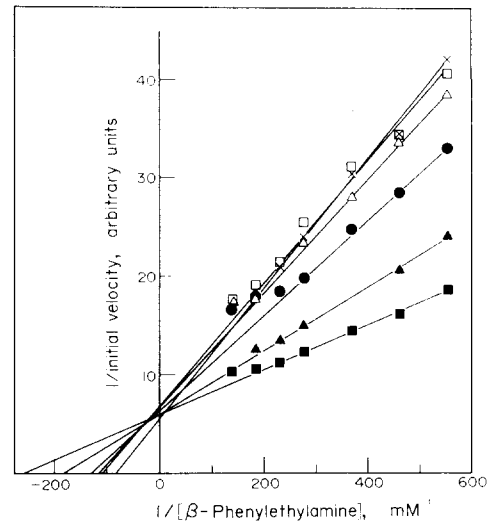

Fig. 6. Inhibition by additional monoamines of β-phenylethylamine oxidation of clorgyline inhibited rat liver monoamine oxidase. Lineweaver Burk plot of initial velocity of β-phenylethylamine oxidation against β-phenylethylamine concentration in the presence of fixed concentrations of additional unlabelled monoamines. Rat liver mitochondria were preincubated in the presence of clorgyline as described in Table 1. The monoamine oxidase activity in samples of the clorgyline inhibited mitochondrial preparation was estimated with various concentrations of β-[^{14}C]phenylethylamine as substrate in the presence of additional unlabelled monoamines. Additional monoamines: × × ×: 375 nmoles of serotonin; · · ·: 150 nmoles of norepinephrine; △ △ △: 75 nmoles of tyramine; ▲ ▲ ▲: 2.5 nmoles of tryptamine; ● ● ●: 30 nmoles of benzylamine; ■ ■ ■: no additional monoamine.

Table 2. K_m values for monoamine oxidase in uninhibited, deprenil inhibited and clorgyline inhibited rat liver mitochondria*

Substrate	Uninhibited mitochondria (μM)	Deprenil inhibited mitochondria (μM)	Clorgyline inhibited mitochondria (μM)
Serotonin	60	62	2500
Tyramine	88	57	102
β-phenylethylamine	14	62	4

* Rat liver mitochondria were preincubated by deprenil and clorgyline as described in Table 1. The monoamine oxidase activity in samples of the deprenil inhibited and the clorgyline inhibited preparation was then estimated with various concentrations of labelled substrates. The K_m values were obtained from Lineweaver Burk plots.

All of the monoamines investigated also inhibited the activity towards β-phenylethylamine, but the degree of inhibition differed considerably between the monoamines (Fig. 2). Thus, the substrates which are mainly oxidized by the 'A form' of monoamine oxidase inhibited the activity towards β-phenylethylamine significantly less than those which are substrates for the 'B form' or for both forms of the enzyme. Even at two hundred times the concentration of β-phenylethylamine norepinephrine and serotonin did not abolish this activity, but it seems likely, with respect to the shape of the curves, that the activity would have approached zero if the concentrations of these amines were further increased.

In order to study the kinetics of the 'A form' of monoamine oxidase separately, the 'B form' of the enzyme was inhibited by deprenil. All of the monoamines investigated inhibited the activity towards serotonin in this preparation in a competitive fashion (Fig. 4). This result contrasts, in part, to the findings of Houslay and Tipton [4] who found that benzylamine inhibited the activity towards serotonin in a mixed fashion in uninhibited rat liver mitochondria.

When the 'B form' of monoamine oxidase was studied after inhibition of the 'A form' by clorgyline, all the monoamines investigated competitively inhibited the activity towards β-phenylethylamine (Fig. 6). This is in agreement with the results of Houslay and Tipton [4] when they inhibited the activity towards benzylamine by serotonin.

To investigate whether or not the 'A form' of monoamine oxidase also has some catalytic activity towards the inhibiting monoamines, the residual activity towards β-phenylethylamine (4 per cent) and tyramine (20 per cent) in the deprenil inhibited preparation was inhibited by various concentrations of clorgyline (Fig. 3). Contrary to the finding in the uninhibited preparation, all the residual activity was sensitive to clorgyline. According to the postulation that the 'A form' of monoamine oxidase is highly sensitive to clorgyline only the 'A form' remained. The 'A form' thus is also able to oxidize β-phenylethylamine, which is usually classified as a 'B form' substrate. It seems likely that this oxidation should also occur to some extent in uninhibited mitochondria, but, the proportion of β-phenylethylamine oxidized by that form seems to be too small to be recorded. The finding that all of the activity towards tyramine was sensitive to clorgyline in the deprenil inhibited preparation is also compatible with the explanation that the 'B form' alone was responsible for the oxidation of tyramine in this preparation.

When the mitochondrial preparation was pretreated with clorgyline, almost all of the residual activity towards serotonin (2 per cent) and tyramine (55 per cent) was sensitive to deprenil (Fig. 5), indicating that almost only the 'B form' of monoamine oxidase remained and that the 'B form' was also able to catalyze the oxidation of serotonin.

Thus, it seems probable that both the 'A' and the 'B form' of monoamine oxidase is able to oxidize a variety of monoamines, but that some of them are predominantly oxidized by one or the other form of the enzyme. However, the proportion of a particular substrate oxidized by the 'A form' to that oxidized by the 'B form' may vary from tissue to tissue. Thus, in pig liver mitochondria, in contrast to most other tissues, there is evidence that serotonin is oxidized by both forms of the enzyme to about the same extent [10]. This may be due to differences between the ratios of the two forms or to different catalytic activities of the enzyme in different tissues.

The K_m values for both serotonin and β-phenylethylamine differed considerably between the two forms of monoamine oxidase in rat liver mitochondria (Table 2), which at least partly explains why serotonin and β-phenylethylamine are mainly oxidized by different forms of the enzyme in that tissue. The finding that the K_m values for both serotonin and β-phenylethylamine in uninhibited mitochondria are comparatively low is also compatible with the postulation that serotonin is mainly oxidized by the 'A form' and β-phenylethylamine by the 'B form' of monoamine oxidase in uninhibited rat liver mitochondria. It may be noted, however, that the activity towards serotonin was estimated at a concentration below the K_m for the 'B form' of the enzyme, and it is possible that the proportion of serotonin oxidized by the 'B form' of the enzyme had been greater, if the activity towards serotonin had been estimated at a higher concentration.

A comparatively small difference between the K_m values of the two forms was obtained for tyramine and the K_m value in the uninhibited preparation was in between those of the 'A' and the 'B form' (Table 2). These findings are in agreement with the assumption that this substrate is oxidized by both forms of the enzyme to about the same extent in uninhibited rat liver mitochondria. (Figs. 3 and 5).

The finding that the 'A form' of the enzyme has about the same affinity for all substrates investigated and that the 'B form' has a higher affinity for the substrates without a *p*-hydroxyl group (i.e. β-phenylethylamine benzylamine and tryptamine) may be compatible with the hypothesis of Severina [11], who proposed that the active site has both a hydrophobic and a polar region, that participate to varying degrees in the binding of different amines. In the binding of substrates without a *p*-hydrozyl group to both the 'A' and the 'B form' of the enzyme only the hydrophobic region of the active site may be involved. On the other hand, in the binding of substrates with a *p*-hydroxyl group (i.e. serotonin, norepinephrine and tyramine) to the 'B form' of the enzyme repulsive forces may act on the *p*-hydroxyl group, while these forces may not be present when these substrates bind to the 'A form' of the enzyme.

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REFERENCES

1. N. H. Neff and H.-Y. Yang, *Life Sci.* **14**, 2061 (1974).
2. J. P. Johnston, *Biochem. Pharmac.* **17**, 1285 (1968).
3. J. Knoll and K. Magyar, *Adv. biochem. Psychopharmac.* **5**, 393 (1972).
4. M. D. Houslay and K. F. Tipton, *Biochem. J.* **139**, 645 (1974).

5. M. D. Houslay and K. F. Tipton, *Biochem. Pharmac.* **24,** 627 (1975).
6. G. Hollunger and L. Oreland, *Archs Biochem. Biophys.* **139,** 320 (1970).
7. M. Jain, F. Sands and R. W. von Korff, *Analyt. Biochem.* **52,** 542 (1973).
8. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
9. B. Ekstedt and L. Oreland, *Biochem. Pharmac.* **25,** 119 (1976).
10. B. Ekstedt and L. Oreland, *Archs int. Pharmacodyn.* in press.
11. I. S. Severina, *Eur. J. Biochem.* **38,** 239 (1973).