Biochemical Pharmacology, Vol. 37, No. 18, pp. 3407–3413, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00
Pergamon Press plc

A COMPARATIVE STUDY OF SOME KINETIC AND MOLECULAR PROPERTIES OF MICROSOMAL AND MITOCHONDRIAL MONOAMINE OXIDASE

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(*Received* 4 *January* 1988; *accepted* 11 *March* 1988)

Abstract—This experimental work tries to characterize the monoamine oxidase of microsomal origin through its kinetic and molecular properties, and to establish a comparative study with the enzyme present in rat liver mitochondria.

The temperature effect upon this catalytic activity was examined and similar behaviour of MAO A and MAO B between both cellular fractions was found. The study of the pH dependence of initial velocity showed similar results both in mitochondria and in microsomes. The FAD cofactor is covalently attached to the MAO of microsomal origin. The FAD containing subunits corresponding to MAO A and MAO B, previous binding of the enzyme with [^{3}H]pargyline and posterior SDS electrophoresis and fluorography, showed molecular weights of 65,900 and 62,400, respectively, in both cellular fractions.

The inhibition curves with clorgyline, deprenyl, semicarbazide and KCN, measuring the remaining activity towards 1 μM of benzilamine, indicated that in mitochondria 5% of the total activity is due to the presence of SSAO activity whereas in microsomes this activity represents about 20%.

From all these results it appears that mitochondrial and microsomal MAO are related enzymes, although further structural studies are necessary to confirm their possible identity.

Monoamine oxidase (MAO) [monoamine : O_2 oxidoreductase (deaminating) (flavin-containing) EC1.4.3.4] plays an important role in the oxidative deamination of biogenic amines, some of which have a neurotransmitter function.

This enzyme is present in many animal tissues and there are two forms of it, which differ in their substrate specificities and inhibitor sensitivities. MAO A is sensitive to inhibition by nanomolar concentrations of the acetylenic inhibitor clorgyline [1] and active towards 5-HT, whereas MAO B is sensitive to nanomolar concentrations of 1-deprenyl [2] and active towards benzylamine and β-phenethylamine as substrates.

Although MAO activity is generally considered to be located in the outer mitochondrial membrane [3, 4], variable amounts of MAO enzyme have been reported to be associated with microsomes [5–9].

In rat liver homogenates, two-thirds of the enzyme activity are present in mitochondria the remainder being included in the microsomal fraction [6, 10].

The close association of MAO with norepinephrine storage particles in some innervated tissues [5], the reduction of the microsomal MAO after denervation, and the difficult separation between them by subcellular fractionation, have thrown some doubts about the actual origin of this microsomal MAO.

On the basis of the different rate of activity recovery in the microsomal and the mitochondrial fraction after administration of irreversible inhibitors, some authors [11–13] have suggested the former could be a precursor of the latter.

At present a serious study on the characterization of MAO activity of microsomal origin has not yet been realized, and consequently this enzyme activity is still open to controversy.

Previous work carried out on MAO activity of microsomal origin [10, 14] indicated a similar kinetic behaviour towards PEA and 5-HT as substrate compared to that in mitochondria. With the aim of further characterization of this microsomal MAO activity, we decided to study its kinetic behaviour as a function of temperature and pH, the possible presence of MAO with the FAD cofactor attached non-covalently, the molecular weight of the subunits containing FAD cofactor corresponding to MAO A and B forms, and the presence of other amino oxidases. The corresponding comparative study was established with mitochondrial MAO activity.

MATERIALS AND METHODS

Subcellular fractionation. Rat liver mitochondria were obtained from 250 g Sprague–Dawley rats fasted overnight. Livers were homogenized (1:10, w/v) in 50 mM potassium phosphate buffer, pH 7.2, containing 0.25 M sucrose. The resultant homogenate was filtered through gauze and centrifuged at 600 *g* for 10 min. The supernatant was centrifuged again under the same conditions and the resultant supernatant was centrifuged at 8000 *g* for 10 min. The resulting pellet containing mitochondria was washed twice, resuspended to a protein concentration of 8 mg/ml in 50 mM potassium phosphate buffer pH 7.2 and stored a −20°. The resulting supernatant was centrifuged at 20,000 *g* for 10 min, the

pellet was discarded and the supernatant was then centrifuged at 200,000 *g* for 30 min in order to sediment the microsomal fraction. This fraction was resuspended in 50 mM potassium phosphate buffer, pH 7.2, to give a final concentration of 8 mg/ml and stored at −20°.

Monoamine oxidase assay. MAO activity was determined radiochemically by a modification [15] of the method of Otsuka and Kobayashi [16], with either 100 μM [^{14}C]-5-nydroxytryptamine (500 μCi/mmol) or 20 μM [^{14}C]-2-phenylethylamine (2.5 mCi/mmol) as substrates. At such concentrations these amines have been shown to behave as essentially specific substrates for the A and B forms of MAO, respectively. The reaction took place in a final volume of 225 μl of 50 mM potassium phosphate buffer, pH 7.2, containing 100–200 μg of enzyme and was stopped by the addition of 100 μl of 2 M citric acid. The products were extracted into toluene:ethyl acetate 1:1 (v/v) containing 0.6% (w/v) PPO and the radioactivity was measured in a scintillation counter.

Kinetic constants at different temperatures were determined from studies of the effects of substrate concentration on the initial velocity using 5-HT or PEA for MAO A or B, respectively, in preparations where the activity of the other form had been inhibited by preincubation with 0.3 μM of 1-deprenyl or clorgyline. These concentrations were found to inhibit the activity of one form of the enzyme completely without significantly affecting the activity of the other [15]. Thus, to each volume of samples containing 7–10 mg of protein the same volume of 100 mM potassium phosphate buffer, pH 7.2, and half volume of clorgyline or 1-deprenyl (a final concentration of 0.3 μM) were added, the mixture being incubated for 2 hr at 37°. The incubation mixtures were then centrifuged at 200,000 *g* for 30 min, and the corresponding pellets resuspended in the initial volume of 50 mM potassium phosphate buffer, pH 7.2. The substrate concentration range used in the kinetics studies was 5–1000 μM for 5-HT and 2.5–50 μM for PEA.

As a consequence of the lack of information about the substrate specificity of MAO activity at different pH, each form of MAO was previously inhibited as described above in order to study separately MAO A and MAO B activities at different pH values. In this case, the final pellets were resuspended in distilled water in order to avoid pH interferences. The different buffers used in this assay were 50 mM potassium phosphate (pH 5–8.5) and 50 mM glycine (pH 9–12).

In order to check the irreversible effect of pH 5 and 12 on MAO activity samples of enzyme (20 μl) maintained at this pH values for 30 min at 37° were added to 180 μl of 50 mM potassium phosphate buffer the activity being subsequently assayed as above.

Inhibition curves with clorgyline, deprenyl, semicarbazide and KCN were carried out by incubating samples of enzyme at different inhibitor concentrations for 30 min at 37°. After this period, the remaining activity was determined towards 1 μM [^{14}C]benzylamine (10 μCi/μmol) as substrate.

Polyacrylamide gel electrophoresis (PAGE–SDS) and fluorography. A modification of the Laemmli method [17] was used for the electrophoresis of [^{3}H]pargyline labelled monoamine oxidase preparation in polyacrylamide gels containing sodium dodecyl sulphate.

MT and MC fractions (480–580 μg per sample) were incubated in the presence of 1.30 μM [^{3}H]pargyline (13 Ci/mmol) in a final volume of 500 μl of 50 mM potassium phosphate buffer, pH 7.5, for 60 min at 37° in a shaking water bath. Some samples were preincubated with 0.3 μM clorgyline or deprenyl for 60 min at 37° before the addition of [^{3}H]pargyline to selectively inhibit either MAO A or B.

Following pargyline binding, samples were diluted with 4 ml of 50 mM potassium phosphate buffer, pH 7.2, and centrifuged twice at 100,000 *g* for 60 min at 4°. Supernatants were discarded and the radiolabelled pellets were resuspended in 156 μl of the loading solution which contained SDS (2%, w/v), Tris–HCl (24.9 μM, pH 6.8), glycerol (0.1%), bromophenol blue (0.0012%) and dithiothreitol (0.1 M). These samples were boiled for 2 min at 100° and 100 μl of them were applied to the gel.

Standard (M_r) markers used were: phosphorylase B (M_r = 92,500), bovine serum albumin (M_r = 66,200), ovoalbumin (M_r = 45,000), carbonic anhydrase (M_r = 31,000) and soybean trypsin inhibitor (M_r = 21,500).

Electrophoresis was performed in a running gel (11.8 × 18 × 0.15 cm) with acrylamide (12.5%), methylene bis-acrylamide (0.155%), Tris–HCl (0.375 M, pH 8.7), and a stacking gel (4.2 × 18 × 0.15 cm) containing acrylamide (5%), methylene bis-acrylamide (0.15%), and Tris–HCl (0.143 M, pH 6.8). Both gels contained SDS (0.1%), ammonium persulphate (3.33%), and *N,N,N′,N′*-tetraethylmethylenediamine (3.3%).

Electrophoresis was carried out at 45 V (constant voltage) and at 15° until the tracer dye had reached the bottom of the running gel (approx. 20 hr). Gels were fixed in methanol:acetic acid:water (40:20:40) (v/v/v) and stained for protein with a solution of Coomassie brilliant blue G-250 (0.25%) in methanol:acetic acid:water (40:20:40) and destained with methanol:acetic acid:water (10:7:83) and then dried. Gels were subsequently prepared for fluorography by the method of Bonner and Laskey [18]. The dried gels were exposed to preflashed Kodak make RP-X7 film by the method of Laskey and Mills [19] for 3 weeks at −80°.

Protein assay. Protein concentration was determined by the Hartree method [20] with bovine serum albumin as standard.

Materials. 5-Hydroxy (side-chain-2-^{14}C) tryptamine-creatinine sulphate and [7–^{14}C]benzylamine hydrochloride, were purchased from Amersham International (Amersham, U.K.); phenylethylamine hydrochloride-(ethyl-1-^{14}C) and [^{3}H]-pargyline-(phenyl-3,benzyl-^{3}H) hydrochloride were obtained from New England Nuclear (Boston, MA). Acrylamide and *N,N′*-bismethylene acrylamide were purchased from Eastman Kodak (F.R.G.). *N,N,N′,N′*-Tetraethyl-methylenediamine was obtained from Sigma (St Louis, MO). All other compounds were standard, analytical-grade laboratory reagents.

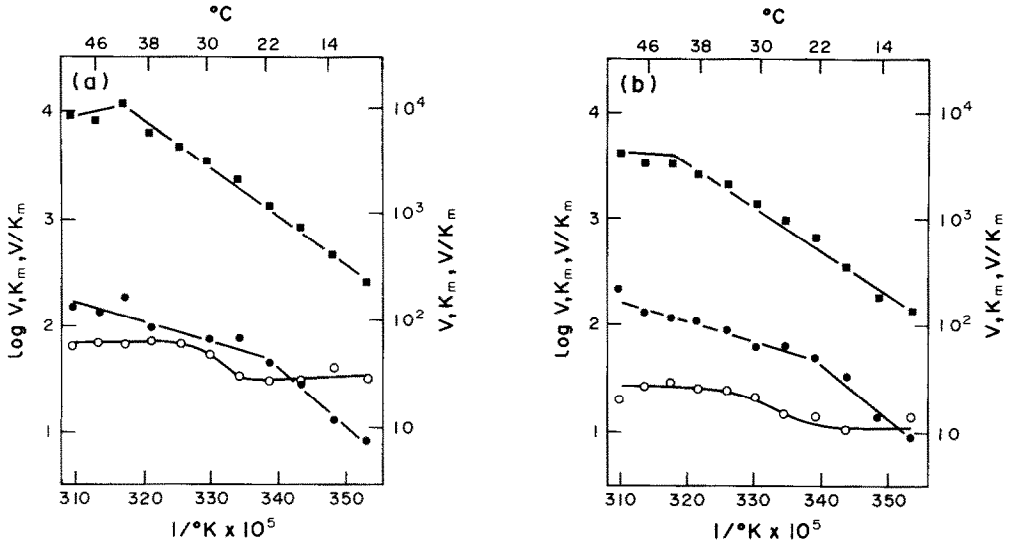

Fig. 1. Effect of temperature on MAO A activity towards 5-HT as substrate in (a) mitochondria and (b) microsomes. Samples were previously incubated with 0.3 μM 1-deprenyl to inhibit the MAO B form: ■, V; ●, K_m; ○, V/K_m.

RESULTS

Temperature-dependence of MAO activity

Arrhenius plots for MAO activity present in mitochondrial and microsomal fractions towards 5-HT and PEA, are given in Figs. 1 and 2 respectively.

In the case of MAO A (Fig. 1), the Arrhenius plot for V values was linear in the interval of 10–42°, and above this the linearity was lost. The corresponding increments of enthalpies, free energies and enthropies of activation, at 30° were calculated for both fractions (Table 1). Nevertheless, V values are higher for mitochondrial MAO, as a result of the greater number of molecules of this enzyme since the k_{cat} value is the same in both fractions [14]. When log K_m values were plotted versus $1/T$, a similar non-linear representation was obtained in both fractions.

A transition temperature at 22° was detected and the corresponding standard increments of enthalpy, free energy and entropy were calculated (Table 2). As a consequence of the different V values in mitochondria and microsomes some differences are observed in V/K_m results in spite of their K_m values. The complexity of the corresponding graphical representation does not allow to calculate the thermodynamic parameters of the catalytic reaction at low substrate concentration. In the case of MAO B (Fig. 2), the Arrhenius plots for V values were linear in the interval of 10–40° and the linearity was lost above 40°. The corresponding thermodynamic parameters

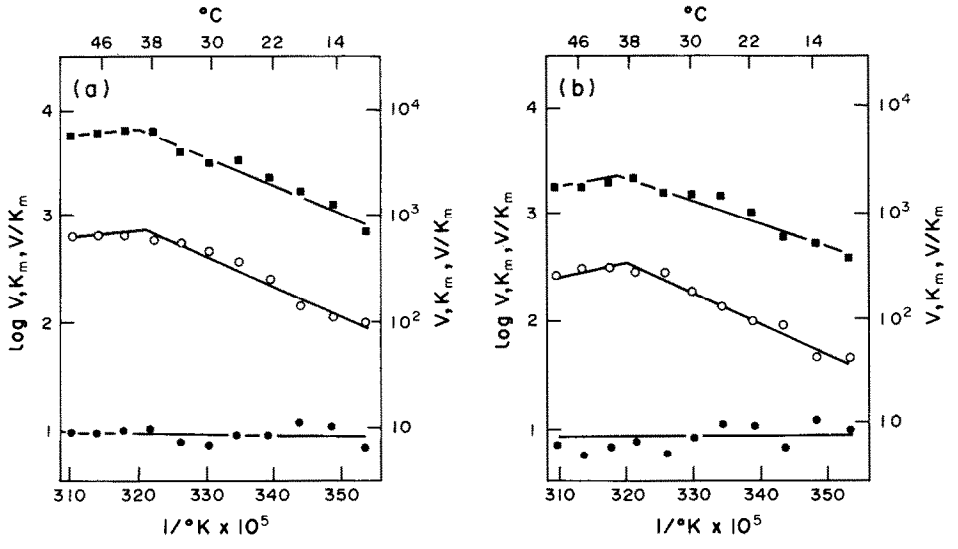

Fig. 2. Effect of temperature on MAO B activity towards PEA as substrate in (a) mitochondria and (b) microsomes. Samples were previously incubated with 0.3 μM clorgyline to inhibit the MAO B form: ■, V; ●, K_m; ○, V/K_m.

Table 1. Thermodynamic parameters calculated from the temperature-dependence of V and V/K_m of MAO activity

			ΔH (cal/mol)	ΔG (cal/mol)	ΔS (cal)/mol.°K)
MAO A	V	MT	21266	16261	16
		MC	18868	16208	9
	V	MT	12172	16324	−14
		MC	11628	16127	−15
MAO B	V/K_m	MT	13072	9218	13
		MC	13850	9081	16

are listed in Table 1. Nevertheless, V values were higher for mitochondrial MAO B for the same reasons described above in the case of MAO A. When log K_m values were plotted vs $1/T$, a linear horizontal representation was obtained in the 10–50° temperature range for both fractions. The graphical representation of the apparent constant of pseudo-first order in front of $1/T$ was non-linear with a transition temperature of 40°. The differences observed in both fractions are a consequence of the different V values. The values of the corresponding thermodynamic parameters are given in Table 1.

Effect of pH on MAO activity

The variation of initial velocity for MAO A and B forms measured with 5-HT and PEA respectively at different pH values is represented in Figs 3 and 4. In mitochondrial as well as in microsomal fraction both forms of MAO exhibit a similar behaviour with an optimum at pH 9. The activity at this pH was taken as 100% of activity. Both above and below the optimum pH MAO activity decreased until being completely abolished at pH 5 and 12.

Table 2. Thermodynamic parameters calculated from the temperature-dependence of K_m of MAO A activity

		ΔH (cal/mol)	ΔG (cal/mol)	ΔS (cal/mol.°K)
K_m	MT	−24677	1896	−91
(10–22°)	MC	−23870	2036	−89
K_m	MT	−8023	2536	−35
(22–50°)	MC	−8550	2489	−36

It has been reported [21, 22] that in some tissues the kinetic constants of MAO activity towards several substrates do not change in the pH range of 6.5–9, and that the real substrate for this enzyme is the non-protonated form of the amine [22–24]. Based upon it, theoretical initial velocities were calculated from the non-protonated amine concentration present in the reaction mixture at each pH studied considering that V and K_m values did not change with pH (these values were taken from Ref. 14). The non-protonated amine concentration at each pH value was calculated by the method of McEwen [23], using the following modification of the Henderson Hasselbach equation

$$[A'] = \frac{[A]}{1 + \text{antilog}\,(pK_a - pH)}$$

where $[A]$ is the total amine concentration, $[A']$ is the concentration of non-protonated amine, and pK_a is 9.8 for both substrates.

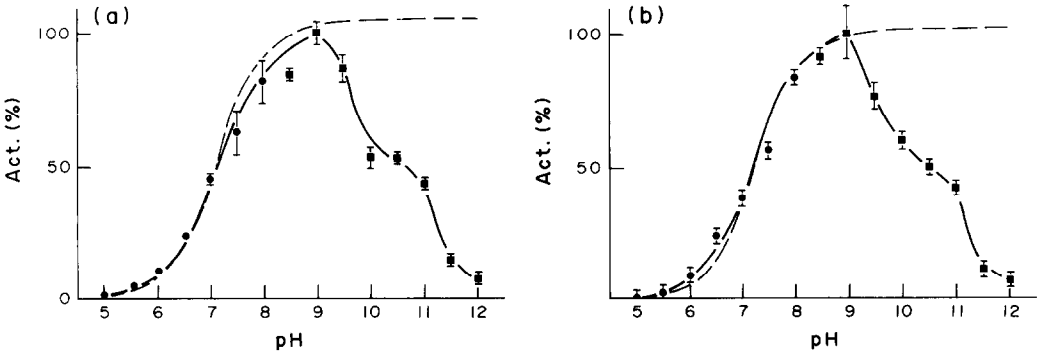

Fig. 3. Effect of different pH on initial velocities (continuous line) of MAO A towards 5-HT as substrate; (a) mitochondria and (b) microsomes. Before activity determination B form was inhibited with 0.3 μM of 1-deprenyl as indicated in the text. The initial velocities at pH 8.5 and 9 were determined with both buffers and not changes was observed. Buffers: 50 mM potassium phosphate (●) and 50 mM glycine (■). The discontinuous line corresponded to the theoretically calculated values such as indicated in the text.

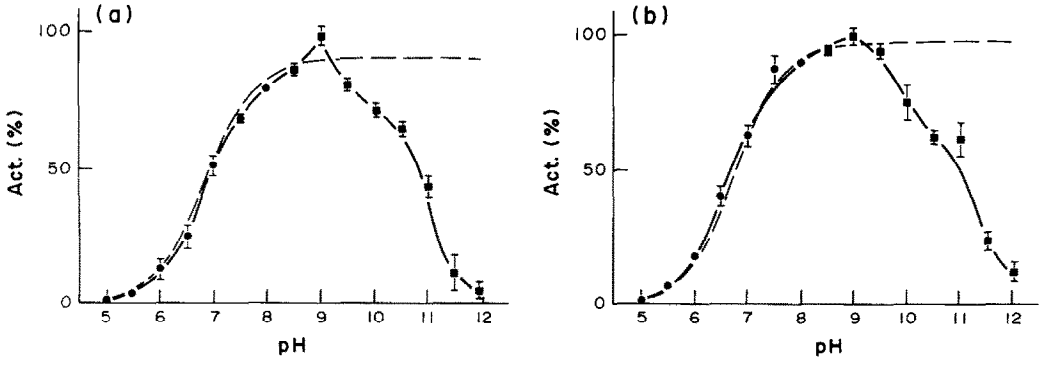

Fig. 4. Effect of different pH on initial velocities (continuous line) of MAO B towards PEA as substrate: (a) mitochondria and (b) microsomes. Before activity determination A form was inhibited with 0.3 μM of clorgyline as indicated in the text. The initial velocities at pH 8.5 and 9 were determined with both buffers and no change was observed. Buffers: 50 mM potassium phosphate (●) and 50 mM glycine (■). The discontinuous line corresponded to the theoretically calculated values such as indicated in the text.

To compare theoretical and experimental curves, the former was drawn considering the activity at pH 7 as the same activity percentage obtained experimentally. Both curves were coincident between a pH range of 5–9 for MAO A and B in the two subcellular fractions studied.

When samples were incubated at pH 5 or 12 for 30 min and then assayed at pH 7.2, the pH effect on MAO activity was reversible in the former case and irreversible in the later (data not shown).

Presence of dissociating or non-dissociating FAD in microsomal MAO

Mitochondrial monoamine oxidase is a protein covalently bound to the cofactor FAD [25]. If the enzyme from microsomal origin is not covalently bound to FAD, then the activity would probably decrease during the subcellular fractionation as a consequence of the dilution-induced cofactor dissociation.

In order to check this hypothesis, the microsomal enzyme was incubated at different FAD concentrations (0–1 mM) and the activity was subsequently assayed towards PEA and 5-HT as substrates. The results showed that microsomal MAO activity does not increase after incubation in the presence of FAD (data not shown).

In order to confirm that microsomal MAO is covalently associated to FAD, a dialysis was carried out for 6 hr against 50 mM potassium phosphate buffer pH 7.2, and the activity measured towards PEA and 5-HT as substrates. Dialysis did not modify the results (data not shown) thus confirming the covalent nature of the link between enzyme and cofactor.

SDS-PAGE and fluorography

In order to find out whether the molecular structure of microsomal MAO was similar to or different from that from the mitochondrial fraction, SDS electrophoresis of samples labelled with [^{3}H]pargyline and subsequent fluorography were carried out. The results obtained are shown in Fig. 5. It can be noticed that in lanes 1 and 6 there is only one band corresponding to MAO A present in mitochondria and microsomes respectively. Lanes 2 and 5 show one band corresponding to MAO B in mitochondria and microsomes respectively, lanes 3 and 4 represent total MAO activity present in both fractions. The M_r values of MAO A and MAO B were 65,900 and 62,400, respectively. These results are similar in both fractions and the molecular weight is that corresponding to the FAD-containing subunit.

Presence of semicarbazide sensitive amine oxidase (SSAO)

The effect of different clorgyline, deprenyl, semicarbazide and KCN concentrations on MAO activity towards 1 μM benzylamine as substrate was examined. This benzylamine concentration was selected because it seems to be a specific substrate of SSAO activity in rat heart [26]. Results obtained are shown in Fig. 6.

When using clorgyline or deprenyl, a simple sigmoidal curve was observed suggesting that benzylamine is metabolized preferentially by MAO B. Nevertheless, there was still a residual activity at 1 mM, which might be due to a plasmatic benzylamine oxidase contamination or to an SSAO activity. In order to discard this latter explanation, incubations were carried out in the presence of different concentrations of KCN (specific inhibitor of plasmatic benzylamine oxidase) and semicarbazide (specific inhibitor of SSAO activity). No inhibition was observed after KCN incubation, indicating the absence of plasmatic benzylamine oxidase. In the inhibition curves with semicarbazide, a partial inactivation was observed at inhibitor concentrations around 1 mM. The inhibited activity is of the same order as the residual activity obtained after incubation with 1 mM clorgyline or deprenyl. From these results it can be concluded that the presence of the SSAO activity is about 5% in the mitochondrial and 20% in the microsomal ones.

DISCUSSION

The variation of the rate constant with the temperature obeys Arrhenius law. In the case of an

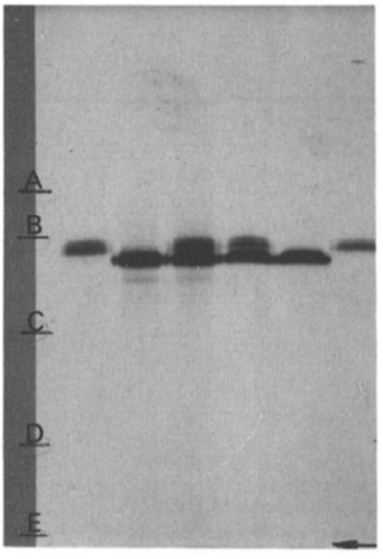

Fig. 5. Fluorography of SDS polyacrylamide gel of [^{3}H]pargyline labelled mitochondrial and microsomal MAO. Both preparations were labelled as described in the text. Results are shown for mitochondria: MAO A (track 1), MAO B (track 2), MAO A + MAO B (track 3), and for microsomes: MAO A + MAO B (track 4), MAO B (track 5) and MAO A (track 6). M_r values were determined by comparison with the mobility of standard proteins: phosphorylase B (92,500), bovine serum albumin (66,200), ovoalbumin (45,000), carbonic anhydrase (31,000) and soybean trypsin inhibitor (21,500); the positions of which (labelled A, B, C, D and E, respectively) were determined by protein-staining of the gel prior to fluorography. The arrow indicates the position of the dye front.

enzymic reaction, the study of the temperature dependence, can lead to important mechanistic information, although in this type of reaction, the situation becomes more complex. This is because in the majority of cases K_m and V values are the result of different individual rate constants and do not obey Arrhenius law, unless an individual rate constant predominates over all the other [27].

As monoamine oxidase activity presents a ping pong mechanism [28] it is impossible to calculate the individual rate constants [29]. There are two main reasons for the non-linear Arrhenius plots. The more common of these is that there is a change in rate-limiting step. Enzyme reactions consist of a number of individual steps each described by rate constants with the corresponding energies of activation. When temperature is altered a change in the rate-limiting step can occur, and Arrhenius plots present two straight lines joined by a curve with higher activation energies dominating at low temperatures. In such a case the non-linearity is due to purely kinetic phenomena. Other reasons can be a temperature-induced conformational change in the enzyme, or a phase change of the medium or both.

In the special situation of a membrane-bound enzyme, a phase change of lipids could be a plausible explanation for the breaks in the Arrhenius plots.

In the study of the effect of temperature on MAO activity, more factors could be taken into account in the interpretation of the Arrhenius plots. For example, solubility of O_2 in aqueous media is altered with temperature, and as oxygen is the second substrate, altered K_m and V values can be found. Although ionization of potassium phosphate does not change with temperature, the concentration of the ionized amines can be altered with temperature and consequently this could be reflected in a change of the kinetic constants.

Denaturation of the enzyme by temperature can be excluded because in this study, velocity was measured at short intervals of time where the reaction is linear.

From the present study it is impossible to determine whether breaks in Arrhenius plots are due to kinetic changes or to a change in the lipid phase. However, they allow us to conclude that both enzymes, mitochondrial and microsomal, have a similar behaviour respect to temperature.

The study of the pH dependence of MAO activity showed a similar behaviour in both subcellular fractions. In the interval of pH from 5 to 9, activities observed in both cases coincide with the theoretical values which means that this behaviour is just a consequence of the presence of non-protonated amine of the substrate and thus the kinetic constants would not be altered in this pH interval. These results are in agreement to those reported by Huszti and Williams [21, 22].

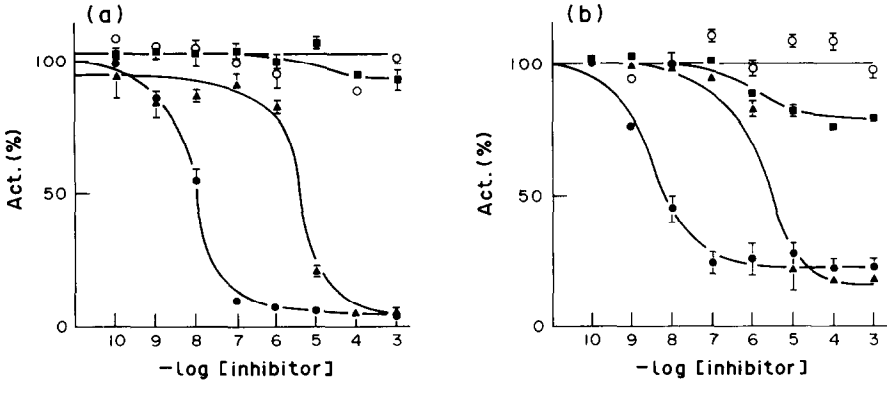

Fig. 6. The effect of clorgyline (▲), deprenyl (●), semicarbazide (■) and KCN (○) towards 1 μM of benzylamine as substrate: (a) mitochondria and (b) microsomes. Each point represents the mean ± SEM of four determinations. Experiments were carried out as is described in Materials and Methods.

In the interval of pH from 9 to 12, a decrease of enzymic velocity was observed. This behaviour cannot be explained if velocity does only depend on concentration of non-protonated amine. There are two possible explanations for these results: either the enzyme becomes ionized with the consequent alteration of kinetic parameters or there is an irreversible inactivation of the enzymic activity. The latter hypothesis is the most plausible in the present study, because if MAO is exposed at pH 12 for 30 min and the assay is subsequently carried out at pH 7.2, the activity was not recovered.

In the case of MAO B, the decrease of activity at this pH, could be due to high PEA concentration [30] depending on the pH and the period of incubation of the enzyme with the substrate. In order to minimize this effect, in the present study, samples were incubated for short periods of time, and a high protein concentration was used. Above pH 9, concentration of non-protonated amine does not increase and, consequently, decrease of MAO activity above that pH is mainly due to the pH effect on the ionized state of the enzyme. In any case, MAO activity present in mitochondria and microsomes shows the same behaviour in respect to pH.

SDS electrophoresis and fluorography prior to binding with [^{3}H]pargyline showed that, MAO A and MAO B had similar M_r values from both subcellular fractions.

Results obtained after incubation of the microsomal MAO in presence of different FAD concentrations and those obtained after dialysis, showed that the FAD cofactor could be attached covalently to the microsomal MAO.

Nevertheless this conclusion is definitively confirmed by the bands obtained after electrophoresis in denaturing conditions, which indicated that FAD is covalently bound to the microsomal subunit. On the other hand, if FAD cofactor was not covalently attached to the enzyme, this group would have been removed after drastic treatment with SDS, and the [^{3}H]pargyline–FAD complex would have run with the dye front.

The inhibition curves with clorgyline, deprenyl, semicarbazide and KCN that measure the remaining activity towards benzylamine 1 μM, indicated that in mitochondria 5% of the total activity is due to the presence of SSAO whereas in microsomes this activity represents about 20%.

From all these results it appears that mitochondrial and microsomal MAO activities correspond to related enzymes, although further structural studies are necessary to confirm their possible identity.

Acknowledgements—The authors would like to express their thanks to Professor C. M. Cuchillo for his help in the review of this manuscript.

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