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5-Hydroxytryptamine is a substrate for both species of monoamine oxidase in beef heart mitochondria

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The activity of beef heart mitochondrial monoamine oxidase towards 5-hydroxytryptamine (5-HT) is inhibited by the selective inhibitors clorgyline, PCO [5-phenyl-3-(*N*-cyclopropyl)-ethylamine-1,2,4-oxadiazole] and Deprenyl with a biphasic dependence on the inhibitor concentration. The activities towards tyramine, dopamine and tryptamine were also inhibited in a biphasic manner, but the apparent proportions of the two enzyme species active on dopamine and tryptamine depended on the inhibitor used. Phenethylamine oxidation was inhibited in a monophasic manner suggesting that only a single enzyme species was responsible for the oxidation of this substrate. The biphasic response of 5-HT oxidation to inhibition by clorgyline persisted when functionally competent mitochondria were used and was unaffected by the soluble amine oxidase inhibitors semicarbazide and aminoguanidine. These results indicate that the behaviour of the beef heart enzyme towards selective inhibitors is considerably different from that of any preparations previously studied and suggest that the classification of monoamine oxidase activities into A and B types may be only of limited usefulness.

The use of selective irreversible inhibitors of monoamine oxidase (monoamine: O_2 oxidoreductase (deaminating) EC 1.4.3.4) has allowed two major species of the enzyme to be detected in preparations from a number of sources (see Squires, 1972; Tipton, Houslay & Mantle, 1976 for reviews). Johnston (1968) showed that the activity of rat brain monoamine oxidase towards 5-hydroxytryptamine (5-HT) was inhibited by much lower concentrations of clorgyline (*N*-methyl-*N*-propargyl-3-(2,4-dichlorophenoxy)propylamine) than were necessary to inhibit the activity towards benzylamine. He termed the more sensitive species the A enzyme and the less sensitive the B enzyme and found that the activity towards tyramine was inhibited in a manner that indicated that both these species were responsible for its oxidation. Subsequent work has shown that the inhibitors Lilly 51641 (*N*-[2-(*O*-chlorophenoxy)-ethyl]-cyclopropylamine) (Fuller, 1972) and PCO (5-phenyl-3-(*N*-cyclopropyl)ethylamine-1,2,4-oxadiazole) (Mantle, Wilson & Long, 1975) behave very similarly to clorgyline, whereas Deprenyl (phenylisopropylmethylpropinylamine) (Knoll & Magyar, 1972) and pargyline(*N*-methyl-*N*-2-propynylbenzylamine) (Fuller, Warren & Molloy, 1970) differ in inhibiting the activity towards benzylamine at lower concentrations than are necessary to inhibit the activity towards 5-HT. The proportions of the two forms of the enzyme that can be detected using such inhibitors have been shown to vary widely in different organs and species (see e.g. Squires, 1972; Tipton & others, 1976), but calculations of the proportions of the two forms using clorgyline and Deprenyl have generally given results that are in good agreement. Squires (1972) however, has reported that clorgyline behaved as a selective inhibitor of the monoamine oxidase activity in a number of organs of the rabbit whereas Deprenyl and pargyline did not, suggesting that the two classes of inhibitors do not always act in a complementary fashion. Despite this anomaly the A and B classification based on inhibitor sensitivity and substrate specificity has found wide use and the specificities of the two forms have been further defined to include noradrenaline as a substrate for the A form, 2-phenethylamine as a substrate for the B form and dopamine as a substrate for both forms (Hall, Logan & Parsons, 1969; Houslay & Tipton, 1974; Neff & Yang, 1974; Mantle & others, 1975).

Many studies have assumed that the specificities of the two forms of the enzyme will apply to enzyme preparations from other sources. However a number of preparations that appear to contain almost exclusively the B form of monoamine oxidase (as defined by clorgyline inhibition) have been shown to be active towards the A-substrate 5-HT (Tipton & Spires, 1968; Hall & others, 1969; Squires, 1972) and Lyles & Callingham (1974) have recently shown that benzylamine is a substrate for both forms of the enzyme in rat heart whereas tyramine is a substrate

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only for the A form. In this paper we report results on the effects of selective inhibitors on the activity of monoamine oxidase from beef heart which suggest that the two forms of the enzyme in this organ differ considerably from those that have previously been studied.

MATERIALS AND METHODS

[1-^{14}C]-Labelled 5-HT creatinine sulphate, tyramine hydrochloride, dopamine hydrochloride and [G-^{3}H]-tryptamine hydrochloride were obtained from The Radiochemical Centre, Amersham, Bucks, U.K., [1-^{14}C]-labelled benzylamine hydrochloride was obtained from ICN Pharmaceuticals, Irvine, Calif. U.S.A. and [1-^{14}C]-labelled 2-phenethylamine hydrochloride was obtained from New England Nuclear, Boston, Mass., U.S.A. Clorgyline (M & B 9302) was a kind gift from May & Baker, Dagenham, Essex, U.K. and Deprenyl and PCO were kind gifts from Roche Products Ltd, Welwyn Garden City, Herts, U.K. All other chemicals were obtained from British Drug Houses, Poole, Dorset, U.K. or Koch-Light, Colnbrook, Bucks, U.K.

Beef heart mitochondria were prepared by the method of Ramsay & Tubbs (1975) and, unless otherwise stated, they were stored in the final-wash buffer at −20° and at a protein concentration of about 40 mg ml^{-1} for at least 18 h before use. In experiments that used mitochondria that had not been pre-frozen, the respiratory control and P:O ratios were determined at 30° using an oxygen electrode (Rank Bros Bottisham, Cambridge, U.K.) in an assay medium that contained (mM) tris 20, KCl 80, potassium phosphate buffer 3·3, $MgCl_2$ 5 and sodium succinate 25 at a final pH of 7·4. The mitochondrial preparation was incubated with this mixture and the respiratory control and P:O ratios were determined following the successive additions of aliquots of 10 mM ADP (see e.g. Estabrook, 1967).

Rat liver parenchymal cells (hepatocytes) were prepared by the method of Howard & Pesch (1968) and mitochondrial fractions prepared from these were stored in 10 mM sodium phosphate buffer, pH 7·2 at −20°.

The activity of monoamine oxidase was assayed at 30° in 100 mM sodium phosphate buffer, pH 7·2 by the method of Otsuka & Kobayashi (1964) except that the products were extracted into toluene–ethyl acetate (1:1; v/v). In experiments where functionally competent mitochondria were used the assays were carried out in the medium used for studying the respiratory properties of the mitochondria except that sodium succinate and ADP were omitted. Inhibition experiments were carried out by incubating the mitochondrial preparation with the stated concentration of the inhibitor at 30° for 30 min before the activity was assayed. Protein concentrations were assayed by the method of Goa (1953) using bovine serum albumin as the standard.

RESULTS

Substrate specificities. The substrate specificities of monoamine oxidase in beef heart mitochondria and rat liver parenchymal cell mitochondria (which have been shown to contain the A and B forms in approximately equal proportions, Tipton & others, 1976) are compared in Table 1. Although beef heart mitochondria are a much poorer source of the enzyme the Km values shown by the two preparations are similar. 2-Phenethylamine is most rapidly oxidized by the beef heart enzyme preparation while the activities towards 5-HT, benzylamine and dopamine are considerably lower.

Inhibitor sensitivities. The concentration-dependences of the inhibition of the activity of beef heart mitochondrial monoamine oxidase towards 5-HT by clorgyline, PCO and Deprenyl are shown in Fig. 1. In each case a biphasic 'dose-response' curve results suggesting that two enzyme species are involved in the oxidation of this substrate, with some 70–80% of the activity being due to the species A-enzyme (as defined by clorgyline- and PCO-sensitivity and

Table 1. *The specificities of monoamine oxidase in mitochondria prepared from beef heart and rat liver parenchymal cells.* Details of the assay methods used are given in the text. Kinetic constants were determined from double-reciprocal plots (Lineweaver & Burk).

	Beef heart		Rat liver parenchyma	
Substrate	K_m (μM)	V_{max}*	K_m (μM)	V_{max}*
Tyramine	125	1·3	140	83
Dopamine	105	0·1	170	24
2-Phenethylamine	26	4·2	22	39·2
5-HT	160	0·2	140	35
Benzylamine	160	0·5	N.D.	N.D.

* The V_{max} values are expressed in nmol min^{-1} mg^{-1}.
N.D. = not determined.

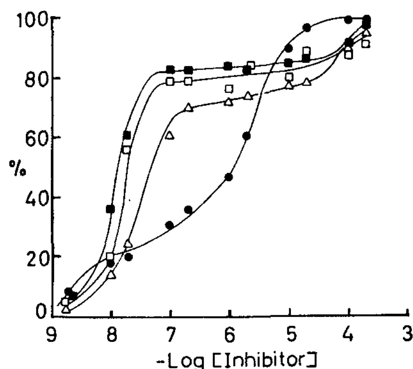

FIG. 1. Inhibition (%) of 5-HT oxidation by beef heart mitochondrial monoamine oxidase by selective inhibitors. Details of the methods used are given in the text. The inhibitors used were Deprenyl (●), PCO (△) and clorgyline (□ and ■). Freeze-thawed mitochindria were used in all experiments except that designated by ■ when functionally competent mitochiondria were used.

Deprenyl-insensitivity). Freshly prepared beef heart mitochondrial preparations which showed respiratory control ratios of between 3 and 7 and P:O ratios of between 1·4 and 1·7 with succinate as the substrate gave a similar biphasic inhibition curve to that given by the frozen preparation when clorgyline was used as the inhibitor and the activities were determined in the medium that was used for estimating the respiratory properties of the mitichondria (Fig. 1).

Biphasic inhibition curves were also given with clorgyline and Deprenyl when tyramine (Fig. 2), dopamine (Fig. 3) and tryptamine (Fig. 4) were used as the substrates. The results indicated that some 80–85% of the activity towards tyramine was due to

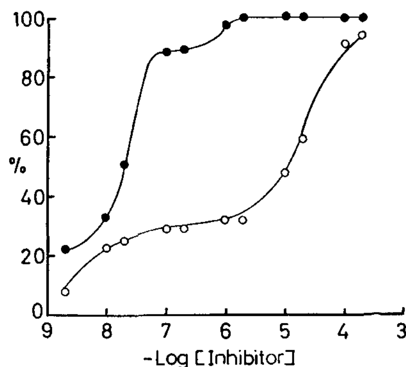

FIG. 3. Inhibition (%) of dopamine oxidation by beef heart mitochondrial monoamine oxidase by selective inhibitors. Details of the methods used are given in the text. The inhibitors used were Deprenyl (●) and clorgyline (○).

the B species (Fig. 2), but the inhibition curves given towards dopamine and tryptamine as substrates were unusual in that there were discrepancies between the proportions of the enzyme that were relatively clorgyline-sensitive and deprenyl-insensitive (Figs 3, 4). By the criterion of clorgyline sensitivity some 30% of the activity towards dopamine and tryptamine would be classified as being due to the A enzyme species whereas the insensitivity towards Deprenyl inhibition indicates that less than 15 % of the activity towards dopamine and some 60% of the activity towards tryptamine is due to the A species.

The activity towards 2-phenethylamine was inhibited in a monophasic manner by increasing concentrations of clorgyline and Deprenyl as shown in Fig. 5. The sensitivity of this activity towards inhibition by Deprenyl and its insensitivity towards

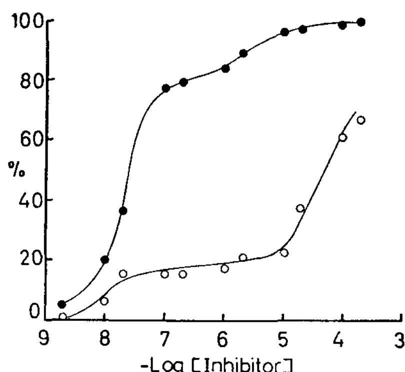

FIG. 2. Inhibition (%) of tyramine oxidation by beef heart mitochondrial monoamine oxidase by selective inhibitors. Details of the methods used are given in the text. The inhibitors used were Deprenyl (●) and clorgyline (○).

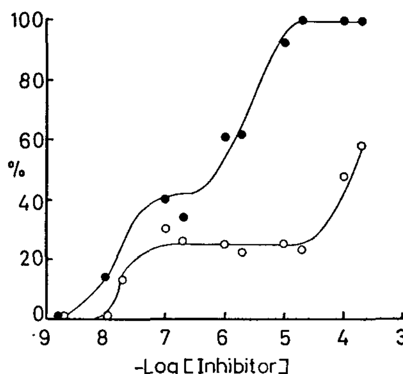

FIG. 4. Inhibition (%) of tryptamine oxidation by beef heart mitochondrial monoamine oxidase by selective inhibitors. Details of the methods used are given in the text. The inhibitors used were Deprenyl (●) and clorgyline (○).

clorgyline inhibition indicates that 2-phenethylamine is a substrate only for the B enzyme species.

The effects of the diamine oxidase inhibitors semicarbazide and aminoguanidine (see e.g. Bardsley, Crabbe & Scott, 1974) were studied by pre-incubation of the mitochondrial preparation with varying concentrations of the inhibitors for 30 min at 30°. No inhibition of the activity towards 5-HT was observed at semicarbazide concentrations up to 31 mM although 0·25 and 2·5 mM aminoguanidine apparently inhibited by 20 and 50% respectively. This apparent inhibition may well result from an effect on the extraction efficiency of the aldehyde-aminoguanidine adduct into toluene-ethyl acetate as 0·5 mM aminoguanidine caused no detectable change in the nature of the biphasic inhibition curve given by clorgyline.

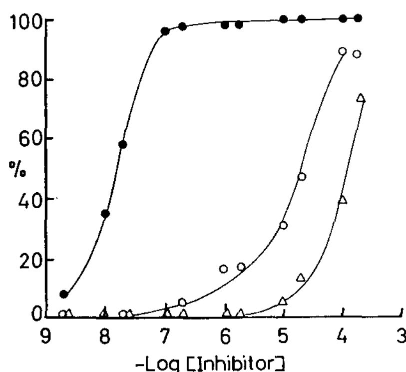

FIG. 5. Inhibition (%) of 2-phenethylamine oxidation by beef heart mitochondrial monoamine oxidase by selective inhibitors. Details of the methods used are given in the text. The inhibitors used were deprenyl (●) and clorgyline (○). Also shown are the effects of clorgyline on functionally competent mitochondria (△).

DISCUSSION

The monoamine oxidase activity in beef heart mitochondria is unique among preparations so far studied in showing evidence for two species of the enzyme being active towards 5-HT, although a number of preparations that contain only the B species have been reported to be active towards this substrate (Tipton & Spires, 1968; Hall & others, 1969; Squires, 1972). The inhibition of the activities towards this substrate, dopamine, tyramine and tryptamine by clorgyline are also unusual in that the differences in the sensitivities between the two enzyme species are much greater than those observed with preparations from a number of other sources (see e.g. Johnston, 1968; Hall & others, 1969; Houslay & Tipton, 1974).

The proportions of the two enzyme forms that can be defined by the use of selective inhibitors do not, of course, have any direct relationship to the molar ratios of the two enzyme species present. Thus the differences between the proportions of the two forms determined from the clorgyline inhibition curves (30% B form determined with 5-HT as the substrate and 85% B form with dopamine and tyramine as the substrates) may result from the two forms showing different relative maximum velocities with 5-HT compared with the other two substrates. The discrepancies between the proportions of the two species that are seen when the results obtained using clorgyline and Deprenyl are compared and dopamine and tryptamine are used as substrates, are difficult to explain on a model involving only two species of the enzyme. However there is evidence that neither of the two major forms that can be distinguished in this way can be regarded as representing a single homogeneous enzyme species (Tipton, 1969; Squires, 1972). The present results would indicate that the components of the two major species do not always behave in a homogeneous way towards clorgyline and Deprenyl, and add support for the suggestion in the work of Squires (1972) that these two inhibitors cannot always be regarded as acting complementarily.

Lyles & Callingham (1974) showed that the monoamine oxidase activity in rat heart showed a biphasic response to inhibition by clorgyline when benzylamine was the substrate and, in addition, they detected the presence of a benzylamine oxidase activity that was resistant to inhibition by clorgyline which they tentatively ascribed to a blood vessel or connective tissue amine oxidase (Lyles & Callingham, 1975). It is unlikely that the preparation used in the present studies would be contaminated with such an enzyme since washed mitochondria were used as the enzyme source, but, since the soluble amine oxidase from beef plasma has been shown to be reversibly inhibited by clorgyline (Houslay & Tipton, 1975) and diamine oxidase from dog intestine has been shown to be active towards 5-HT (Kusche, Lorenz & Schmidt, 1975), it was necessary to consider the possibility that a proportion of the activity towards 5-HT was due to the presence of a soluble 'diamine oxidase'. The failure of semicarbazide which is known to be a powerful inhibitor of the soluble amine oxidases (see e.g. Lyles & Callingham, 1975) to cause significant inhibition of the activity towards 5-HT and the biphasic inhibition of 5-HT deamination by clorgyline in the presence of 0·5 mM aminoguanidine indicates that the effects reported here are solely due to the mitochondrial monoamine oxidase.

The apparent multiplicity of monoamine oxidase appears to be due to a single enzyme existing in different environments within the mitochondrial membrane rather than being due to the presence of two distinct enzymes (Houslay & Tipton, 1973; Tipton, Houslay & Garrett, 1973) and thus differences in the substrate specificities and inhibitor sensitivities between organs and species could reflect differences in the compositions of the mitochondrial outer membranes. The properties of the enzymes have been shown to change when it is solubilized from the mitochondrial outer membrane (Tipton & others, 1976) and Achee, Togulga & Gabay (1974) have stressed the importance of working with intact mitochondria in order to assess the behaviour of the enzyme in its native environment. The fact that the biphasic response of the activity towards 5-HT to clorgyline inhibition when functionally competent beef heart mitochondria were used, suggests that these effects cannot be ascribed to changes in mitochondrial structure. A model suggested by Houslay & Tipton (1974) to explain why certain substrates were grouped as A and/or B substrates predicted that 5-HT would be a substrate for both species and had to invoke steric restraints to explain the experimental observations. In this respect the beef heart enzyme would thus appear to conform to the model in its simplest form.

The present results provide further evidence that the assumption of the existence of two forms of monoamine oxidase that differ in a similar way in all sources in their specificities and inhibitor sensitivities is an over simplification and that the, frequently used, classification into A and B enzyme types may be of little value in some tissues.

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