Biochemical Pharmacology, Vol. 35, No. 24, pp. 4467–4472, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00
Pergamon Journals Ltd.

DETERMINATION OF MONOAMINE OXIDASE CONCENTRATIONS IN RAT LIVER BY INHIBITOR BINDING

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(*Received* 4 *March* 1986; *accepted* 7 *July* 1986)

Abstract—The concentrations of monoamine oxidase-A and -B have been determined in mitochondria, mitochondrial outer membranes and microsomes from Sprague–Dawley and Wistar rats by determining the binding of tritium-labelled pargyline. Although the amounts of each form present depended on the source and the preparation method, this was paralleled by the specific activity such that the molecular turnover number was found to remain constant. The catalytic constants, k_{cat}/K_m, which represents the apparent second-order rate constant for the combination of enzyme and substrate, were about 0.13 and 2.1 $sec^{-1} \cdot \mu M^{-1}$ for 5-hydroxytryptamine and 2-phenethylamine, respectively, regardless of the source. Estimations of the amounts of the two forms by determining the concentrations of the inhibitors clorgyline, (−)-deprenyl, J-508 or pargyline necessary to give complete inhibition were shown to give overestimates of the true values because of the non-specific binding of these inhibitors to sites other than the monoamine oxidase active site.

The enzyme monoamine oxidase (amine:O_2 oxidoreductase (deaminating) (flavin-containing) EC 1.4.3.4) exists in two forms that differ in their substrate specificities and inhibitor sensitivities. The A-form (MAO-A) is sensitive to inhibition by low concentrations of clorgyline and active towards 5-hydroxytryptamine (5-HT) whereas the B-form (MAO-B) is sensitive to inhibition by l-deprenyl and active towards benzylamine and 2-phenethylamine [for reviews see 1–3].

The activities towards these specific substrates have been used in several studies to provide an indication of the relative amounts of the two forms present in different tissues. The results from such an approach would, however, only reflect the molar proportions of the two forms present if they had identical specific activities towards their specific substrates. Furthermore, recent studies have shown that neither 5-HT nor 2-phenethylamine are entirely specific substrates for the A- and B-forms respectively [4, 5]. An alternative procedure has used a compound, such as tyramine, that is a substrate for both forms of the enzyme, in conjunction with a selective inhibitor such as clorgyline. This approach would only be valid if the two forms had the same molecular activities and K_m values for the substrate. With tyramine, however, it has been shown that both the K_m and maximum velocity values for the two forms differ so that the apparent contributions of the two forms will depend on the substrate concentration [6]. The reports that there may be endogenous activators and inhibitors of monoamine oxidase in the tissues and body fluids [7–10] also suggest that attempts to determine the proportions of the two forms from activity measurements alone may give misleading results.

Two more direct methods have been used to determine the molar amounts of the two forms present in tissue preparations. The first of these involves measurement of the binding of the radioactively labelled enzyme-activated irreversible inhibitor pargyline to the active site of the enzyme [11–13]. With suitable correction for any non-specific binding, this procedure should give a direct measure of the amount of enzyme present. The alternative approach involves determination of the concentration of an irreversible inhibitor, such as the MAO-A selective inhibitor clorgyline or the MAO-B-selective inhibitor J-508, necessary to inhibit the activity completely [14, 15]. The validity of this activity titration approach depends on the assumptions that the inhibitor reacts stoichiometrically with the enzyme and that non-specific inhibitor binding does not occur. The latter assumption has not been rigorously established although indirect evidence has been interpreted that this is so in some tissue preparations [14, 15].

This paper reports the development of inhibitor binding assays for the determination of the absolute concentration of the monoamine oxidase forms in rat liver and a comparison with the results from activity titration experiments which show that non-specific binding results in substantial overestimates of the concentrations of the two forms when the latter method is used.

MATERIALS AND METHODS

Rat liver mitochondria were obtained from either Wistar or Sprague–Dawley rats that had been fasted overnight. With the former, the method of Kearney

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et al. [16] was used and the crude mitochondrial fraction obtained was washed twice, by suspension in 10 mM Tris–HCl buffer, pH 7.2, containing 0.25 M sucrose and centrifugation at 8500 *g* for 15 min. The resultant mitochondrial pellet was then either suspended to a protein concentration of 5 mg/ml in 0.1 M potassium phosphate buffer pH 7.2, stored at −20° or used to prepare mitochondrial outer membranes. These were prepared by the shrink-swell-sonicate method of Sottocasa *et al.* [17] followed by ammonium sulphate precipitation of the outer membrane fraction [18]. The preparation was then dialysed against 0.1 M potassium phosphate buffer pH 7.2, and stored at −20°.

The procedure for preparing mitochondria from the Sprague–Dawley rats was similar [19] except that 8000 *g* rather than 8500 *g* was used to sediment the mitochondria. The supernatant from this was centrifuged at 20,000 *g* for 10 min and the supernatant was then centrifuged at 200,000 *g* for 30 min in order to sediment the microsomal fraction. Both the mitochondrial and microsomal fractions were washed twice, by resuspension and sedimentation, resuspended in 50 mM potassium phosphate buffer, pH 7.2, to give final concentrations of 10–15 mg/ml and stored at −20°.

Monoamine oxidase activity was determined radiochemically by a modification [6] of the method of Otsuka and Kobayashi [20] with either 100 μM 5-hydroxytryptamine or 20 μM 2-phenethylamine as the substrates. At these concentrations these amines have been shown to behave as essentially specific substrates for the A and B-forms of MAO, respectively [5].

Inhibitor binding studies with [^{3}H]-labelled pargyline were performed either by filtration or centrifugation. In the latter procedure which is a modification of the method described by Parkinson and Callingham [13], samples were incubated in 0.1 M potassium phosphate buffer, pH 7.8, with [^{3}H]-labelled pargyline (50 mCi/mmol) in amounts ranging from 25 to 400 pmol for 60 min at 37°. The total incubation volume was 0.1 ml. The samples were then cooled on ice and 1.0 ml of 0.1 M potassium phosphate was added and the mixtures were centrifuged at 16,000 *g* for 1.5 min. The pellet obtained was resuspended in 1.0 ml of the same buffer and allowed to stand at room temperature for 60 min before centrifugation as before. The pellet was then resuspended in 1.0 ml of the same buffer and transferred to a scintillation vial containing 10 ml toluene: Triton X-100 (2:1, v/v) containing 0.4% (w/v) PPO and the radioactivity was determined by liquid scintillation counting. Non-specific binding was determined with samples that had been pretreated for 60 min at 37° with 2 mM unlabelled pargyline. Control experiments with untreated enzyme preparations showed all activity to be sedimented under these centrifuge conditions.

In the filtration assay the samples were incubated with varying amounts of labelled pargyline (3.2 Ci/mmol) in 50 mM potassium phosphate buffer, pH 7.2, as before. Ice-cold potassium phosphate buffer (4 ml) was then added and the mixtures were filtered, under vacuum, through Whatman GF-C filter papers. The papers were then washed once with the same buffer, dried and the radioactivity was determined by liquid scintillation counting in 4 ml of toluene: Triton X-100 (2:1 v/v) containing 0.4% (w/v) PPO.

For determination of the concentration of MAO-A separately the samples were preincubated at 37° for 60 min with 0.5 μM (pH 7.8) or 120 min with 0.3 μM (pH 7.2) l-deprenyl before the binding studies. MAO-B was determined after preincubation at 37° for 120 min (pH 7.2) or 60 min with 0.3 μM clorgyline. These concentrations were found to inhibit the activity of one form of the enzyme completely without significantly affecting the activity of the other (see ref. 6). Control samples were then taken through the same procedure but with the inhibitor replaced by water. Preincubation of the samples with 1 mM clorgyline completely inhibited the activity of both forms of the enzyme and was used as an alternative method for determining non-specific pargyline binding.

Activity titrations were carred out essentially as described by Fowler *et al.* [14, 15]. After preincubation with clorgyline or l-deprenyl as described above, to inhibit MAO-A or -B activity respectively, the enzyme preparation was incubated with inhibitor at concentrations ranging from 0 to 100 nM for 180 min at 37° before the activity remaining was determined. In an alternative procedure the enzyme preparation was preincubated for 180 min at 37° with the appropriate concentrations of clorgyline or l-deprenyl plus the titration inhibitor and the activity remaining was determined and expressed as the percentage activity remaining with respect to samples that had been incubated for the same length of time with either clorgyline or l-deprenyl alone. Similar results were obtained with both these procedures. The titration inhibitors used were clorgyline for MAO-A and pargyline l-deprenyl or J-508 (*N*-methyl-*n*-propargyl-1-aminoindane hydrochloride) for MAO-B. J-508 is a racaemic compound and only the l-enantiomer has been shown to be responsible for the inhibition produced. Thus the concentration of this compound required to give complete inhibition has to be divided by 2 to give the concentration of the active enantiomer required [15].

Protein concentration was determined by the method of Markwell *et al.* [21] or Hartree [22] with bovine serum albumin as the standard. 5-Hydroxytryptamine-[side chain-2-^{14}C] creatinine sulphate was obtained from Amersham International (Amersham, Bucks., U.K.). Pargyline-[phenyl-3, benzyl-^{3}H] hydrochloride was from New England Nuclear (Boston, MA) and 2-phenethylamine-[ethyl 1-^{14}C]-hydrochloride was obtained from both of these sources.

RESULTS

The effects on the activities towards 5-HT and 2-phenethylamine of preincubation of rat liver mitochondria with varying concentrations of pargyline shown in Fig. 1. The results are consistent with this compound showing some selectivity as an inhibitor of the B-form of monoamine oxidase [23, 24]. The small proportion of MAO-B activity towards 5-HT and possibly MAO-A activity towards 2-phen-

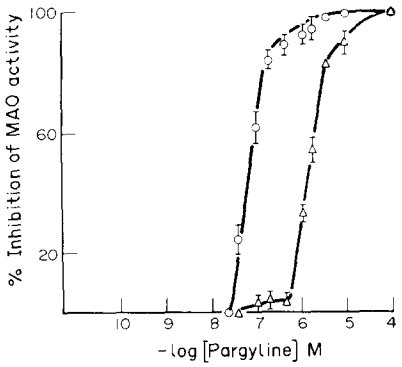

Fig. 1. The effects of pargyline on the deamination of 100 μM 5-hydroxytryptamine (△) and 20 μM-phenethylamine (○) by monoamine oxidase in rat liver mitochondria. The enzyme preparation was incubated for 60 min at 37° with the indicated inhibitor concentrations before addition of substrate to assay for activity. Results are given as means ± range of duplicate determinations of the percentage inhibition, with respect to control samples preincubated for the same time with an equivalent volume of distilled water, in three different mitochondrial preparations from Wistar rats.

ethylamine that can be seen are in agreement with previous reports that these substrates are not completely specific for only one form of the enzyme [4, 5].

When mitochondrial samples were incubated with 2.5 μM pargyline for varying times before the extent of binding was determined by centrifugation assay, the binding process was found to be time-dependent (Fig. 2) reaching a plateau value after 60 min. Similar results were obtained when the filtration assays was used.

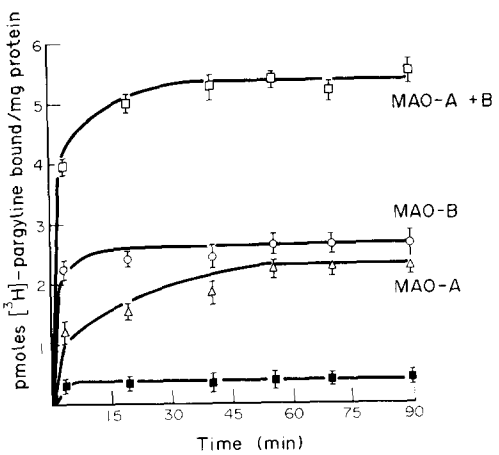

Fig. 2. Time courses of [^{3}H]-pargyline binding to rat liver mitochondrial monoamine oxidase. Mitochondrial homogenates (800 μg protein) from Wistar rats were incubated for 60 min at 37° with either distilled water (□), 0.5 μM l-deprenyl (△) or 0.3 μM clorgyline (○) before addition of [^{3}H]-pargyline (2.5 μM, 50 mCi/mmol) and further incubation for periods of up to 90 min before binding was determined by the centrifugation assay. Each value, which represents the mean ± S.E.M. of triplicate determinations in three mitochondrial preparations. Non-specific (■) binding was determined as described in the text.

Binding curves obtained when the enzyme preparations were preincubated with varying concentrations of [^{3}H]-labelled pargyline are shown in Fig. 3. The total binding observed represents the sum of specific and non-specific binding and the broken line shows the difference between these two quantities.

Table 1 shows the values obtained for the binding of the labelled pargyline to the mitochondrial, mitochondrial outer membrane and microsomal fractions. Determination of the maximum velocities for the oxidation of 5-HT and 2-phenethylamine by MAO-A and -B respectively allowed the turnover numbers and catalytic constants to be calculated. These values are shown in Tables 2 and 3. The differences in the amounts of monoamine oxidase determined in the mitochondrial preparations from Wistar and Sprague–Dawley rats can be seen to be a reflection of differences in the activities of the two preparations with the values obtained for their turnover numbers being similar.

Titration of the activities of monoamine oxidase with the unlabelled inhibitors clorgyline, l-deprenyl and J-508 gave significantly different results. Figure 4 shows the results of titrations of the activities in rat liver mitochondria and mitochondrial outer

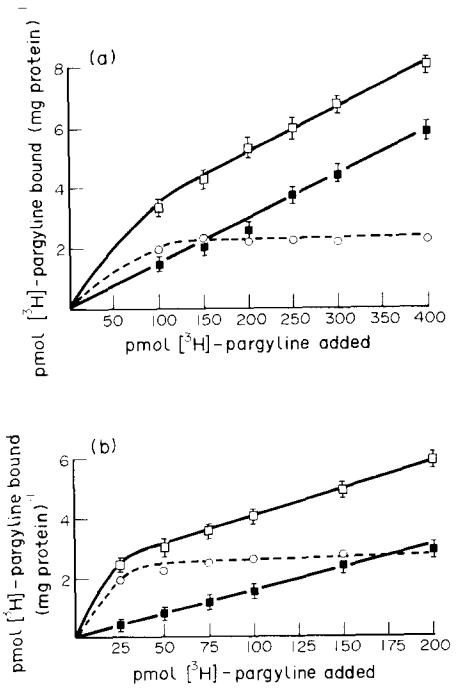

Fig. 3. The binding of [^{3}H]-pargyline to rat liver mitochondria. Mitochondria from Wistar rats (160 μg protein) were preincubated with 0.5 μM l-deprenyl (a) or 0.3 μM clorgyline (b) before the binding of ratioactively labelled pargyline was determined by the centrifugation assays as described in the text. Values shown are means ± S.E.M. of triplicate determinations in three different mitochondrial preparations. Specific binding to rat liver mitochondrial monoamine oxidase (○) was defined as the difference between the total binding (□) and the non-specific binding (■) curves.

Table 1. The concentration of the two forms of MAO in rat liver fractions determined by [^{3}H]-pargyline binding

Fraction	MAO-form	Concentration (pmol/mg protein	
		Sprague–Dawley	Wistar
Mitochondria	A plus B	11.3 ± 1.9	5.5 ± 0.3
	A	5.4 ± 0.8	2.4 ± 0.1
	B	5.7 ± 0.9	2.7 ± 0.1
Mitochondrial outer membranes	A plus B	—	23.0 ± 5.0
	A	—	11.0 ± 2.0
	B	—	10.4 ± 0.4
Microsomes	A plus B	4.4 ± 0.6	—
	A	2.0 ± 0.3	—
	B	2.3 ± 0.3	—

Values obtained were corrected for non-specific binding as described in the text.
Values are means ± S.E. for determinations in three preparations.

membranes. The apparent enzyme concentrations determined by this procedure are given in Table 4.

DISCUSSION

The acetylenic inhibitors clorgyline, l-deprenyl, J-508 and pargyline have been shown to react stoichiometrically with monoamine oxidase to form an adduct with the covalently-bound flavin group in a 1:1 ratio [16, 24–26]. When the monoamine oxidase preparations were pretreated with 1 mM clorgyline or 0.3 μM clorgyline plus 0.3 μM l-deprenyl to inhibit all the monoamine oxidase activity, the subsequent binding of radioactively-labelled pargyline was non-saturable and similar to that observed after pretreatment with 2 mM unlabelled pargyline (data not shown—see Fig. 3), consistent with the specific binding of these inhibitors being at the same site on the enzyme.

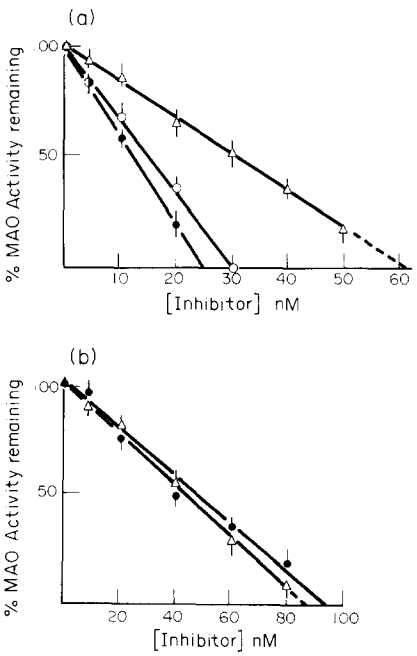

Fig. 4. Titration of rat liver mitochondrial (a) and mitochondrial outer membrane (b) monoamine oxidase activities with clorgyline, J-508 and l-deprenyl. The preparations from Wistar rats and at protein concentrations of 2 mg/ml were preincubated with 0.5 μM l-deprenyl, to inhibit MAO-B, or 0.3 μM clorgyline, to inhibit MAO-A, and the indicated concentrations of clorgyline (△), J-508 (●) or l-deprenyl (○) before the assay for activity with either 100 μM 5-hydroxytyptamine (△) or 20 μM 2-phenethylamine (●, ○). Points are mean values ± S.E.M. of triplicate determinations, each carried out with three preparations, of the percentage activity remaining with respect to control samples, preincubated with 0.5 μM l-deprenyl or 0.3 μM clorgyline alone.

Although the total amounts of the two forms of monoamine oxidase in the liver mitochondria from Wistar and Sprague–Dawley rats were different (Table 1). Their turnover numbers were similar (Table 2) indicating that their molecular activities were the same in the preparations from both sources. The absolute concentration of monoamine oxidase will depend on the purity of the mitochondrial preparation. The procedure used in the present work [16] yields a relatively impure mitochondrial fraction. In experiments with more highly purified mitochondrial preparations from Wistar rats [18] values for the total monoamine oxidase of up to 21 ± 3 pmol/mg·protein could be obtained. However, there was a parallel increase in the specific activity so that the turnover numbers remained similar to those shown in Table 2. Clearly the absolute concentration of monoamine oxidase and its specific activity will depend on the nature of the mitochondrial preparation used but the ratio of these values will remain constant.

The higher value for the monoamine oxidase content would correspond to the two forms of the enzyme representing about 0.13% of the total mitochondrial protein if a minimum molecular weight of 60,000 is assumed for the flavin-containing sub-unit of the enzyme [27, 28]. This value is somewhat lower than the value of 0.9% that can be calculated for the data of Callingham and Parkinson [28] and very much below that of 2.5% reported by McCauley [29]. Unfortunately these workers did not give sufficient activity data to allow the turnover numbers to be

Table 2. Molecular turnover numbers of the two forms of MAO in rat liver fractions

Source	Fraction	Turnover number*	
		MAO-A	MAO-B
Sprague–Dawley	Mitochondria	1022 ± 219	883 ± 195
Sprague–Dawley	Microsomes	802 ± 183	585 ± 106
Wistar	Mitochondria	810 ± 23	852 ± 35
Wistar	Mitochondrial outer membranes	845 ± 211	769 ± 113

Maximum velocities were determined from studies of the effects of substrate concentration on the initial velocity using either 5-hydroxytryptamine or 2-phenethylamine for MAO-A or MAO-B, respectively [4–6], in preparations where the activity of the other form had been inhibited by preincubation with low concentrations of l-deprenyl or clorgyline as described in the text. Values are mean ± S.E. for determinations carried out with at least three different preparations.

* mol product formed · mol enzyme form^{-1} · min^{-1}.

calculated for their preparations. The enrichment of monoamine oxidase concentration obtained by preparing mitochondrial outer membrane fractions (Table 1) is consistent with the known localisation of this enzyme (see ref. 30).

The nature of the monoamine oxidase activity associated with the microsomal fraction is uncertain. It had been claimed that this might represent a precursor of the enzyme associated with the mitochondria [31] but more recent work has indicated the enzyme to be synthesised on free, rather than membrane-bound polysomes [32]. The substrate specificities and inhibitor sensitivities of the two forms in the microsomal fraction appear to be rather similar to those in the mitochondrial outer membrane [33] but their rates of degradation *in vivo* are different [31]. The results shown in Table 2 indicate the molecular turnover numbers of both forms in the microsomal fractions are simlar to those in the mitochondria. Thus in all these fractions, the molecular turnover of MAO-A towards 5-HT is similar to that of MAO-B towards 2-phenethylamine (Table 2).

However, the values of k_{cat}/K_m, average 0.13 ± 0.05 and 2.10 ± 0.76 ($S^{-1} \cdot \mu M^{-1}$) for MAO-A and -B respectively, were considerably higher for the latter form. The value of k_{cat}/K_m corresponds to the apparent second-order rate constant for the combination of enzyme and amine at very low concentrations of the latter. If the rate limiting step in the enzyme-catalysed reaction were the binding of substrate to the enzyme, this would represent the rate constant for this reaction. Otherwise it will represent the lower limit (see e.g. ref. 34). The values shown in Table 3 are considerably lower than would be expected for a simple diffusion-controlled reaction (see ref. 34). However, such a simple relationship might not be expected for an enzyme catalysing a reaction involving two substrates that has been shown to obey a double-displacement mechanism [35, 36]. Furthermore the K_m values used in this calculation were determined from the total amine concentration whereas it appears that only the unprotonated amine serves as a substrate for the enzyme [36, 37]. Although it would be possible to express this value in terms of the unprotonated species only, it would not be appropriate to do so without information on whether the protonated form was inhibitory.

Attempts to determine the molar quantities of monoamine oxidase by activity titration gave values that were considered greater than those determined by binding methods. In view of the high levels of non-specific binding shown in Fig. 3, which are not allowed for in this procedure, this is not surprising and neither is the observation that markedly different values were obtained with mitochondrial fractions

Table 3. Kinetic parameters of the two forms of MAO in rat liver fractions

Source	Fraction	Substrate	MAO-form	k_{cat} (sec^{-1})	K_m (μM)	k_{cat}/K_m ($sec^{-1} \cdot M^{-1}$)
Sprague–Dawley	Mitochondria	5-HT	A	17 ± 1.1	84.2 ± 5.9	2.0×10^5
Sprague–Dawley	Mitochondria	PEA	B	14.7 ± 0.1	9.7 ± 0.5	1.5×10^6
Wistar	Mitochondria	5-HT	A	13.5 ± 1.0	137 ± 15	9.8×10^4
Wistar	Mitochondria	PEA	B	14.2 ± 2.1	5 ± 2	2.8×10^6
Sprague–Dawley	Microsomes	5-HT	A	13.4 ± 1.6	106 ± 62	1.3×10^5
Sprague–Dawley	Microsomes	PEA	B	9.8 ± 1.4	7.5 ± 0.1	1.3×10^6
Wistar	Mitochondrial	5-HT	A	14.1 ± 3.2	160 ± 31	8.8×10^4
Wistar	Outer membranes	PEA	B	12.8 ± 1.9	6 ± 3	2.6×10^6

Values, shown as means ± S.E. were determined with samples that have been pretreated with low concentrations of l-deprenyl or clorgyline, for determination of MAO-A or MAO-B activities, respectively, as described in the text.

Table 4. Apparent monoamine oxidase concentrations determined in rat liver fractions by titration of the activity with irreversible inhibitors

Source	Fraction	Enzyme form	Titration value (pmol MAO/mg protein)			
			Clorgyline	l-Deprenyl	Pargyline	J-508
Sprague–Dawley	Mitochondria	A	8.8 ± 0.5	—	—	—
Sprague–Dawley	Mitochondria	B	—	31.1 ± 4.8	42.9 ± 1.7	—
Sprague–Dawley	Microsomes	A	5.7 ± 0.2	—	—	—
Sprague–Dawley	Microsomes	B	—	17.9 ± 2.0	24.8 ± 0.2	—
Wistar	Mitochondria	A	31.0 ± 2	—	—	—
Wistar	Mitochondria	B	—	14.4 ± 0.7	—	12.7 ± 0.1
Wistar	Mitochondrial	A	44.0 ± 6	—	—	—
Wistar	Outer membranes	B	—	—	—	47.0 ± 3.0

Activities were determined with 5-hydroxytryptamine or 2-phenethylamine for MAO-A and MAO-B, respectively.

prepared in different ways and from different breeds of rats. These results indicate that the activity titration method will not yield meaningful values for the monoamine oxidase content of rat liver mitochondria or mitochondrial outer membranes.

Acknowledgements—A.M.O'C. and K.F.T. are grateful to the Medical Research Council of Ireland for a grant in support of part of this work. N.G. and M.U. thank CAICYT for a grant (No. 1195/81) in support of part of this work.

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