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The binding of [^{3}H] pargyline to rat liver mitochondrial monoamine oxidase

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The synthesis and purification of tritium labelled *N*-desmethylpargyline and pargyline are described. The suitability of these irreversible suicide inhibitors of monoamine oxidase (MAO) as ligands in binding studies to rat liver mitochondrial MAO has been evaluated. [^{3}H] Pargyline was found to be more satisfactory than its *N*-desmethyl analogue because of its greater potency and lower proportion of non-specific binding. The binding of pargyline reached saturation when about 31 pmol mg protein^{-1} was bound. It was not possible to explain the time course of the binding by either simple first or second-order kinetics. [^{3}H]-Pargyline is a potentially valuable ligand for the estimation of the concentration of MAO active centres without the need to remove the enzyme from the mitochondrial membrane.

Irreversible inhibitors of monoamine oxidase (MAO: EC 1.4.3.4), have been used extensively to study the nature of this mitochondrial enzyme. While some inhibitors show little selectivity between the two forms of this enzyme, MAO-A and MAO-B, others are much more effective against one or other (see Fowler et al 1978). For example, clorgyline (Johnston 1968) is more potent against MAO-A while deprenyl (Knoll & Magyar 1972) is more potent against MAO-B.

The mechanism and kinetics of the interaction between the acetylenic (*N*-substituted propargylamine) inhibitors and MAO has been studied by a variety of methods, employing radioactively-labelled inhibitors. Covalent binding of the inhibitor to the enzyme has been demonstrated with [^{14}C]pargyline (Hellerman & Erwin 1968), which correlated with the degree of inhibition of the enzymic activity (Erwin & Deitrich 1971). Maycock et al (1976) suggested that the inhibition of bovine liver MAO by [^{14}C]dimethylaminopropyne involved the metabolism of the inhibitor by the MAO followed by Michael addition of the intermediate to the flavine moiety. [^{14}C]Labelled inhibitors have also been used to determine the molecular weights of MAO fragments (Collins & Youdim 1975; Ekstedt & Oreland 1976; McCauley 1978). Although suitable for many purposes [^{14}C]-labelled inhibitors usually do not possess sufficient specific activity to enable ligand binding studies to be undertaken with mitochondrial fractions. However, Edwards & Pak (1979) have successfully used [^{3}H]pargyline. We have also attempted to overcome the limitations caused by low specific activity on the use of labelled inhibitors as ligands for the active centre of MAO by synthesizing [^{3}H]*N*-desmethylpargyline (N-DMP) and [^{3}H]pargyline from [^{3}H]-benzylamine, by methods based on those described for the synthesis of the unlabelled compounds (Corrigan 1963) and on a method for the synthesis of [^{14}C]pargyline kindly supplied by Dr C. H. Williams, Queens' University, Belfast. These inhibitors were chosen since their synthesis is straightforward, and pargyline is known to be a potent irreversible inhibitor of MAO (Swett et al 1963). Some characteristics of the binding of these agents to mitochondrial fractions from rat liver are presented here.

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MATERIALS AND METHODS

Materials

The following drugs, reagents and other supplies were obtained as indicated: benzylamine and Merck silica gel G (British Drug Houses Ltd., Poole, U.K.), 3-chlorobenzylamine (Aldrich Chemical Co. Ltd., Gillingham, Dorset, U.K.), pargyline hydrochloride, tranylcypromine, and kynuramine dihydrobromide (Sigma London Ltd., Poole, U.K.), propargyl bromide (Fluka) from Fluorochem Ltd., Glossop, U.K.), Soluene-350 (Packard Instrument Ltd., Caversham, U.K.), dimethyl sulphate and all other reagents were of analytical grade where possible, and were obtained from Fisons Scientific Apparatus, Loughborough, U.K. *N*-Desmethylpargyline and 2-chloropargyline were gifts from Dr A. O. Geiszler, Abbott Laboratories, Chicago, U.S.A. Male Wistar rats were obtained from A. J. Tuck & Son, Rayleigh, U.K.

METHODS

Thin layer chromatography

Separation of chemical mixtures was by t.l.c. on 20 × 20 cm glass plates, coated with a 0·5 mm thick layer of silica gel. Solvent systems used were: I. butanol–acetic acid–water (12 : 5 : 3); II. butanol–acetic acid–water (4 : 1 : 1); III. methylethyl ketone–acetone–formic acid–water (46 : 2 : 1 : 6); IV. chloroform–acetone (9 : 1).

Individual compounds were identified by running authentic samples in separate tracks on each plate. These were located either under a u.v. lamp or by spraying with 0·2% ninhydrin in 95% ethanol for amines, and 2% potassium permanganate: 0·1M sulphuric acid (1 : 1) for propargylamines. Corresponding bands of radioactively labelled material were scraped from the plates and eluted with two 1 ml aliquots of 0·1M HCl. The washings were pooled and made alkaline (to a pH of greater than 10), and extracted with 4 aliquots of diethyl ether. The ether was then removed under a stream of nitrogen.

Synthesis of [^3H]N-desmethylpargyline ([^3H]N-DMP)

3-[^{3}H]Benzylamine was prepared at the Radiochemical Centre, Amersham, U.K., by catalytic reduction of 3-chlorobenzylamine by tritium gas (code T.R.3). After removal of the catalyst and exchangeable tritium, the labelled material was supplied as a solution in 0·1M HCl–ethanol (1 : 1).

A 25 ml aliquot of this solution (2 Ci) was concentrated under vacuum, and purified by t.l.c. with system I (R_F: benzylamine, 0·75; 3-chlorobenzylamine, 0·85). This purification stage is essential since 3-chloropargyline is more potent than pargyline as an inhibitor of MAO (unpublished observations). [^{3}H]benzylamine was extracted into 500 μl of ethanol and 20 μmol of propargyl bromide in 100 μl of ethanol added. The reaction mixture was left overnight at room temperature (20 °C) under nitrogen in a sealed tube. The tube was then heated on a water bath at 80 °C for 2 h. After cooling, the products were separated by t.l.c. with system III (R_F: benzylamine 0·3: N-DMP, 0·4).

A small quantity of [^{3}H]N-DMP was purified further by t.l.c. with system II (R_F: 0·69) to provide a sample for investigation of its binding to mitochondrial fractions

Synthesis of [^3H]pargyline

[^{3}H]N-DMP was extracted into 200 μl of diethyl-ether before addition of 10 μmol of dimethyl sulphate in 100 μl of diethyl-ether. The reaction mixture was left overnight at room temperature (20 °C) under nitrogen in a sealed vessel. The products were separated by t.l.c. with system II (R_F: pargyline, 0·62). The pargyline was purified further by t.l.c. with system IV (R_F: pargyline base, 0·7). The product from this final step was extracted with ether, back-extracted in 0·1M HCl, and stored at −20 °C in 1 ml 0·1M HCl–ethanol (1 : 1).

The purity of the product was established by high voltage paper electrophoresis (Shandon Southern, Model L24) on Whatman No. 1 paper, with 0·1 M sodium acetate buffer pH 5·3. After electrophoresis at 3 kV (40 v cm^{-1}) for 30 min, the product was found to be isographic with authentic pargyline, which had migrated approximately 13 cm towards the cathode. The [^{3}H]pargyline as well as the [^{3}H]N-DMP was assayed for inhibitory activity towards the MAO of rat liver mitochondria (kynuramine as substrate), with authentic samples of each inhibitor as standards. The final sample contained 50 nmol of [^{3}H]pargyline at a specific activity of 22·9 Ci mmol^{-1}.

Preparation of rat liver mitochondrial binding fraction

Livers from 6 male Wistar rats (body weight 240–250 g) were pooled and homogenized with an MSE Atomix for 30 s in 0·25 M sucrose-10mM potassium phosphate buffer, pH 7·8 (1 : 10 w/v). After centrifugation at 600 *g* for 15 min to remove broken cells, nuclei and debris, the supernatant was centrifuged at 7000 *g* for 20 min to sediment a crude mitochondrial fraction. This pellet was resuspended in sucrose buffer and centrifuged at 7000 *g* again. The pellet was resuspended in 50 mM potassium phosphate buffer, pH 7·8 (1:2 w/v, referred to the original weight of tissue), which gave a protein content of 14 mg ml^{-1}. This suspension was then divided into aliquots, and stored at −20 °C until needed for the preparation of binding fractions. One ml of 50 mM potassium phosphate buffer was added to an aliquot containing 4 mg of protein, and centrifuged in an Eppendorf Zentrifuge 3200 at approximately 12000 *g* for 1·5 min. The pellet was resuspended in buffer and sedimented again. Final resuspension was made in 50 mM buffer for use, at a final protein concentration of 4 mg ml^{-1}. This washed fraction was also stored at −20 °C in small aliquots, for use in both binding and MAO assays.

Binding assays with [^3H]propargylamines

Before the addition of the [^{3}H]propargylamine (either N-DMP or pargyline), 25 μl of washed mitochondrial fraction (100 μg protein), 25 μl of 0·1 M potassium phosphate buffer, pH 7·8 and 25 μl of distilled water or inhibitor solution were incubated

at 37 °C for 40 min and then cooled on ice. Then 25 μl of [³H]propargylamine in 50 mM potassium phosphate buffer was added at an appropriate specific activity, before incubation at 37 °C for various time intervals. The tubes were cooled on ice and the reaction mixture transferred quantitatively to Eppendorf centrifuge tubes containing 1·2 ml of 50 mM buffer. The mitochondrial fraction was centrifuged at 12000 *g* for 1·5 min. The pellet was resuspended in 1·2 ml of buffer and allowed to stand at room temperature for 1 h, before centrifugation. The pellet was dissolved in 50 μl of Soluene-350 at room temperature for 1 h. The solution was transferred to a plastic scintillation vial with two 1 ml aliquots of 2-ethoxyethanol. After the addition of 10 ml of toluene containing 0·5% PPO and 0·01% POPOP (w/v), radioactivity was measured by liquid scintillation in a Packard 3320 spectrometer with channels ratio external standardization.

Assay of MAO activity

MAO activity was measured fluorimetrically with kynuramine as substrate (Kraml 1965; Squires 1968). The assay mixture consisted of 25 μl of washed mitochondrial fraction, 25 μl of 0·1 M potassium phosphate buffer, pH 7·8 and 25 μl of distilled water or inhibitor solution. After preincubation at 37 °C for up to 40 min, the assay tubes were cooled on ice and 25 μl of 2 mM kynuramine in 50 mM buffer added. The enzyme reaction was allowed to proceed for 5 min at 37 °C before addition of 250 μl of 1 M NaOH. 4-Hydroxyquinoline content was estimated in a Perkin Elmer MPF-4 spectrophotofluorimeter, with 3 mm quartz cells at an excitation wavelength of 315 nm and an emission wavelength of 380 nm (uncorrected). Control experiments indicated that the reaction did not deviate from linearity at incubation times of up to 10 min.

Protein determination was by the method of Lowry et al (1951), with bovine serum albumin as standard.

RESULTS

Inhibition of MAO by propargylamines

The inhibition of rat liver MAO by both [³H]-pargyline and [³H]N-DMP increased in proportion with the length of preincubation with the inhibitor, reaching a maximum at about 40 min. Pargyline was about 50 times more potent than either N-DMP or tranylcypromine as an MAO inhibitor, but 2-chloropargyline was the most potent compound (Fig. 1).

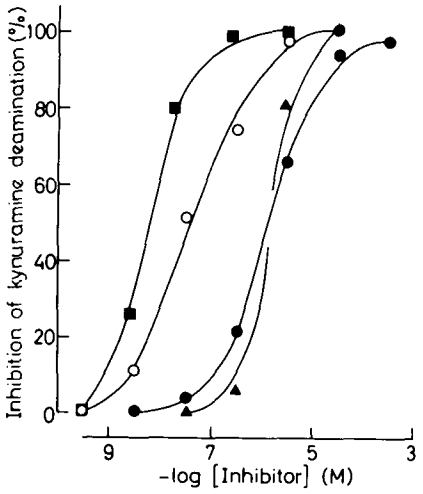

FIG. 1. The inhibition of rat liver mitochondrial MAO by propargylamines. Aliquots of mitochondrial suspensions were incubated for 40 min at 37 °C with: 2-chloropargyline; ■—■, pargyline; ○—○, *N*-desmethylpargyline (N-DMP); ●—●, and for comparison, tranylcypromine; ▲—▲. The substrate used was kynuramine (0·5 mM).

Specific binding of [³H]propargylamines

The binding of both [³H]N-DMP and [³H]pargyline were investigated at concentrations where only small amounts of MAO activity would be inhibited, in order to limit non-specific binding. Preincubation of rat liver mitochondria for 40 min with increasing concentrations of pargyline progressively prevented the subsequent binding of [³H]N-DMP at a concentration of $1{\cdot}25 \times 10^{-6}$ M. This protective effect did not appear to increase above 10^{-4} M pargyline when about 20% of the total ligand binding had been prevented. When $2{\cdot}5 \times 10^{-8}$ M [³H]pargyline was used as the ligand, 70% of the total binding could be prevented by prior incubation with tranylcypromine (Fig. 2). Identical results were obtained when tranylcypromine was replaced by 2-chloropargyline. If the [³H]pargyline was added to the incubation mixture and then immediately centrifuged about 30% of the control amount bound was recovered in the pellet.

Therefore, [³H]pargyline was used for subsequent experiments because of its greater potency and the lower proportion of non-specific binding compared with [³H]N-DMP.

Time course of [³H]pargyline binding

The binding of [³H]pargyline was studied at several

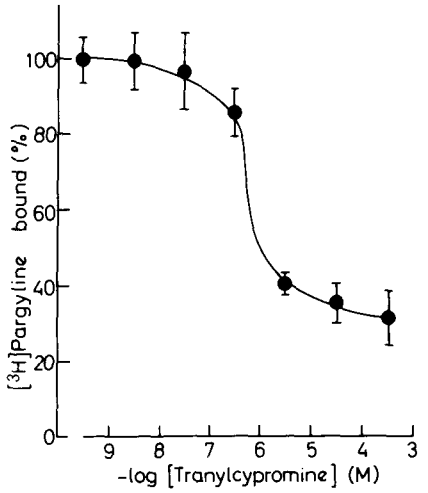

FIG. 2. The inhibition of [^{3}H]pargyline binding to rat liver mitochondrial fractions by tranylcypromine. Aliquots of mitochondrial fractions corresponding to 100 μg protein were incubated with various concentrations of inhibitor for 40 min at 37 °C before the addition of [^{3}H]pargyline ($2{\cdot}5 \times 10^{-8}$ M, 22·9 Ci mmol^{-1}) and further incubation for 30 min. Each point is the mean ± s.e. ratio (n = 8).

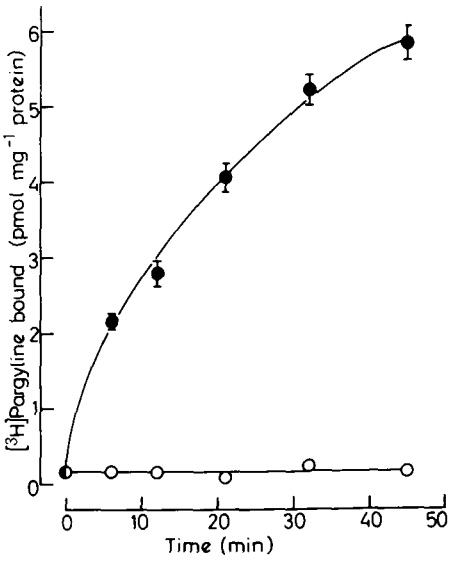

FIG. 3. The binding of [^{3}H]pargyline to rat liver mitochondrial fractions. Mitochondrial fractions (100 μg protein) were incubated for 40 min at 37 °C in the absence (●—●) or presence (○—○) of $3{\cdot}3 \times 10^{-7}$ M 2-chloropargyline before the addition of [^{3}H]pargyline ($8{\cdot}25 \times 10^{-8}$ M, 1 Ci mmol^{-1}) and further incubation for periods up to 45 min before washing off unbound ligand. Each point represents the mean ± s.e. of 4 observations. Where no error bar is shown the s.e. was smaller than the symbol.

concentrations with incubation times up to 50 min. Non-specific binding was estimated after incubation with $3{\cdot}3 \times 10^{-7}$ M 2-chloropargyline for 40 min at 37 °C.

In the presence of $8{\cdot}25 \times 10^{-8}$ M [^{3}H]pargyline, the total binding increased in a non-linear manner with time, while the non-specific component was constant over the whole incubation period (Fig. 3). Specific binding of [^{3}H]pargyline, defined as the difference between the total binding and the non-specific component at each time point, at three concentrations of ligand is shown in Fig. 4. Attempts to fit these non-linear curves to either first or second order kinetics were not successful.

Saturable binding of [^{3}H]pargyline

The incorporation of total tritium label into the mitochondrial fractions increased in a non-linear manner with increasing concentrations of [^{3}H]-pargyline. However, after preincubation with 2-chloropargyline ($3{\cdot}3 \times 10^{-7}$ M) for 40 min, this relationship became linear (Fig. 5). The concentration-dependent specific binding appeared to reach saturation at about 3×10^{-6} M free ligand and was equivalent to a maximum binding of 31 pmol (mg protein)$^{-1}$.

DISCUSSION

These results indicate that the binding of [^{3}H]-pargyline to rat liver mitochondrial fractions becomes saturated at a concentration of 3×10^{-6} M of the free ligand. At this concentration, MAO activity was completely inhibited when 31 pmol (mg protein)$^{-1}$ of [^{3}H]pargyline were bound at sites that could be blocked by prior incubation with 2-chloropargyline. While the amount of non-specific binding found with [^{3}H]pargyline was within acceptable limits, the very high amount of non-specific binding with [^{3}H]N-DMP, coupled with its relatively low potency as an inhibitor of MAO precludes the use of this latter compound in this type of binding study.

In any study of ligand binding it is vital to determine what proportion of the total binding takes place at the site of interest. Since propargylamines irreversibly inactivate MAO by a mechanism which is thought to require metabolism of the compound by the enzyme to its active form (Rando 1974: Maycock et al 1976), it would be expected that pargyline would bind to MAO in a highly specific manner. However, pargyline will also inhibit mitochondrial aldehyde dehydrogenase (Lebsack et al 1977), and together with N-DMP, will interfere with several hepatic microsomal enzymes (Valerino et al 1978). 2-

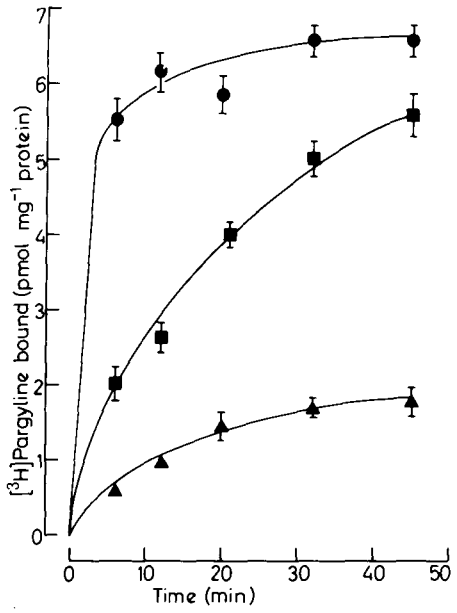

FIG. 4. Specific [^{3}H]pargyline binding to rat liver mitochondrial MAO. MAO-specific binding is defined as the difference between the binding of [^{3}H]pargyline alone, and after incubation for 40 min at 37 °C with $3{\cdot}3 \times 10^{-7}$ M 2-chloropargyline at each time point. The concentrations of [^{3}H]pargyline were: $1{\cdot}25 \times 10^{-8}$ M (▲—▲), $8{\cdot}25 \times 10^{-8}$ M (■—■) and $2{\cdot}5 \times 10^{-7}$ M (●—●); specific activity was 1 Ci mmol^{-1} for all concentrations. Each point is the mean ± s.e. of 4 observations.

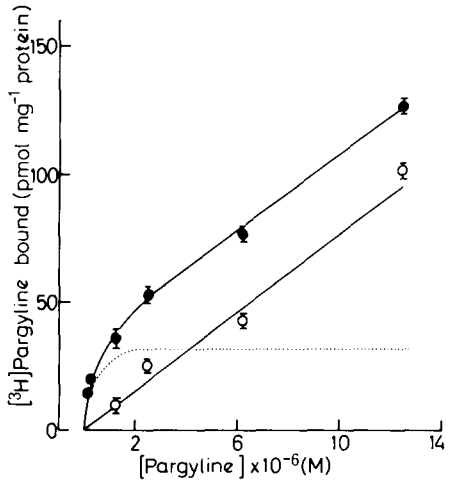

FIG. 5. The binding of [^{3}H]pargyline to rat liver mitochondrial fractions. After incubation for 40 min at 37 °C in the absence (●—●) or presence (○—○) of $3{\cdot}3 \times 10^{-7}$ M 2-chloropargyline, the aliquots were further incubated with various concentrations of [^{3}H]-pargyline (200 mCi mmol^{-1} for 1 h before washing off unbound ligand. The dotted line represents the difference between the two curves. Each point is the mean ± s.e. for 4 observations.

Chloropargyline, which is a more potent MAO inhibitor than pargyline itself (Swett et al 1963), was used to determine the amount of non-specific binding in these experiments, because there would be less chance that the similarity in structures would prevent the binding of [^{3}H]pargyline to non-specific sites, as well as to MAO. If this were to occur an erroneously low estimate of non-specific binding would result. Tranylcypromine, an MAO inhibitor that does not inhibit aldehyde dehydrogenase (Lovenberg et al 1962) and is not a propargylamine, gives the same value for non-specific binding of [^{3}H]pargyline as that found with 2-chloropargyline. It would seem unlikely that both these inhibitors would prevent the binding of pargyline to non-specific sites to exactly the same degree. Furthermore, the fact that the level of non-specific binding at low concentrations of [^{3}H]pargyline is not different from unincubated samples and is not time-dependent when incubated at 37 °C, suggests that entrapment of the radioligand in the centrifuged pellet is the major source of the non-specific binding.

In consequence, it appears likely that the value for the maximum binding of [^{3}H]pargyline to the mitochondrial fractions of 31 pmol (mg protein)$^{-1}$, represents binding to the MAO itself. This value would be significantly increased if highly purified preparations of mitochondrial outer membranes were used, but it is much lower than the value of 117 p mol (mg protein)$^{-1}$ for intact mitochondria reported by Edwards & Pak (1979).

Nelson et al (1979) have recently reported the use of the reversible MAO-A selective inhibitor [^{3}H]-harmaline as a suitable ligand for the detection of MAO-A sites. Although, unlike reversible inhibitors [^{3}H]pargyline cannot readily be used to investigate the kinetics of the interaction of MAO with its substrates and other inhibitors, it does offer certain advantages. In particular, both MAO-A and MAO-B can be detected and the concentration of both MAO active sites measured by the use of very simple methods. Once the inhibitor has interacted with the enzyme there is little chance that it will dissociate during the extraction and washing procedures. The prior incubation of MAO with selective irreversible inhibitors before the addition of [^{3}H]-pargyline provides a method for the estimation of the relative proportions of MAO-A and MAO-B in tissues. This technique, followed by SDS-polyacrylamide gel electrophoresis (Edwards & Pak 1979) has revealed that sub-units of MAO-A and MAO-B are different proteins (Cawthon & Breakefield 1979; Callingham & Parkinson 1979).

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