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7[^{14}C]pargyline binding to mitochondrial outer membranes*,†

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Studies *in vitro* using a highly purified bovine kidney enzyme have shown that 7[^{14}C]pargyline binds irreversibly and quite probably covalently to monoamine oxidase (MAO). The bound [^{14}C]pargyline was not extracted by a number of procedures including trichloroacetic acid (TCA) washes and chloroform: methanol extraction [1]. Under appropriate conditions, the radioactive inhibitor binds in a ratio of 1 mole/mole of MAO flavin, and the [^{14}C]pargyline can be recovered bound to a flavopeptide isolated from proteolytic digests of the inhibited enzyme [2]. It has also been reported that [^{14}C]pargyline is specific for MAO in the sense that it will not bind to other purified flavoproteins [1]. This argument has been extended by Erwin and Deitrich [3] who have shown that after its administration *in vivo* radioactive pargyline is distributed among the rat liver organelles roughly in parallel with their MAO content. These data and data *in vitro* suggest at least a degree of specificity in the binding of [^{14}C]pargyline to mitochondrial MAO.

In this report, the specificity of the binding has been further investigated using the interaction *in vitro* of [^{14}C]pargyline with mitochondrial outer membranes. The outer membrane preparations were isolated from the pooled livers of several 300 g male albino rats as described by Sottocasa *et al.* [4]. 7[^{14}C]pargyline‡ (7.03 μCi/mg) was the kind gift of Dr. A. O. Geiszler of Abbott Laboratories, North Chicago, Ill.

Usually about 0.4 to 0.8 mg/ml of outer membrane protein [5] was incubated at 35° in 50 mM Tris–HCl (pH 7.5) and varying concentrations of radioactive pargyline. In some experiments, the incubation was sampled and MAO activity was estimated at room temperature using a modification of the method of Tabor *et al.* [6]. In this assay, the benzylamine concentration was 1 mM and the buffer was 50 mM Tris–HCl (pH 7.5). One unit of enzyme activity is defined as 1 nmole benzaldehyde formed/min using a mM extinction coefficient for benzaldehyde of 13.8. The binding of [^{14}C]pargyline was estimated by measuring the

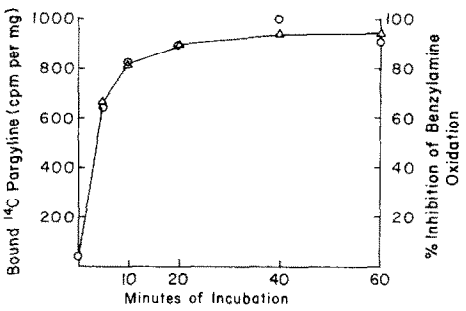

Fig. 1. Outer membranes (0.60 mg protein/ml) were incubated at 35° in 4 ml of a medium containing 50 mM Tris–HCl (pH 7.5) and 10^{-6} M 7[^{14}C]pargyline. At the indicated intervals, 0.1 ml was removed to measure the deamination of benzylamine (△—△) and 0.5 ml was removed to estimate bound radioactivity (○—○) by the TCA method (see text). Initially the specific enzymatic activity of MAO in the outer membranes was 93 units/mg of protein.

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‡ Radiochemical analysis by Abbott Laboratories showed that greater than 99.5 per cent of the radioactivity was present as pargyline.

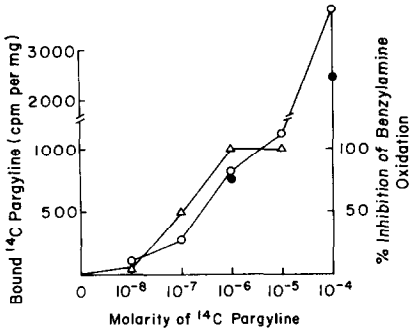

Fig. 2. Outer membranes (0.44 mg protein/ml) were incubated for 20 min at 35° in 1 ml of 50 mM Tris–HCl (pH 7.5) and the indicated concentrations of 7[^{14}C]pargyline; 0.05 ml was removed to estimate MAO (△—△) and the remainder was used to estimate bound radioactivity by the TCA procedure (○—○) or the pargyline procedure (●). The specific enzymatic activity of outer membranes incubated in the absence of inhibitor was 119 units/mg of protein.

radioactivity remaining bound after the membranes had been precipitated and then washed three times with 2 ml of 5% TCA. The final precipitate was dissolved in a small volume of 0.5 N NaOH and transferred to scintillation vials where the mixture was neutralized with 1 N HCl, prior to the addition of Aquasol (New England Nuclear Corp.). As an alternative washing procedure, the membranes were sedimented at 104,000 *g* after the incubation mixture had been brought to a final concentration of 10^{-2} M with unlabeled pargyline–HCl. These membranes were then resuspended and sedimented three more times in 2 ml of 10^{-2} M pargyline. The pargyline-washed membranes were counted directly in Aquasol. This procedure was as efficient as the TCA washes (see Fig. 2), and was used prior to the gel electrophoresis experiments. Electrophoresis with polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS) was performed essentially as described by Weber and Osborn [7] but using gels containing 0.3 g bis acrylamide/22.2 g acrylamide and a total monomer content of 7.5 per cent. Gels used to estimate the distribution of radioactivity were sliced with a wire device into approximately 1-mm slices. Two slices were incubated overnight at 35° with 10 ml of a mixture of Omnifluor-3% Protosol (New England Nuclear Corp.) in glass scintillation vials. One drop of concentrated acetic acid was added prior to scintillation counting. Radioactivity was estimated in a Beckman LSC 100 at an efficiency of 80–85 per cent for ^{14}C.

In Fig. 1, the binding of [^{14}C]pargyline to outer membranes and the inhibition of benzylamine oxidation as a function of time after the addition of 10^{-6} M radioactive pargyline are compared. Both inhibitor binding and enzyme inhibition had very similar time courses, and were both essentially complete by 20 min. This incubation interval was used in the subsequent experiments in Fig. 2, a typical experiment comparing the binding and enzyme inhibition as a function of [^{14}C]pargyline concentration is shown. Although MAO was completely inhibited by 10^{-6} M radioactive inhibitor, the binding of [^{14}C]pargyline continued through 10^{-4} M. It seems that at lower concentrations inhibitor binding is related to enzyme inhibition, but at higher concentrations the radioactive inhibitor binds to other sites as well.

This possibility was tested directly by comparing the distribution of radioactivity after SDS polyacrylamide gel electrophoresis (Fig. 3). After electrophoresis of outer membranes incubated with 10^{-6} M [^{14}C]pargyline, radioactivity was only found in one area of the gel, presumably

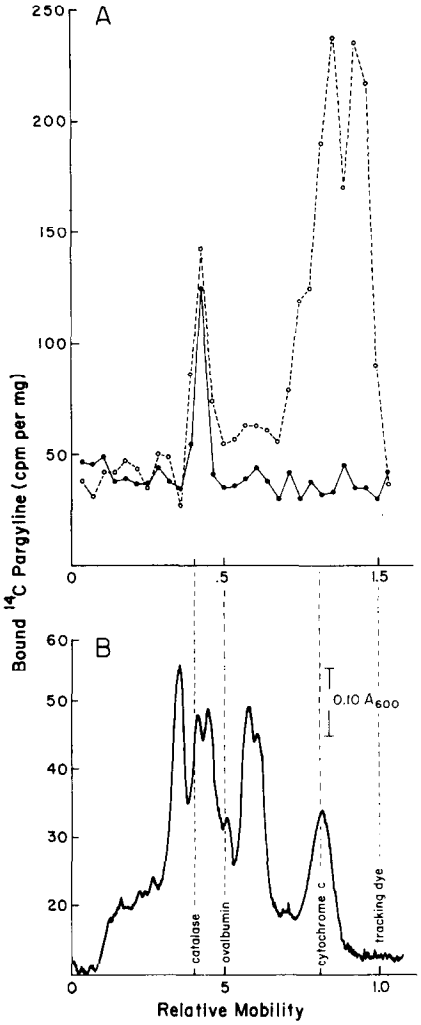

Fig. 3. Outer membranes (0.80 mg/ml) were incubated with 10^{-6} M and 10^{-4} M 7[^{14}C]pargyline as described in the legend of Fig. 2. The membranes were washed by the pargyline procedure and finally dissolved in a buffer containing 1% SDS, 10 mM mercaptoethanol, and 0.003% bromphenyl blue at 60° for 30 min. One hundred μg of each was subjected to SDS polyacrylamide gel electrophoresis. The distribution of radioactivity in the membranes from 10^{-6} M (1,000 cpm/mg; solid line) and the 10^{-4} M (13,700 cpm/mg; dashed line) incubations is shown in panel A. The relative mobility of proteins of known molecular weight is indicated on the abcissa. Panel B shows a densitometer tracing of a gel stained with Coomassie brilliant blue [7], after electrophoresis with 20 μg SDS–mercaptoethanol-treated outer membranes.

a subunit of MAO, with an apparent molecular weight of about 60,000 daltons. This observation is consistent with the findings by Oreland *et al.* [8] who have reported that highly purified pig liver MAO contains two subunits of about 60,000 daltons, both of which bind pargyline. Recent observations [9] indicate that radioactive phenethyhydrazine binds to a 60,000 dalton protein present in partially purified rat liver MAO. The distribution of radioactivity after electrophoresis of outer membranes incubated with 10^{-4} M pargyline was more complicated. Radioactivity was found not only in the higher molecular weight region, but was also distributed in at least two other regions of

the gel. Both of these lower molecular weight regions had a greater electrophoretic mobility than cytochrome *c* but less than the tracking dye. The nature of the pargyline binding in these regions is not known. In several experiments (not shown), about 50 per cent of the radioactivity bound at 10^{-4} M could be extracted with a mixture of ethanol and ether suggesting that pargyline may, in high concentration, bind to membrane lipids.

While other investigators have shown that [^{14}C]pargyline binds specifically to highly purified MAO with a predictable stoichiometry based on cofactor content or minimum molecular weight [1, 2, 8], the situation is more complicated while MAO is still incorporated in the mitochondrial outer membrane. As judged by SDS polyacrylamide gel electrophoresis, the specificity of the binding to MAO depends on the concentration of [^{14}C]pargyline. At 10^{-4} M, there are several binding sites, while at 10^{-6} M the radioactive inhibitor binds exclusively to a subunit of MAO with an apparent molecular weight of 60,000 daltons. In three assays with outer membranes incubated with 10^{-6} M [^{14}C]pargyline under the conditions described in Fig. 2, benzylamine oxidation (originally 97 ± 12 units/mg of protein) was inhibited by 96 ± 3 per cent, and 0.38 ± 0.06 nmole [^{14}C]pargyline was bound/mg of outer membrane protein. Assuming that 1 mole of inhibitor binds/mole of enzyme [1, 2], it can be calculated that the 60,000 dalton MAO subunit comprises about 2.5 per cent of the outer membrane protein. Since pargyline binds to or very near the covalently linked MAO flavin [1, 2, 10], it seems likely that the 60,000 dalton subunit is a flavoprotein. On the other hand, the molecular weight of catalytically active rat liver MAO has been estimated at 150,000 daltons [11]. Using this molecular weight and again assuming 1 mole of inhibitor binds to 1 mole of enzyme, MAO may be calculated to represent about 6 per cent of the outer membrane protein. As estimated by the specific binding of [^{14}C]pargyline, MAO comprises a substantial portion of the protein in the mitochondrial outer membrane.

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