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MONOAMINE OXIDASE ACTIVITIES IN LYMPHOCYTES AND GRANULOCYTES TAKEN FROM PIG BLOOD

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Abstract—The characterisation of monoamine oxidase activities in lymphocytes and granulocytes was studied using cells prepared from pig blood. The specific activities against β-phenylethylamine, benzylamine, tyramine and 5-hydroxytryptamine as substrates in granulocytes (G) were approximately twice those found in lymphocytes (L).

The absence of the semicarbazide-sensitive amine oxidase (SSAO) was confirmed by insensitivity of the latter to semicarbazide as inhibitor with benzylamine as substrate. MAO activity present in (G) and (L) was selectively inhibited by low deprenyl concentrations; this fact, in addition to the simple sigmoid inhibition curves obtained with increasing concentrations of clorgyline with tyramine as substrate, suggests that the MAO activity present both in (G) and (L) is predominantly of the MAO-B form. The absence of any contamination with plasma amine oxidase (EC 1.4.3.6) was confirmed by the fact that activity towards benzylamine (Bz) was insensitive to KCN-induced inhibition.

Kinetic constants were determined for each fraction towards β-phenylethylamine (PEA) and Bz as substrates.

MAO-B was titrated with unlabelled pargyline, deprenyl and [^{3}H]-pargyline; the corresponding K_{cat} values, turnover number and the active concentrations were then determined. The molecular weight of MAO-B present in both cellular fractions was calculated by SDS-electrophoresis and fluorography, after reaction with [^{3}H]-pargyline. Some of these results are compared with those obtained with human blood leucocytes.

Monoamine oxidase (MAO) [monoamine: O_2 oxidoreductase (deaminating) (flavine-containing) EC 1.4.3.4] is responsible for the metabolism of biogenic amines in the peripheral and central nervous system and is therefore important in the etiology of affective disorders. This enzyme is present in many animal tissues [1–3] and has two forms, MAO-A and MAO-B, differing both in their response towards the acetylenic inhibitors clorgyline and deprenyl [4], as well as in their substrate specificities [5, 6].

Clinical investigations of MAO in blood samples from humans have been restricted to platelets, with few studies reported for human white cells [7–9]. Low MAO activity in lymphocytes has been reported to be correlated with low activity in platelets in schizophrenic patients [9].

Thus, a study of white cells might allow some relationships among low MAO activities, abnormal immunological mechanisms and affective disorders to be established [9]. The scope for such clinical investigation of MAO levels would be enlarged by characterisation of the amine oxidase activity present in white cells.

The aims of this work were to characterise the MAO activity in lymphocytes and granulocytes taken from pig blood, in terms of specific activities, sensitivity towards acetylenic inhibitors, kinetic constants, molecular concentrations, and the molecular weight of its subunits, and then to compare these results with those found in human white cells.

MATERIALS AND METHODS

Separation of blood leucocytes. Leucocytes were separated from the same blood sample by the Pegueroles method [10]. The procedure used is summarised in Fig. 1. Venous blood from a human donor (400 ml) and from male pigs (2000 ml), with EDTA (100 mg/ml) as anticoagulant, was mixed with 5% dextran, 0.9% NaCl (4 parts of blood +1 part of the ery-

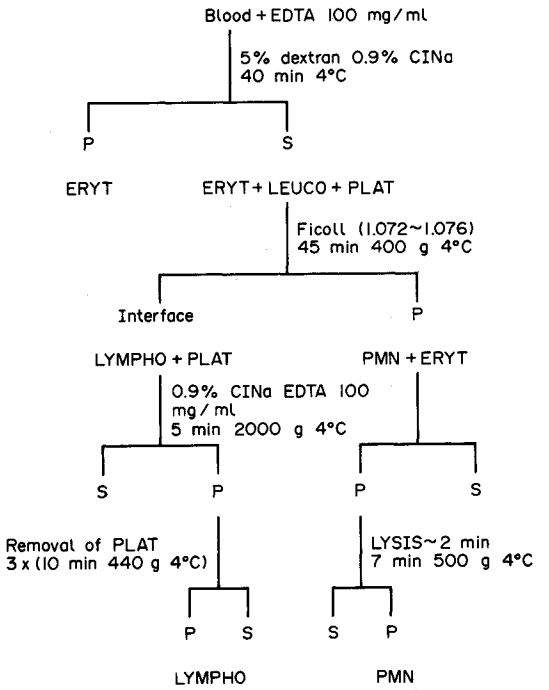

Fig. 1. Separation of blood lymphocytes and granulocytes by the Pegueroles procedure [10].

throcyte-aggregating agent). After mixing, the red cells were allowed to settle for 40 min in an ice-cooled plastic container, and the leucocyte-rich plasma was then collected. One-hundred-and-sixty ml of this fraction were carefully layered into 120 ml of the Ficoll-urografin density fluid ($d = 1.072$–1.076) in a plastic tube (6 × 11 cm). After centrifugation at 400 *g* for 45 min at 4°, lymphocytes appeared as a narrow white layer immediately above the interface, and granulocytes and a few contaminating erythrocytes appeared at the bottom of the tube.

After removing the granulocyte fraction and washing it once with 0.9% NaCl, 1 mg/ml EDTA pH 7.4 via suspension and centrifugation (2000 *g* for 5 min), the pelleted cells (granulocytes and erythrocytes) were resuspended in 0.3% NaCl, 1 mg/ml EDTA, pH 7.4, and incubated for 2 min at 4°. Hypotonic lysis was stopped by the addition of an equal volume of 1.5% NaCl, 1 mg/ml EDTA, pH 7.4. The cells were then centrifuged for 7 min at 500 *g* and the supernatant removed.

Lymphocytes were removed and washed two or three times to free them from platelets, via suspension in 0.9% NaCl, 1 mg/ml EDTA, pH 7.4, and centrifugation at 440 *g* for 10 min at 4° in a total volume of 30 ml.

Cells were counted in a Coulter counter model ZB1 coupled to a multiple channel analyser and also in a Neubauer chamber. They were then resuspended in 50 mM phosphate buffer, pH 7.2, and stored in aliquots at −80°. Before each assay, the preparations were sonicated at low frequency for 30 sec.

Monoamine oxidase assay. Monoamine oxidase activity was determined radiochemically by a modification [11] of the method of Otsuka and Kobayashi [12] with (100 μM) 5-hydroxytryptamine (5-HT), (20 μM) 2-phenethylamine (PEA), (1 μM and 1 mM) benzylamine (Bz) or (100 μM) tyramine (TYR) as substrate in a final volume of 225 μl of phosphate buffer (50 mM, pH 7.2) at 37°, containing 6–7 × 10^6 cells for each assay. The reaction was terminated by the addition of 100 μl 2 M citric acid and the products were extracted into (1:1, v/v) containing 0.6% (w/v) toluene/ethyl-acetate/PPO for scintillation counting.

[^{3}H]-*Pargyline binding assay.* Inhibitor binding studies with [^{3}H]-pargyline were performed by filtration assay, in which 6–7 × 10^6 cells of each fraction were incubated for 60 min at 37° in potassium phosphate buffer (0.05 M pH 7.2) with [^{3}H]-pargyline (3.2 Ci/mmol) in concentrations ranging from 25 to 300 pmol. The total incubation volume was 0.1 ml. 4 ml of ice-cold potassium phosphate buffer (50 mM, pH 7.2) were then added and the mixtures were filtered under vacuum through Whatman GF-C filter papers. The filters were then washed twice 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.

Preincubation of the samples with clorgyline (10^{-3} M) completely inhibited the activity of the enzyme and was used as a method for determining nonspecific binding. Samples, taken through the same procedure but with the inhibitor replaced by water, were used for the total binding determination.

Polyacrilamide gel electrophoresis. A modification of the Laemli method [13] was used for the electrophoresis of [^{3}H]-pargyline labelled monoamine oxidase preparation in polyacrylamide gels containing sodium dodecyl sulphate.

L and G preparations (100 μg of protein = 13 × 10^6 cells per sample) were incubated with 0.7 nmol of [^{3}H]-pargyline (13 Ci/mmol) in a final volume of 500 μl of Tris–HCl (50 mM, pH 7.5) for 60 min at 37° in a shaking water bath.

Following pargyline binding, samples were centrifuged twice at 100,000 *g* for 60 min at 4°. Supernatants were discarded and the radiolabelled pellets were resuspended in 150 μl of a loading solution containing SDS (2%, w/v), Tris–HCl (2.49 × 10^{-5} M, pH 6.8), glycerol (0.1%), Bromophenol blue (0.0012%), and dithiothreitol (DTT) (0.1 M), and these samples were solubilised by heating at 100° for 2 min.

Standard molecular weight markers used were: bovine serum albumin, ovalbumin, carbonic anhydrase and trypsin inhibitor.

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 [14]. The dried gels were exposed to preflashed Kodak mafe RP-x7 film by the method of Laskey and Mills [15] for 3 weeks at −80°.

MATERIALS

5-Hydroxy (side-chain-2-^{14}C) Tryptamine-creatinine sulphate, was 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 (Germany). *N,N,N,N*-Tetraethylmethylenediamine was obtained from Sigma. All other compounds were standard, analytical-grade laboratory reagents.

RESULTS

Isolation of lymphocytes (L) and granulocytes (G)

L and G were separated by Ficoll-urografin density gradient centrifugation by the Pegueroles method [10]. The purity of both fractions was checked with the Coulter-counter and by microscopy after Turk-

Table 1. Monoamine oxidase specific activities in G and L fractions taken from pig blood expressed in pmol/min 10^6 cells

	Specific activities			
	PEA	TYR	Bz	5-HT
G	15.82 ± 1.8	9.21 ± 0.007	3.73 ± 0.48	0.330
L	7.54 ± 2.6	4.81 ± 1.39	1.90 ± 0.11	0.138

pmol/min.10^6 cells.
Each value is the mean ± SEM of four experiments.

stain, and shown to be about 90–95% in both cases. The platelet contamination was negligible in both cases.

All the results presented in this work were referred to 10^6 cells because of the lack of correlation between protein content and the amount of cells, resulting, in turn, from aggregation problems and residual erythrocyte contamination. The absence of amine oxidase activity in erythrocytes insured that there would be no problems with interference from that source.

Specific activities

Linearity of the amount of product formed with time was checked and shown to be maintained under these assay conditions for 8 min with PEA, 45 min with TYR and Bz, and 30 min with 5-HT. The linearity of the initial rate with the amount of cells was also established. The corresponding specific activities are summarised in Table 1. It can be seen that the enzyme in white cells is more reactive towards PEA, a specific substrate of the MAO-B form, but has a very low activity against 5-HT, a specific substrate of A form. These results would indicate the possibility that only the B form of MAO is present in both cellular preparations. The activity of the G fraction was about twice that found in the L fraction towards all substrates.

Sensitivity to clorgyline

The inhibitory effect of different clorgyline concentrations (10^{-4}–10^{-11} M) on MAO activity towards TYR, a common substrate of both MAO forms, was also studied. The results obtained are shown in Fig. 2. A simple sigmoid curve was observed and total inhibition was presented only at high clorgyline concentration (10^{-5} M).

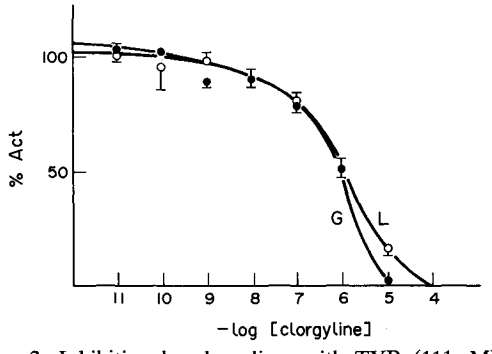

Fig. 2. Inhibition by clorgyline, with TYR (111 μM) as substrate, of amine oxidase in G (●) and L (○) fractions taken from pig blood. Cells were preincubated for 30 min at 37° with different inhibitor concentrations (10^{-4}–10^{-11} M). The remaining activity is expressed as per cent of the control activity determined in the absence of the inhibitor. Each value is the mean ± SEM of four determinations.

Presence of semicarbazide-sensitive amine oxidase (SSAO)

The effects of different deprenyl concentrations (10^{-3}–10^{-11} M) on MAO activity towards Bz (1 μM) as substrate were also studied. Activation at low inhibitor concentrations was observed in the G fraction (Fig. 3a).

In order to discard definitively the presence of SSAO activity in white cells taken from pig blood, the effects of different semicarbazide concentrations (10^{-11}–10^{-3} M) on Bz oxidation were investigated. No inhibition was observed at any semicarbazide concentration (Fig. 3a) with either 1 μM or 1 mM (data not shown).

The effects of different clorgyline concentrations (10^{-11}–10^{-3} M) on MAO activity against Bz as substrate were also studied (Fig. 3b). If any SSAO activity had been present, there would have been a residual activity towards Bz after treatment with clorgyline, but none was detected at either 1 μM or 1 mM (data not shown).

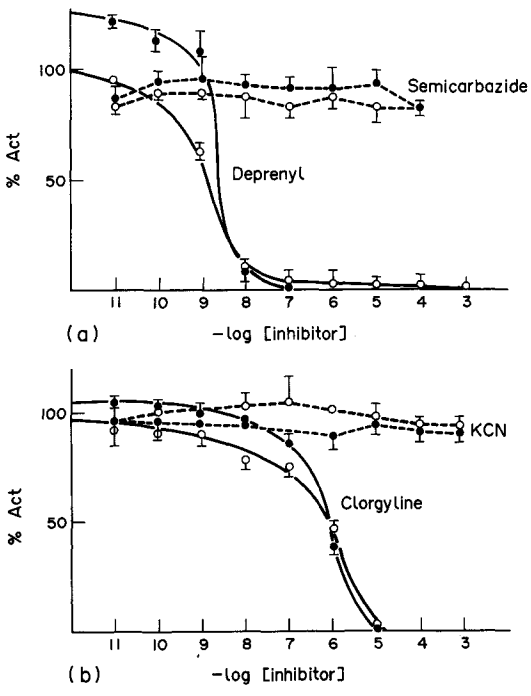

Fig. 3. (a) The effects of deprenyl and semicarbazide on the amine oxidase activities in G (●) and L (○) fractions taken from pig blood. White cells were preincubated at 37° for 30 min with either deprenyl (——) and semicarbazide (– –). The remaining activity was assayed towards 1 μM Bz as substrate and expressed as per cent of the control activity, determined in the absence of either inhibitor. Each point represents the mean ± SEM of four determinations. (b) The effects of clorgyline (——) and KCN (– –) on the amine oxidase activities in G (● and L (○) fractions taken from pig blood with 1 μM Bz as substrate. Experiments were carried out as is described above. Each value is the mean ± SEM of four determinations.

Table 2. Kinetic parameters of MAO activity in G and L fractions taken from pig blood

	PEA		Bz	
	K_m	V_{max}	K_m	V_{max}
G	6.87	25.08	87.69	72.84
L	3.28	8.76	100.43	12.25

K_m: μM.
V_{max}: pmol/min . 10^6 cells.
MAO activity was assayed using five different concentrations of PEA (2.5, 5, 10, 15 and 20 μM) and Bz (10, 15, 25, 50 and 100 μM), and expressed as pmols/min 10^6 cells. Correlation coefficients of each linear regression of Lineweaver–Burk plots were higher than 0.999.

Presence of AO (EC 1.4.3.6)

The absence of amine oxidase (EC 1.4.3.6) contamination from plasma was demonstrated by the lack of inhibition by different KCN concentrations (10^{-4}–10^{-11} M) with Bz as substrate (Fig. 3b).

Determination of kinetic parameters

The K_m and V_{max} values of MAO activity present in the G and L fractions towards Bz and PEA as substrates were estimated from Lineweaver–Burk plots and the results obtained are summarised in Table 2.

Titration of MAO activity

When the G and L samples were incubated with 300 pmol of [^{3}H]-pargyline for different periods of time, the binding process was time-dependent, reaching a plateau at 60 min for the G fraction and 30 min for the L fraction (data not shown).

Binding curves, obtained when samples were incubated at different [^{3}H]-pargyline concentrations, are shown in Fig. 4a, which shows both the total and non-specific binding as well as the difference between them.

From this figure it is possible to calculate the specifically bound pmol/10^6 cells of [^{3}H]-pargyline specifically bound and consequently, the number of active centers in each case (Table 3). The enzyme concentration was also estimated by titration with deprenyl. Total inhibition was obtained with 40 nM deprenyl in the case of the G and 23 nM in the case of the L fraction (Fig. 4b). The corresponding enzyme concentration was calculated from this graph and these results, together with those calculated from titration with unlabelled pargyline, are summarised in Table 3.

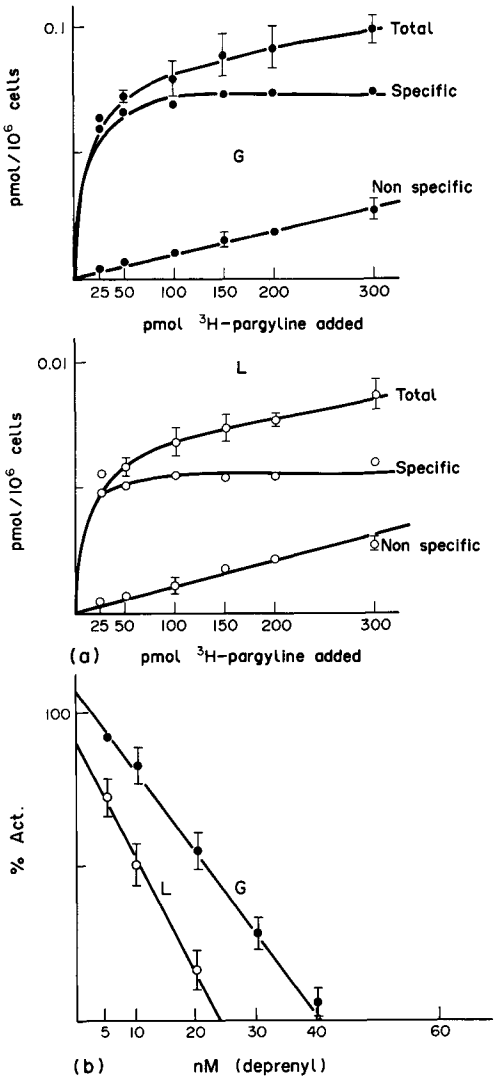

Fig. 4. (a) Binding of [^{3}H]-pargyline to G (●) and L (○) fractions taken from pig blood. The binding of radioactively labelled pargyline was determined by the filtration assay as described in the text. Values shown are the mean ± SEM of triplicate determinations. Specific binding to G and L monoamine oxidase was defined as the difference between the total binding and the non-specific binding curves. (b) Titration of G (●) and L (○) MAO with deprenyl. White cells were preincubated for 3 hr at 37° with different concentrations of deprenyl before the assay for activity with 20 μM PEA. Points are the mean ± SEM of four assays. Values are expressed as the percentage of activity remaining with respect to control samples, which were incubated with phosphate buffer (50 mM pH 7.2) instead of with the inhibitor. Regression lines of best fit are shown.

Table 3. MAO concentration in G and L fractions taken from pig blood determined by titration of the activity with irreversible inhibitors and by the binding to [^{3}H]-pargyline

	Deprenyl (pmol/10^6 cells)	Pargyline (pmol/10^6 cells)	^{3}H-Pargyline (pmol/10^6 cells)	K_{cat} (s^{-1})		K_{cat}/K_m (s^{-1} μM^{-1})		Turnover number	
				PEA	BZ	PEA	BZ	PEA	BZ
G	1.92	1.24	0.072	5.8	16.8	0.84	0.19	384	1010
L	1.34	0.94	0.017	8.6	11.9	2.6	0.12	516	716

Each value is the mean ± SEM of four experiments.

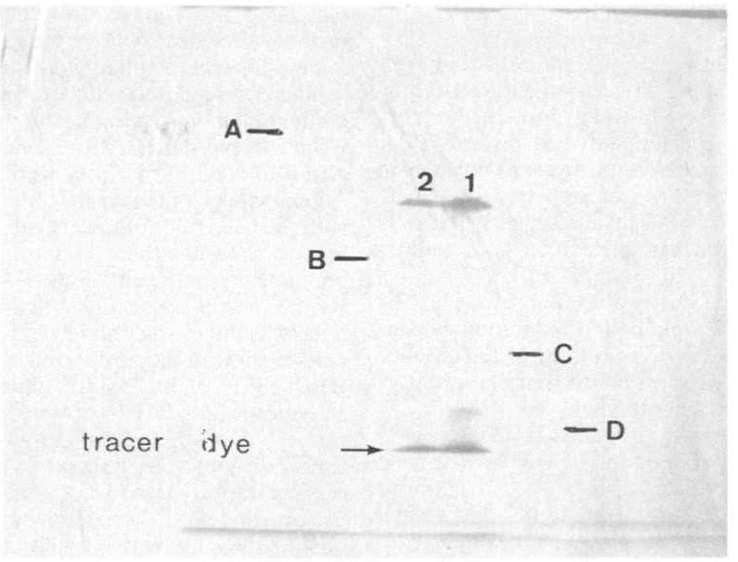

Fig. 5. Fluorography of SDS polyacrylamide gel of [^{3}H]-pargyline-labelled MAO. G and L MAO was labelled as described in the text. Results are shown for G (track 1) and L (track 2). Molecular weights were determined by comparison with the mobility of standard proteins: bovine serum albumin (67,000), ovalbumin (45,000), carbonic anhydrase (31,000), and trypsin inhibitor (21,000); the positions of which (labelled A, B, C, and D, respectively) were determined by protein-staining of the gel prior to fluorography. The arrow indicates the position of the dye front.

SDS-electrophoresis and fluorography

In order to show whether the MAO enzyme found in the G fraction was similar to or different from that in the L fraction, at the molecular level, both SDS electrophoresis of a sample labelled with [^{3}H]-pargyline as well as subsequent fluorography were carried out. The results obtained are shown in Fig. 5. The same molecular weight: 59,800 was calculated for the only pargyline binding subunit present in the G and L fractions, by comparison with different protein markers.

MAO activity in the L and G fractions taken from human blood

In order to compare the results obtained from pig blood with those in humans, a single sample of 400 ml of human blood was utilised to obtain the G and L fractions as described above [10]. Specific activities were calculated towards Bz, PEA, 5-HT and TYR as substrates, and these preliminary results are summarised in Table 4. The activity in the L fraction against PEA and TYR as substrates was higher than that in the G fraction. No activity was, however, detected with 5-HT as substrate in either preparation, because it was below the detection limit of the assay method used. Lymphocytes presented some activity towards 1 μM Bz, whereas no activity was detected in the case of granulocytes.

In order to confirm the presence of only one form of MAO and the absence of SSAO activity, inhibitory curves with different concentrations (10^{-3}–10^{-11} M) of semicarbazide against Bz (1 μM) as substrate, and of clorgyline and deprenyl towards TYR as substrate, where determined for both cell preparations. The results obtained are also summarised in Table 4. In the experiments with clorgyline towards TYR as substrate, a unimodal curve was obtained which showed a total inhibition at 10^{-4} M, the activity being preserved at 10^{-7} M. This low value for the activity calculated from linearity is due probably to the lability of the enzyme because of the long incubation period.

An inhibitory unimodal curve was also obtained with deprenyl, showing total inhibition at 10^{-7} M concentration. In the case of semicarbazide, no inhibition was detected at any inhibitor concentration.

Table 4. Specific activities of MAO present in G and L fractions taken from human blood

	PEA	5-HT	TYR	Bz	Bz + 10^{-3} Sc	TYR + DEP (10^{-7} M)	TYR + CLOR (10^{-7} M)
G	0.29	ND	0.32	ND	ND	0	0.23
L	0.98	ND	0.76	0.47	0.35	0	0.4

In the case of Bz, specific activity was determined in the absence (−) and presence (+) of semicarbazide (10^{-3} M), whereas TYR deamination was calculated in the absence (−) and presence (+) of deprenyl (10^{-7} M) and clorgyline (10^{-7} M) after preincubation of the enzyme with the inhibitor for 30 min at 37°. Specific activities are expressed in pmol/min 10^6 cells.

DISCUSSION

MAO activity present in granulocytes and lymphocytes taken from pig blood was characterised by the affinities for three substrates and susceptibility to inhibition. The specific activities towards PEA and Bz were, in the G fraction, twice as much as in the L fraction. Only very low activities towards 5-HT were detected. These results suggest that only the B form of MAO was present in both cellular fractions.

According to the criteria of Johnston [4], the simple sigmoid curve obtained for inhibition by clorgyline with TYR as substrate confirmed the absence of MAO A, which is sensitive to inhibition by low concentrations of this compound.

Thus, MAO in lymphocytes and granulocytes consists predominantly of one form of MAO resembling in terms of its substrates specificity and inhibitor sensitivity, the MAO B form found in other animal tissues.

SSAO activity is sensitive to inhibition by low semicarbazide concentrations, whereas MAO activity is not affected. The fact that activity against Bz (tested at 1 μM and 1 mM), common substrate for MAO and SSAO enzymes, remained constant after incubation with semicarbazide demonstrated the total absence of semicarbazide-sensitive amine oxidase activity in the G and L fractions. The absence of any significant clorgyline-resistant activity towards this substrate also supported this conclusion. The absence of SSAO activity is also confirmed by the lack of deviation in the Lineweaver–Burk plots, which indicated that only one enzymatic form was present.

The lack of inhibition by KCN, with Bz as substrate, showed that no contamination with plasma amine oxidase (EC 1.4.3.6.) was present.

The kinetic parameters summarised in Table 2 show that the V_{max} value in the G fraction was three and six times that found in the L fraction with PEA and Bz, respectively.

The K_m values for each substrate were similar in both cell preparations, with that towards PEA being considerably lower than the value for Bz.

The acetylenic inhibitors pargyline and deprenyl were utilized to titrate the amount of MAO present in the G and L fractions, since they react stoichiometrically with the covalently-bound flavine group in the enzyme in a 1:1 ratio [16, 17].

There were differences between the total amounts of MAO determined in both cell preparations: those obtained by titration with unlabelled deprenyl and pargyline, and those obtained by binding assay with [^{3}H]-pargyline (Table 3). In the former case the non-specific binding is not controlled, and this is the reason for the fact that the results do not coincide with those using labelled pargyline [18]. Despite the different molecular concentrations of MAO in the G and L fractions, the molecular turnover numbers and K_{cat} values (Table 3) suggest that their activities are similar.

The results described above suggest that the MAO forms present in the G and L fractions taken from pig blood are similar. In order to examine this further, SDS-electrophoresis and fluorography prior to binding with [^{3}H]-pargyline were performed. The results obtained showed both activities to contain pargyline-binding subunits of the same molecular weight. Nevertheless, more analysis would be necessary to confirm that they were identical.

Comparison of the results obtained using pig blood with the behaviour of the enzyme taken from human blood showed that human lymphocytes and granulocytes both contain the B form of MAO only. Neither MAO A nor SSAO activities were detected in any cellular fraction. These results are not in agreement with those reported by Bond *et al.* [7] and Banchelli *et al.* [8], where some SSAO and some activity towards 5-HT were present. The reason for this could be that these authors utilised different concentrations of Bz and protein as well as a different cell separation method.

Nevertheless, these preliminary results are obtained from only one human blood sample. Care must be taken with the final interpretation due to the possible variability present. At this moment we are carrying out additional experiments in order to reach conclusive results.

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