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DETERMINATION OF THE ABSOLUTE CONCENTRATIONS OF MONOAMINE OXIDASE A AND B IN HUMAN TISSUES

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Abstract—The concentrations of monoamine oxidase-A and -B were determined in homogenates of human cerebral cortex, caudatus and placenta and in human platelet-rich plasma and platelet membranes by determining the specific binding of tritium-labelled pargyline. The concentrations of the two enzyme forms were similar in both human brain regions examined. Determinations of the minimum quantities of clorgyline, (−)-deprenyl, J-508 or pargyline necessary to give complete inhibition of enzyme activity was found to give overestimates of the amounts of monoamine oxidase-A and -B present due to nonspecific binding of these inhibitors.

The A- and B-forms of monoamine oxidase (MAO) differ in their substrate specificities and inhibitor sensitivies (see Refs 1–3 for review). A number of studies have indicated that the levels of these enzymes in human tissues may be useful markers for specific psychiatric disorders [4–6]. The activities towards specific substrates, such as 5-hydroxytryptamine for MAO-A and benzylamine or 2-phenethylamine for MAO-B, have been frequently used to estimate the amounts of enzyme present. The results of such studies would, however, only give accurate estimates of the quantities of the two enzymes present if their specificities were absolute and they had identical specific activities towards their respective substrates. However, it has been shown that neither 5-hydroxytryptamine nor 2-phenethylamine are entirely specific substrates for A- or B-forms of the enzyme, respectively [7, 8].

The use of a substrate that is oxidised by both forms of the enzyme together with a selective inhibitor for MAO-B [1–3] offers an alternative approach to estimating the proportions of the two forms present in tissues. However, such an approach would only be valid if the two forms had identical activities towards the substrate. In the case of tyramine it has been shown that the K_m and V_{max} values of the two forms differ such that the relative proportions determined will depend on the substrate concentration used [9]. There have also been reports of the presence of endogenous activators [10, 11] or inhibitors [11–14] of monoamine oxidase in the tissues which may be important in some psychiatric conditions. Thus, in addition to the methodological problems described above, the determination of enzyme activities in tissue preparations may give misleading estimates of the total amounts of the two forms present. A full understanding of the relative importance of differences in the amounts of the two enzymes present and the possible modulation of their activities by endogenous factors will necessitate comparisons of the determined tissue activities with measurements of the absolute concentrations present.

We have developed an approach to the determination of the two forms in rat liver based on the binding of radioactively labelled pargyline [15], which is a mechanism-based irreversible inhibitor of the enzyme [16–18]. In the present work we have used this approach to determine the concentrations of the two forms of MAO in human brain, platelets and placenta and compared the results obtained with direct activity determinations.

MATERIALS AND METHODS

Homogenates of human cerebral cortex and caudatus were prepared from autopsy material as described previously [19]. Human placenta samples were obtained immediately on delivery and transported to the laboratory on ice. Samples were cleaned, washed in 0.1 M potassium phosphate buffer, pH 7.2, blotted dry and homogenised in 10 vol. of the same buffer in a Thomas Homogeniser with a Teflon pestle with a 0.012-inch clearance. Platelet-rich plasma was prepared from 20 ml samples of human blood, which were collected in plastic tubes containing 2.0 ml of a solution containing 20 mg disodium EDTA, 14 mg NaCl and 2.2 mg glucose. The mixture was centrifuged at 200 *g* for 15 min to remove the blood cells and the supernatant platelet-rich plasma was retained. A preparation of platelet membranes was obtained by the procedure of Cesura *et al.* [20].

The tissue samples were obtained from subjects without histories of psychiatric diseases. All samples were stored at −20° until required.

The activities of monoamine oxidase towards 100 μM 5-hydroxytryptamine and 20 μM 2-phenethylamine were determined radiochemically as previously described [21]. At these assay concentrations these two substrates behave as essentially specific substrates for MAO-A and MAO-B respectively.

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For studies on the activities and binding properties of the two forms in the cerebral cortex samples they were preincubated for 60 min with either 0.3 μM clorgyline or 0.5 μM (−)-deprenyl which have been previously shown to inhibit the activity of the A- or B-form, respectively without significantly affecting the activity of the other [19].

Binding of the radioactively-labelled inhibitor pargyline was determined by a modification of the procedure of Parkinson and Callingham [22] as previously reported [15]. Samples were preincubated for 1 hr at 37° in 0.1 M potassium phosphate buffer, pH 7.8, containing 0.3 μM clorgyline, 0.5 μM (−)-deprenyl or an equivalent volume of water. [^{3}H]-pargyline (50 Ci mol^{-1}) in amounts ranging from 25 to 400 pmol was added to give a total volume of 0.1 ml and the mixture was incubated at 37° for 1 hr. The mixtures were then cooled on ice and 1.0 ml of potassium phosphate buffer, pH 7.8, was added before centrifugation at 16,000 *g* for 1.5 min. The pellet was suspended in 1.0 ml of the same buffer and allowed to stand at room temperature for 1 hr before centrifugation as before. The pellet was resuspended in 1.0 ml of phosphate buffer and transferred to a vial containing 10 ml toluene–Triton X-100 (2:1 v/v) containing 0.4% (w/v) 2,5-diphenyloxazole and the radioactivity was determined by liquid scintillation counting. Non-specific binding was determined using samples that had been preincubated for 1 hr at 37° with 2 mM unlabelled pargyline.

Activity titrations, to determine the minimum amounts of the inhibitors clorgyline, J-508 (*N*-methyl-*n*-propargyl-1-aminoindane hydrochloride), (−)-deprenyl and pargyline necessary to inhibit the activity completely, were carried out by the procedure based on that of Fowler *et al.* [23, 24] as previously described [15, 19]. Protein concentrations were determined by the method Markwell *et al.* [25] with bovine serum albumin as the standard.

5-Hydroxytryptamine-[side-chain-2-^{14}C]creatinine sulphate and 2-phenethylamine-[ethyl-1-^{14}C]hydrochloride were obtained from Amersham International (Amersham, Bucks., U.K.). Pargyline-[phenyl-3,benzyl-^{3}H]hydrochloride was from New England Nuclear (Boston, MA). Clorgyline and (−)-deprenyl were kind gifts from May & Baker (Dagenham, Essex, U.K.) and Professor J. Knoll, Department of Pharmacology, Semmelweis University of Medicine, Budapest, Hungary, respectively.

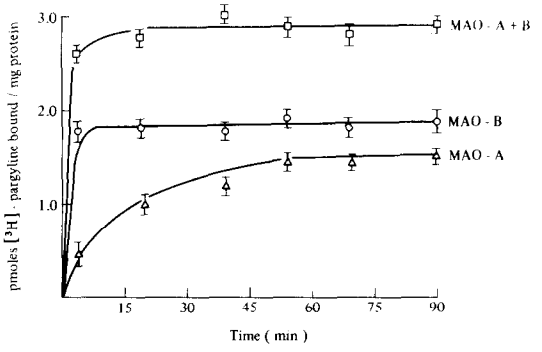

Fig. 1. Time course of the binding of [^{3}H]-pargyline to monoamine oxidase homogenates of human cerebral cortex. Samples containing 800 μg of protein were incubated for 1 hr at 37° in 0.1 M phosphate buffer, pH 7.8, with distilled water (□), 0.5 μM (−)-deprenyl (△) or 0.3 μM clorgyline (○) before addition of 2.5 μM [^{3}H]-pargyline (50 Ci·mol^{-1}) and further incubation for periods of up to 90 min. The extent of binding was determined by the procedure described in the text. Each experimental point represents the mean ± SEM of triplicate determinations in three separate tissue homogenates.

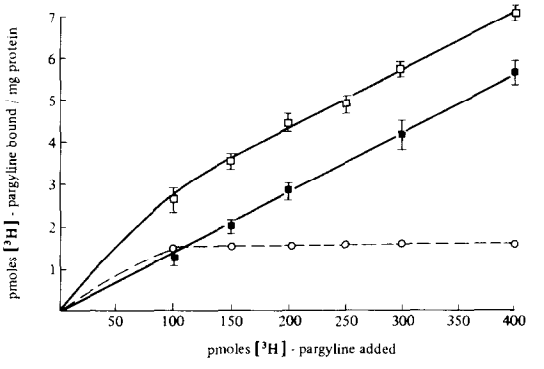

Fig. 2. The binding of [^{3}H]-pargyline to monoamine oxidase-A in homogenates of human cerebral cortex. Samples containing 160 μg of protein were preincubated for 1 hr at 37° in 0.1 M phosphate buffer, pH 7.8, containing 0.5 μM (−)-deprenyl prior to incubation for a further 1 hr in the presence (■) or absence (□) 2 mM unlabelled pargyline. [^{3}H]-Pargyline was then added and, after incubation for 1 hr at 37°, the extent of binding was determined as described in the text. Specific binding (○) was calculated as the difference between the binding determined in the presence and absence of pretreatment with 2 mM unlabelled pargyline. Values shown are means ± SEM of triplicate determinations in three separate tissue homogenates.

RESULTS AND DISCUSSION

In agreement with previous studies pargyline was found to be a selective inhibitor of MAO-B in human cerebral cortex homogenates. After incubation for 60 min at 37° and at pH 7.2 with different concentrations of pargyline the inhibitor concentrations necessary to give 50% inhibition were approximately 1.5 μM for MAO-A, assayed with 100 mM 5-hydroxytryptamine, and 0.1 μM for MAO-B, assayed with 20 μM 2-phenethylamine. Time courses for the binding of [^{3}H]-labelled pargyline to the human cerebral cortex homogenates, shown in Fig. 1, indicated the process to be complete within 60 min.

The total and nonspecific binding of the labelled pargyline to MAO-A and MAO-B in the human cerebral cortex homogenate are shown in Figs 2 and 3. The specific binding to the enzyme in homogenates from human cerebral cortex and caudatus, calculated by subtracting the nonspecific values from the total binding, are shown in Table 1. Determination of the nonspecific binding using enzyme samples that had been pretreated with 0.3 μM clorgyline plus 0.5 μM (−)-deprenyl gave similar results to those obtained

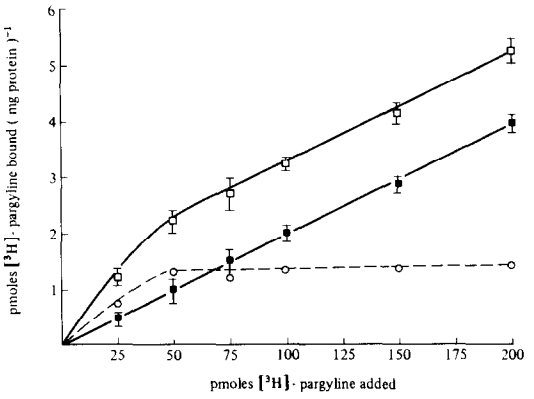

Fig. 3. The binding of [^{3}H]-pargyline to monoamine oxidase-B in homogenates of human cerebral cortex. Procedures and symbols were as described in Fig. 2 except that the samples were pretreated with 0.3 μM clorgyline to inhibit the MAO-A activity.

when pretreatment with an excess of unlabelled pargyline (2 mM) was used.

Human platelets contain no detectable MAO-A activity. The concentrations of MAO-B determined in the platelet-rich plasma and the platelet membrane preparations are shown in Table 1. The low concentrations of enzyme in the platelet-rich plasma limited the accuracy of determinations in those preparations. The MAO-A concentrations in the placental preparations, which contain no detectable MAO-B, are also shown in Table 1.

The results of an experiment in which the concentrations of the two forms of MAO were estimated by titration of the activity with the irreversible inhibitors clorgyline, for MAO-A, and J-508, for MAO-B respectively, are shown in Fig. 4. This procedure, originally devised by Fowler *et al.* [23, 24] cannot take account of the possibility that a proportion of the added inhibitor may bind nonspecifically to other components of the preparation and thus be unavailable to inhibit the enzyme [15]. The apparent enzyme concentrations for the cerebral cortex, platelet and placenta preparations determined by titration with clorgyline, (−)-deprenyl, pargyline and J-508 are shown in Table 2. Comparison of these values with those in Table 1 indicates that the nonspecific binding of these inhibitors results in the titration procedure giving a considerable overestimation of the enzyme concentrations. Thus in the activity titrations of the cerebral cortex preparations with clorgyline for MAO-A and J-508 for MAO-B the specific binding to the enzyme accounts for only 18% and 56% of the total binding, respectively. The corresponding values for human placental MAO-A and platelet-rich plasma MAO-B were 28% and 11%, respectively.

The data for the apparent enzyme concentration determined by activity titration of the preparations with pargyline was considerably greater than would be expected from the total binding obtained in the experiments with [^{3}H]-labelled pargyline, as can be

Table 1. The concentrations of the two forms of MAO in some human tissues

Source	Concentration (pmol·mg protein^{-1})	
	MAO-A	MAO-B
Cerebral cortex	1.6 ± 0.3	1.8 ± 0.3
Caudatus	1.8 ± 0.6	2.0 ± 0.5
Placenta	3.9 ± 0.8	N.D.
Platelet-rich plasma	N.D.	0.048 ± 0.004
Platelet membranes	N.D.	5.7 ± 0.5

Values were determined by measuring the specific binding of [^{3}H]-pargyline as described in the text. Each value represents the mean ± SEM of determinations in three separate preparations. N.D. = not detected.

Table 2. Apparent monoamine oxidase concentrations in human tissue preparations determined from activity titrations with irreversible inhibitions

Source	Enzyme	Inhibitor	Concentration (pmol·mg protein^{-1})
Cerebral cortex	A	Clorgyline	9.0 ± 2.0
Cerebral cortex	A	Pargyline	29.0 ± 7.0
Cerebral cortex	B	(−)-Deprenyl	5.5 ± 0.2
Cerebral cortex	B	J-508	3.2 ± 0.2
Cerebral cortex	B	Pargyline	21.0 ± 4.0
Placenta	A	Clorgyline	13.7 ± 0.1
Platelet-rich plasma	B	J-508	0.44 ± 0.04

Activities were determined with 5-hydroxytryptamine or 2-phenethylamine for MAO-A and -B, respectively. Values are means ± SEM from three separate preparations. Values obtained with J-508 have been divided by 2 since this is a racaemic compound but only the 1-enantiomer is responsible for the inhibition produced [24].

Table 3. Activity titrations and inhibitor binding studies with pargyline in homogenates from human cerebral cortex

Titrant	Enzyme	Method*	Total binding (pmol·mg protein^{-1})
Pargyline	A	Titration	29.0 ± 7.0
	B	Titration	21.0 ± 4.0
[^{3}H]-Pargyline	A	Titration	39.0 ± 3.0
	B	Titration	25.0 ± 3.0
[^{3}H]-Pargyline	A	Binding	3.5 ± 0.5
	B	Binding	2.5 ± 0.4

* Titration refers to the enzyme activity titration procedure and binding refers to the determination of the total amount of radioactive pargyline bound to the pellet. Both determinations were carried out at pH 7.2 as described in the text and the values are means ± SEM of triplicate determinations in three separate determinations. The specific component of the [^{3}H]-pargyline binding was as shown in Table 1.

seen from a comparison between Fig. 2 and Tables 1 and 2. In order to ensure that this was not an artefact arising from differences between the labelled and unlabelled pargyline preparations used, the activity titration was performed with the [^{3}H]-labelled inhibitor and samples were also removed to determine the total radioactivity bound by the procedure described earlier. The results shown in Table 3, indicate that activity titrations, with either labelled or unlabelled pargyline, give very much higher binding values than are obtained in the direct binding studies with [^{3}H]-pargyline.

This large difference between the apparent total binding values suggests that a substantial proportion of the added inhibitor is bound nonspecifically but reversibly to components of the preparation. Such material would not be detected in the binding studies with labelled pargyline, since it would be removed in the washing procedure, but it would contribute to the values obtained in the activity measurements as no such washing was carried out in the latter procedure. The binding of [^{3}H]-labelled pargyline was determined as previously described except that surface washing of the enzyme pellet was employed instead of resuspension and centrifugation. This procedure was based on that used in a number of ligand-binding studies [26]. It involved addition of 1.0 ml of 0.1 M potassium phosphate buffer, pH 7.8, to the pellet and inversion of the tube before aspiration of the buffer, resuspension of the pellet and scintillation counting as before. This procedure gave very much higher nonspecific binding values, representing about 90% of the total. Thus it appears that the washing procedure is important in removing reversibly-bound pargyline and that this would be sufficient to account for the difference between the results obtained in the binding and activity titration procedures.

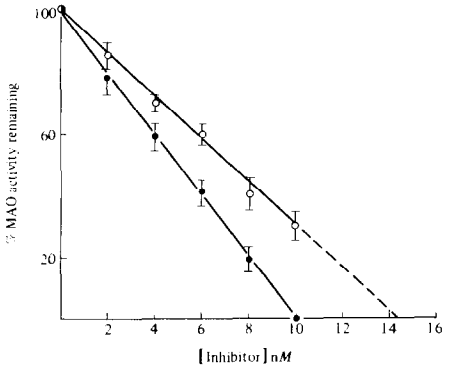

Fig. 4. Activity titration of monoamine oxidase activities in homogenates of human cerebral cortex. Samples at protein concentrations of 1.6 mg·ml^{-1} were preincubated in 0.1 M phosphate buffer, pH 7.2, with 0.5 μM (−)-deprenyl, to inhibit MAO-B, or 0.3 μM clorgyline, to inhibit MAO-A, and the indicated concentrations of either clorgyline (○) or J-508 (●) before assay for activity with either 100 μM 5-hydroxytryptamine (○) or 20 μM 2-phenethylamine (●). Points are means ± SEM of determinations in three separate homogenates of the % MAO activity remaining with respect to samples that had been incubated with either 0.5 μM (−)-deprenyl or 0.3 μM clorgyline.

The finding that the molecular quantities of the two forms of MAO in human cerebral cortex and caudatus were similar contrasts with the data of Brown *et al.* [27] who reported that the molecular concentration of MAO-B was about four times higher than that of MAO-A in human brain. This was based on the measurement of the binding at relatively low ratios of [^{3}H]-pargyline to human brain mitochondria after incubation for 45 min at 37° [28]. The data presented here, however, indicate that the pargyline concentration and incubation time used in that study would be insufficient for complete labelling of MAO-A to occur.

The results of the present work show that determination of the minimum amount of mechanism-based inhibitor, such as clorgyline, (−)-deprenyl, J-508 or pargyline, necessary to inhibit the activity of the enzyme completely will give overestimates of the quantities of the monoamine oxidase forms present due to the nonspecific binding of these inhibitors to other components of the preparation.

The dilution of tissue samples in preparation for the binding studies would be expected to reduce the effects of any endogenous reversible inhibitors or activators. Irreversible inhibitors would be expected to reduce the amount of enzyme that would be

detected by the direct binding procedure whereas competitive inhibitors would slow the rate of reaction between pargyline and the enzyme without affecting the final extent of binding. The use of [^{3}H]-pargyline to determine the absolute concentrations of the two forms of MAO together with direct determination of their activities in human tissue preparations should allow the possible involvement of endogenous inhibitors and activators in psychiatric conditions to be assessed.

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