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Electrophoretic Characterization of Monoamine Oxidase by [^{3}H]Pargyline Binding in Rat Hepatoma Cells with A and B Activity

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SUMMARY

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[^{3}H]Pargyline bound to active sites of both A and B types of monoamine oxidase (MAO) activity in a clonal line of rat hepatoma cells. Crude mitochondrial preparations were incubated with this drug, washed and solubilized, then electrophoresis was carried out in sodium dodecyl sulfate (SDS) polyacrylamide gels. At [^{3}H]pargyline concentrations up to 2 nmoles/mg mitochondrial protein (0.8 μM), a single major protein species of molecular weight 57,000 was identified by autoradiography. The dose-dependent inhibition of labeling of this protein band by clorgyline and deprenyl was consistent with the presence of both A and B sites of MAO. Moreover, both A and B types of activity were inhibited under conditions giving saturated binding of [^{3}H]pargyline to the 57,000 dalton protein. Our results support the hypothesis that MAO activity resides in one protein or several proteins of similar molecular weight.

INTRODUCTION

Monoamine oxidase (MAO, monoamine: O_2 oxidoreductase EC 1.4.3.4)[2] deaminates biogenic amines throughout the body. This enzyme is located in the outer mitochondrial membrane (1) and depends on a covalently bound flavin cofactor for activity (2, 3). Its affinity for different substrates varies widely among species and in various tissues within a species. A classification of enzyme activity has been adopted based on substrate specificity and drug sensitivity (4, 5). The A type of MAO preferentially deaminates 5-hydroxytryptamine (serotonin) and is blocked by low concentrations of clorgyline (4). The B type of MAO has a high affinity for phenylethylamine (6) and is inhibited by low concentrations of deprenyl (7). Some amines such as tryptamine and tyramine are substrates for both types of activity (6). Some drugs such as pargyline can block both types of activity. This drug is slightly more effective against the B type activity (5). Studies using highly purified preparations of MAO from bovine kidney have shown that [^{14}C]pargyline inhibits the enzyme stoichiometrically and irreversibly through formation of a stable adduct with the flavin residue (8). This reaction did not occur between [^{14}C]pargyline and purified preparations of other flavin-containing enzymes (8). [^{14}C]Pargyline has been used also to specifically label MAO in partially

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[2] The abbreviations used are: MAO, monoamine oxidase; SDS, sodium dodecyl sulfate; NEN, New England Nuclear.

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purified enzyme and crude mitochondrial preparations from several tissues (9, 10).

In spite of extensive research, the molecular basis of differences between the A and B types of MAO activity is still not clear (11). Some workers have suggested that both types of activity are due to a single enzyme species with different active sites (12) or with varying allotopic properties conferred by the membrane environment (13, 14). Others have postulated two distinct enzyme species (15–17). Because MAO is tightly bound to the membrane, its solubilization and purification bring considerable loss of activity and alter its kinetic properties (14, 18). Earlier studies on the electrophoretic characterization of partially solubilized MAO revealed several large molecular weight protein bands (>100,000 daltons) which retained enzymatic activity (19–21). Subsequently, with the use of the adequate solubilization procedures it has been shown that highly purified preparations of catalytically active enzyme contain 1 mole of flavin per 120,000 g protein (2, 13). The minimal molecular weight estimates for completely solubilized MAO range from 55,000 to 63,000, as determined by gel filtration, analytical centrifugation and SDS-polyacrylamide electrophoresis (10, 17). However, the kinetic properties of this purified enzyme are different from those of the source tissue and suggest that the purified enzyme activity is solely of the B type.

We have reported previously that cultured rat hepatoma cells of line MH_1C_1 have both types of MAO activity, about 70% type A and 30% type B, as judged by the dose-dependent inhibition of deamination of A and B substrates by clorgyline and deprenyl (22, 23). The types of MAO activity present in crude mitochondrial preparations of these cells are similar to those in living cells (23). In the present study [^{3}H]pargyline has been used as a radioactive probe to characterize A and B types of MAO in crude mitochondrial preparations of MH_1C_1 cells by SDS polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Cell culture and harvesting. Rat hepatoma line MH_1C_1 was obtained from the American Type Culture Collection (Cat #CCL144). Cells were grown as monolayers on plastic tissue culture dishes (Falcon plastics) in Dulbecco's modified Eagle's medium (DMEM, #H21, Grand Island Biologicals) supplemented with 10% fetal calf serum (Flow) without antibiotics. Cultures were maintained at 37° in a humidified atmosphere of 5% CO_2 and 95% air. Cells were fed every 3–4 days and subcultured (1:5 ratio) at confluency following treatment with 1X pancreatin (Grand Island Biologicals) for 3 min at 37° and resuspension with gentle trituration, as described (22).

Cultures were harvested at confluency. Monolayers were rinsed 3 times with isotonic phosphate buffered saline and scraped off the dish with a teflon-coated straight edge into a small volume of this buffer (5–10 mg protein/ml), as described (22). Cell suspensions were frozen immediately on dry ice and stored in the vapor phase of liquid nitrogen (−70°).

Isolation of crude mitochondrial preparation. Frozen cell suspensions were thawed and maintained at 0–4° throughout the isolation procedure. Cells were collected by centrifugation at 1,160 × *g* for 5 min and resuspended in 10 ml of swelling buffer (1.5 mM calcium chloride, 10 mM sodium chloride and 10 mM Tris-HCl, pH 7.5) per g wet weight of cells. The suspension was held for 30 min and then homogenized using a Potter-Elvehjem glass homogenizer (15–20 strokes). Next the tonicity of the suspension was increased by adding one seventh volume of 7× Tris-EDTA-Sucrose (TES) buffer (2 M sucrose, 35 mM EDTA, 50 mM Tris-HCl, pH 7.5). Unbroken cells and nuclei were collected by centrifugation at 1,160 × *g* for 5 min. The pellet was resuspended in 1× TES buffer and the centrifugation repeated. The two supernatants were pooled and centrifuged at 8,100 × *g* for 15 min. The pellet containing the crude mitochondrial preparation was resuspended in 50 mM Tris-HCl, pH 7.5 (2–3 mg protein/ml).

Binding and electrophoretic characterization of mitochondrial proteins using [^{3}H]pargyline. Freshly prepared crude mitochondrial preparations (0.4 mg protein/

ml, final concentration) were incubated for 60 min at 37° with varying concentrations of [benzylmethylene ^{3}H]-pargyline (6.86 Ci/mmole, NEN) in 50 mM Tris-HCl, pH 7.5, as previously described (9). When unlabeled inhibitors, clorgyline (Dr. Sabit Gabay, V.A. Hospital, Boston, MA) and deprenyl (Prof. J. Knoll, Sammelweis University of Medicine, Budapest, Hungary) were used, mitochondrial preparations were preincubated with them for 30 min at 37° prior to addition of [^{3}H]pargyline. At the end of the binding incubation the samples were centrifuged at 100,000 × *g* for 60 min at 4°; the pellets were washed by resuspending in 1 ml 50 mM Tris-HCl, pH 7.5 and the centrifugation was repeated. The pellet was resuspended in 50 μl of a solution containing 3% SDS, 0.15 M sucrose, 1% β mercaptoethanol, 0.05% bromophenol blue and 0.03 M Tris-HCl pH 7.5, and proteins were solubilized by boiling for 3 min, as described (24).

Polyacrylamide slab gel electrophoresis was performed in 7.5% acrylamide (7.3% acrylamide, 0.2% methylene bis acrylamide) and 0.1% SDS using a modification (24) of the procedure of Maizel (25). Gels were stained for protein with a solution containing 0.1% Coomassie blue R, 25% 2-propanol and 10% acetic acid; destained in a solution containing 10% 2-propanol and 10% acetic acid; and held for several days in 10% acetic acid, as described (26). Gels were then treated for fluorography (27), dried and exposed to preflashed Kodak X-Omatic R film for 1–3 weeks at −70°. Quantitative distribution of the radioactivity in the gels was determined using a modification of the method of Horvitz (28), as recommended by New England Nuclear. The area of the gel to be analyzed was located by superimposition of the autoradiogram. Individual lanes were sliced into 2–3 mm lengths, each slice was placed in a glass scintillation vial and allowed to swell in 100 μl of distilled water for 1 hr at room temperature. Then 10 ml of a solution containing 3% Protosol (NEN) and 7.5% Liquifluor (NEN) in toluene was added, and samples were incubated overnight at 37°.

The following protein standards were used for molecular weight determinations: phosphorylase, bovine albumin, glutamic acid dehydrogenase, ovalbumin, trypsin (Sigma) and catalase (Calbiochem.). Standards were run in separate lanes concomitantly with samples.

Assays. Monoamine oxidase activity was measured in crude mitochondrial preparations containing 0.4 mg protein/ml, after preincubation with various concentrations of [^{3}H]pargyline for 60 min at 37°. Activity was measured against 5-hydroxy [side chain 2-^{14}C]tryptamine creatinine sulfate (52 mCi/mmole, Amersham/Searle), β[ethyl-2^{14}C]phenylethylamine HCl (48.25 mCi/mmole, NEN) and [2-^{14}C]tryptamine bisuccinate (49.6 mCi/mmole, NEN) using a modification of the method of Groshong *et al.* (29). Reaction mixtures (100 μl total volume) contained 50 mM potassium phosphate buffer, pH 7.4 and 100 μM [^{14}C]5-hydroxytryptamine (10.4 μCi/μmole), 44 μM [^{14}C]phenylethylamine (20.7 μCi/μmole) or 50 μM [^{14}C]tryptamine (10 μCi/μmole). Incubations were carried out at 37° for 15 min and reactions were terminated by addition of 200 μl 2N HCl. Deaminated products were extracted into 1 ml of ethylacetate:benzene (1:1) for 5-hydroxytryptamine, toluene for phenylethylamine, or ether for tryptamine.

Activity against [1-^{14}C]tyramine HCl (56.2 mCi/mmole, NEN) was measured by the method of Wurtman and Axelrod (30). The reaction mixture (100 μl total volume) contained 100 mM potassium phosphate buffer, pH 7.4, and 37.8 μM [^{14}C]tyramine (26.5 μCi/μmole). Incubations were carried out and reactions terminated as above. Deaminated products were extracted into 4 ml of toluene:liquifluor (25:1). Assays were linear with respect to time and protein for all substrates. Substrates were not at saturating concentrations.

Protein concentrations were determined by a modification of the method of Lowry *et al.* (31) using bovine serum albumin as standard. Radioactivity was measured by liquid scintillation spectrometry.

RESULTS

Crude mitochondrial preparations of MH$_1$C$_1$ cells were incubated with increasing amounts of [^{3}H]pargyline and washed to

remove unbound label. The proteins were then solubilized and subjected to SDS-polyacrylamide slab gel electrophoresis. The distribution of proteins and radioactivity are shown in Fig. 1. When the concentration of [^{3}H]pargyline in the incubation mixture was between 0.375 and 1.275 nmoles/mg mitochondrial protein (0.15–0.51 μM), only a single labeled band was detected by autoradiography. This band coincided with a distinct band darkly stained by Coomassie blue. It had a molecular weight of 57,000 daltons, as estimated in linear 7.5% acrylamide gels by the migration of known molecular weight proteins. At concentrations above 3.75 nmoles [^{3}H]pargyline/mg protein, several other labeled bands appeared throughout the gels, these bands increased in density proportionally with increasing concentrations of drug. The degree of binding of [^{3}H]pargyline to both mitochondria and the 57,000 dalton protein band was highest at concentrations less than 0.5 nmoles/mg protein (Fig. 2A and B). With increasing concentrations of [^{3}H]pargyline the binding to this protein band approached saturation at 2–4 nmoles/mg protein. However, the total amount of label bound to mitochondrial preparations continued to increase up to the highest concentration tested, i.e., 7.3 nmoles/mg protein.

The specificity of labeling of the 57,000 dalton protein with [^{3}H]pargyline was tested by preincubating mitochondrial preparations with varying concentrations of clorgyline and deprenyl. These drugs also bind irreversibly to MAO (12), apparently to the same site as pargyline (8, 13). [^{3}H]Pargyline labeling of the 57,000 dalton protein was blocked in a dose-dependent manner with both inhibitors (Fig. 3). Blockage of binding with clorgyline appeared to be biphasic, with about 70% inhibition at 10 nM and 90% at 0.1 mM. Blockage of binding with deprenyl was monophasic with 80% inhibition at 0.1 mM. (A monophasic pattern of deprenyl inhibition was also seen against enzyme activity in homogenates [*in vitro*], mitochondrial preparations and living MH_1C_1 cells [22, 23].) The labeling of the other protein bands was not affected by 0.1 mM clorgyline or 1 mM deprenyl, but was slightly decreased by 1 mM clorgyline (data not shown).

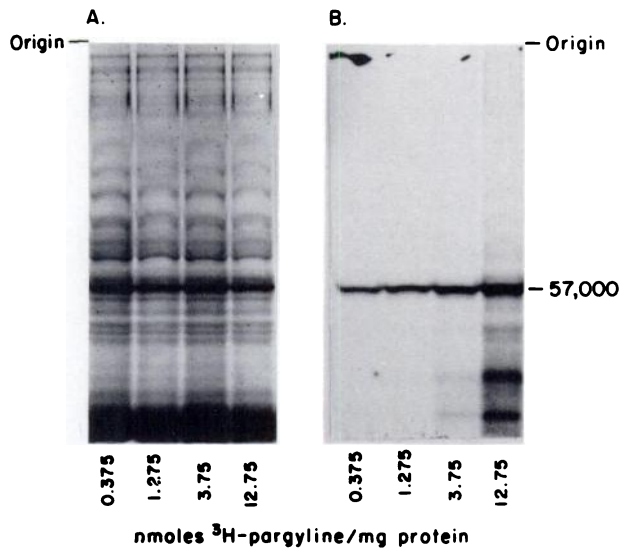

FIG. 1. *SDS-polyacrylamide slab gel electrophoresis of [^{3}H]pargyline bound to crude mitochondrial preparations*

Suspensions of crude mitochondria were incubated with increasing amounts of [^{3}H]pargyline (0.375–12.75 nmoles/mg protein, 0.15–5.1 μM) for 60 min at 37° and then washed. Proteins were solubilized and electrophoresis was carried out as described in MATERIALS AND METHODS. A. Coomassie blue banding pattern. B. Autoradiogram. Gel was treated for fluorography and exposed to pre-flashed X-Omatic R film for 10 days at −70°.

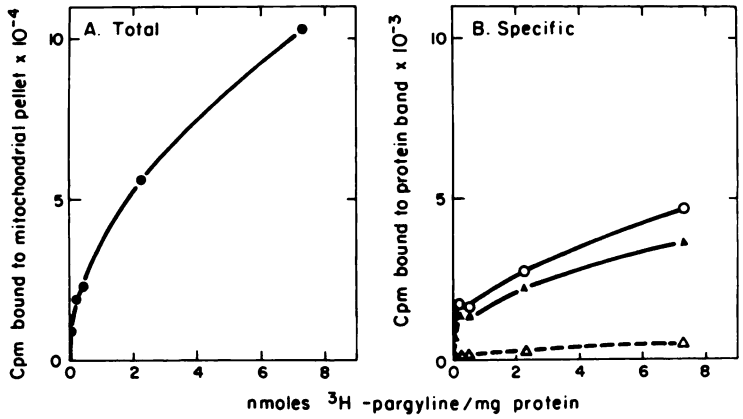

FIG. 2. *Binding of [³H]pargyline to mitochondrial preparations and the 57,000 dalton protein*

Suspensions of crude mitochondria (0.4 mg protein/ml in 50 mM Tris-HCl, pH 7.5) were incubated with increasing amounts of [³H]pargyline (0.02-2.92 μM) and the samples prepared for SDS-polyacrylamide gel electrophoresis as described in the legend to Fig. 1. A. Radioactivity was measured in aliquots of solubilized mitochondria. B. Electrophoresis of the remaining solubilized mitochondria was carried out on SDS-polyacrylamide gels. The gels were then treated for fluorography, dried and exposed to X-Omatic R film for 12 days at −70°. The specific labeled band was located, sliced, solubilized and counted. 1) (○——○) cpm in the gel slice corresponding to the specifically labeled band; 2) (△- - -△) cpm in a gel slice that did not produce an image on the X-ray film (used as background), located 8 mm ahead of the specifically labeled band; 3) (▲——▲) difference between cpm in (1) and (2). Pretreatment of mitochondria with 0.1 mM clorgyline blocked 93% of binding of [³H]pargyline to the specifically labeled band (7.3 nmoles/mg protein). Results shown are from one of two similar experiments.

Specific [³H]pargyline binding was also correlated with the loss of both types of activity in crude mitochondrial preparations of MH_1C_1 cells (Fig. 4). Four nmoles of [³H]pargyline/mg protein (saturating for 57,000 dalton protein) blocked 92% of activity against phenylethylamine, 80% against tyramine and 45% against both 5-hydroxytryptamine and tryptamine. [³H]Pargyline at 22 nmoles/mg protein inhibited 80% of 5-hydroxytryptamine deamination.

DISCUSSION

These studies show that in crude mitochondrial preparations from rat hepatoma cells inhibition of A and B types of MAO activity correlates with binding of [³H]pargyline to a 57,000 dalton protein species, as identified by SDS-polyacrylamide gel electrophoresis. Using outer mitochondrial membranes from rat liver, McCauley (9) also found that [¹⁴C]pargyline bound to a single protein species with a molecular weight of around 60,000. In our studies [³H]pargyline binding to both forms of the enzyme was established by using selective substrates and inhibitors. Concentrations of [³H]pargyline which saturated binding to the 57,000 dalton protein also inhibited deamination of 5-hydroxytryptamine phenylethylamine, tryptamine and tyramine in crude mitochondrial preparations. Partial inhibition of serotonin and tryptamine deamination by [³H]pargyline at these concentrations suggests that, under our binding conditions, only a fraction of the total A sites may be labeled. However this may constitute a significant portion of the total labeled enzyme, due to the predominance of the A type of activity in this preparation. Further, binding to the 57,000 dalton protein was blocked selectively by both A and B inhibitors. The dose-dependent inhibition by clorgyline and deprenyl closely resembled that seen for inhibition of tryptamine deamination in living MH_1C_1 cells (22) and in mitochondrial preparations (23).

As reported by others (10, 17), whenever β-mercaptoethanol was omitted during solubilization and electrophoresis, two labeled bands appeared near each other with ap-

parent molecular weights of 54,000 and 58,000 daltons (data not shown). Labeling of both bands was blocked to the same extent by clorgyline and deprenyl at two concentrations tested, 10 nM and 0.1 mM, failing to support any correlation between these bands and the distinction between A and B types of MAO activity.

In the presence of high concentrations of [^{3}H]pargyline (above 3.75 nmoles ^{3}H-pargyline/mg protein), several other protein bands were also labeled. This labeling was only partially inhibited by 1 mM clorgyline, suggesting it was not specific for MAO. It may be that pargyline, which is unstable in aqueous solutions, complexes with other membrane components. McCauley (9), using even higher concentrations of [^{14}C]pargyline, found a large amount of label migrating at the gel front. In our studies even with the lowest concentration of [^{3}H]pargyline tested (0.05 nmoles/mg protein),

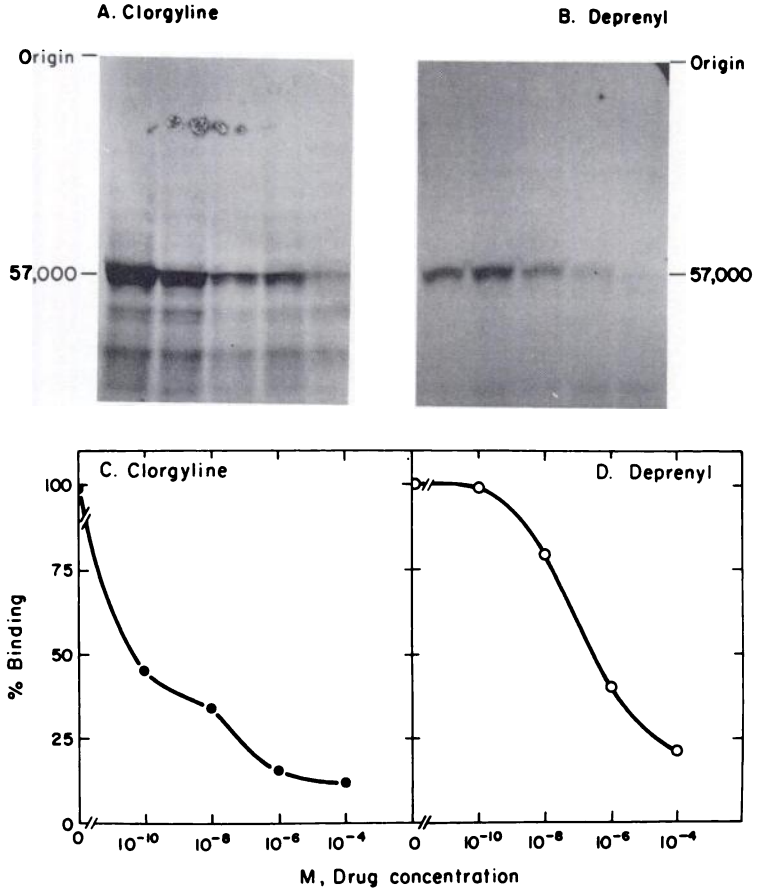

FIG. 3. *Inhibition of [^{3}H]pargyline binding to the 57,000 dalton protein by clorgyline and deprenyl*

Following pre-incubation with varying concentrations of clorgyline or deprenyl for 30 min at 37°, crude mitochondrial preparations were incubated with [^{3}H]pargyline (3.75 nmoles/mg protein, 1.5 μM). Samples were solubilized and electrophoresis was carried out on SDS-polyacrylamide slab gels. Gels were exposed to pre-flashed X-Omatic R film for 12 days at −70°. Autoradiograms of the dose-dependent inhibition of [^{3}H]pargyline binding by clorgyline and deprenyl, are shown in A and B, respectively. The radioactive content of the 57,000 dalton protein band was determined as described in legend to Fig. 2. C shows quantitation of inhibition from the gel in A; at 100% binding (no clorgyline) the 57,000 dalton protein band contained 5.7×10^3 cpm, i.e., 0.73 pmoles of [^{3}H]pargyline. D shows quantitation of inhibition from the gel in B; at 100% binding (no deprenyl) the 57,000 dalton protein band contained 5.2×10^3 cpm, i.e., 0.65 pmoles [^{3}H]pargyline. Results shown are from one of two similar experiments.

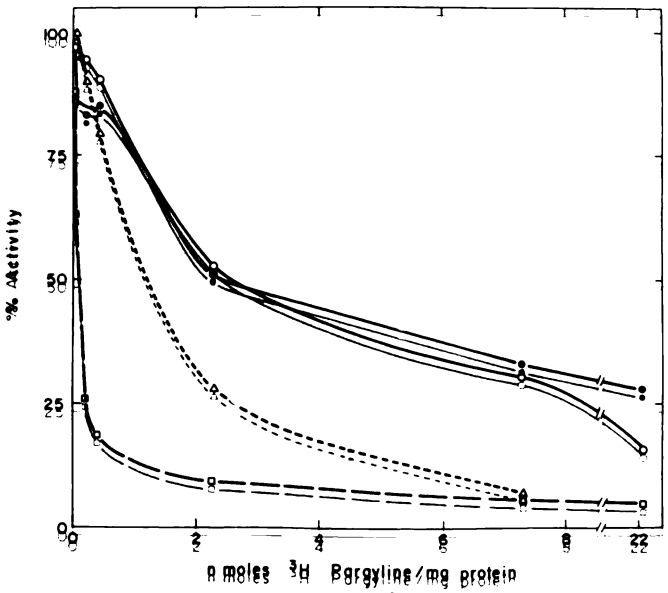

FIG. 4. *Inhibition of A and B types of MAO activity by [³H]pargyline*

Suspensions of crude mitochondrial preparations were incubated with varying concentration of [³H]pargyline (0.02–8.13 μM) under the same conditions as in binding experiments, see legend to Fig. 1. Immediately after this incubation, MAO activity was assayed as described in MATERIALS AND METHODS using the following substrates: (●—●) tryptamine; (○—○) 5-hydroxytryptamine; (□—□) phenylethylamine; (△—△) tyramine. 100% Activity represents 1.7 nmoles/min/mg protein for tryptamine, 2.5 for 5-hydroxytryptamine, 1.2 for phenylethylamine and 0.5 for tyramine. Results shown are from one of two similar experiments.

which is well below saturation for specific binding to the 57,000 dalton protein, only about 15% of the added label was bound to the mitochondria and only about 1% to the 57,000 protein band. These results contrast with 100% efficiency of binding of [¹⁴C]pargyline to highly purified preparations of MAO (8).

In conclusion, [³H]pargyline can be used to specifically identify MAO from crude mitochondrial preparations on SDS-polyacrylamide gels. The 57,000 dalton protein labeled with [³H]pargyline contains both A and B sites for MAO activity. This suggests the involvement in A and B activity of either a single protein species or different protein species with similar molecular weights.

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