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# OBSERVATIONS ON THE INHIBITION OF RAT LIVER MONOAMINE OXIDASE BY CLORGYLINE

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**Abstract**—Two forms of monoamine oxidase (MAO) defined as MAO A and B by others differ in their specificities to substrates and their sensitivities to the irreversible inhibitor clorgyline. From studies using the substrates 5-HT, tyramine and benzylamine, the presence of both MAO forms in rat liver mitochondria has been confirmed and some characteristics of their inhibition by varying concentrations of clorgyline investigated. Although both MAO forms showed time-dependent inhibition, this process occurred, in general, at a qualitatively slower rate for MAO B, despite the fact that this enzyme form requires higher concentrations of clorgyline than MAO A for inhibition of its activity. However, factors such as the concentration of enzyme, the concentration of clorgyline and the enzyme: drug ratio employed in the assay all influence the resultant time-course and the final degree of the inhibition observed. The possible importance of the lipid environment of the outer mitochondrial membrane in generating multiple MAO forms and in regulating the inhibition kinetics of these forms is discussed. The results indicate that the effects of pre-incubation time and the enzyme: drug ratio on inhibition of MAO by clorgyline should be fully recognized when using the drug to indicate multiple forms in animal tissues.

Evidence has accumulated in recent years indicating that the enzyme monoamine oxidase (MAO) exists in multiple forms. These forms are reported to vary with respect to heat and inhibitor sensitivity, substrate specificity, and mobility on polyacrilamide gels [1].

Studies using the irreversible inhibitor, clorgyline, have been particularly useful in suggesting the presence of multiple forms of MAO, even within the same animal tissue. Early investigations with this drug suggested the existence of two forms of the enzyme (MAO A and B) in mitochondria from rat brain and liver [2, 3]. These forms differed in their sensitivity towards clorgyline, and also in their ability to metabolize various amine substrates. For instance, data suggested that 5-hydroxytryptamine (5-HT) was a substrate solely for MAO A, benzylamine was a substrate solely for MAO B, whereas tyramine was metabolized by both enzyme forms. Similar results confirming these substrate specificities for the two enzyme forms have been obtained in studies using a number of animal sources. However, it appears from recent findings that there are some exceptions to generally accepted substrate preferences of MAO A and B [4, 5].

The mechanism of inhibition of MAO by clorgyline has not been characterized. However, by analogy with pargyline and other related propargylamine derivatives which are irreversible inhibitors of MAO, clorgyline may combine covalently and in a stoichiometric fashion with the flavin cofactor moiety near the active site of the enzyme [6, 7]. The degree of inhibition of MAO by clorgyline has been reported to be unchanged after 10 min pre-incubation [8], but more recent studies have indicated time-dependent inhibition during short (up to 5 min) pre-incubation periods [9] and also during much longer pre-incubation conditions [10]. Most investigations into the sensitivity of MAO towards clorgyline are performed after fixed pre-incubation times which vary from author to author. Thus, if a mechanism of inhibition were time-dependent, this fact could alter the apparent sensitivity of the enzyme activity being studied and could accordingly affect the inhibition curves obtained over a range of drug concentrations.

The present results describe the effects of varying both pre-incubation time and quantity of enzyme in the assay on inhibition of rat liver mitochondrial MAO by clorgyline. Three substrates, 5-HT, tyramine and benzylamine were used to investigate the possibility of selective effects on MAO A or B.

## MATERIALS AND METHODS

Radioactive substrates used to assay MAO activity were [methylene-$^{14}$C] benzylamine hydrochloride (ICN Pharmaceuticals, Inc. Irvine, California), [generally labelled-$^{3}$H] 5-hydroxytryptamine creatinine sulphate (Amersham–Searle, Arlington Heights, Illinois) and [generally labelled-$^{3}$H] tyramine hydrochloride (New England Nuclear, Boston, MA).

Clorgyline hydrochloride [*N*-methyl-*N*-propargyl-3-(2,4-dichlorophenoxy) propylamine, M + B 9302] was a generous gift from May and Baker Ltd., Dagenham, Essex, U.K.

Mitochondria were prepared from adult, male, Charles River rats by a previously described method [11] in an isolation medium containing sucrose (70 mM), D-mannitol (220 mM), bovine serum albumin (0.5 mg/ml), HEPES (*N*-2-hydroxyethyl piperazine-N′-2-ethanesulphonic acid) buffer (2.0 mM, pH 7.4). The mitochondrial preparation, which had a protein content of 40 mg/ml was stored at −20° and thawed before use. In order to vary protein contents of MAO assays, aliquots of this preparation were diluted with appropriate volumes of isolation medium. Actual quantities of assay protein studied were 1.6, 8.0 and 40.0 μg i.e. a 25-fold range. Metabo-

lite production remained linear with respect to both protein content and assay time in these experiments. Results described in this paper were obtained with a single mitochondrial preparation, but are representative of similar results obtained with other preparations.

*MAO assay.* The assay of MAO activity was based on the radiochemical method described by Callingham and Laverty [12]. The three substrates, benzylamine, tyramine, and 5-HT, were used at final specific activities of 0.5, 1.0 and 2.0 $\mu$Ci/$\mu$mole, respectively, and at final assay concentrations of 1 mM. This concentration was chosen as a result of $K_m$ determinations for the three substrates, with rat liver mitochondrial MAO. The Lineweaver–Burk plots obtained (not shown) were completely linear over a range of substrate concentrations from 5 $\mu$M to 2.5 mM, with no evidence of substrate inhibition at the higher concentrations. Estimated $K_m$ values were $1.1 \times 10^{-4}$ M (5-HT), $0.9 \times 10^{-4}$ M (tyramine) and $1.3 \times 10^{-4}$ M (benzylamine) and thus subsequent experimental assays at 1 mM represented approximately $10 \times K_m$ concentrations.

It has been suggested from 'curved' Lineweaver–Burk plots for tyramine metabolism by MAO in rat superior cervical ganglion, that the affinities of MAO A and B for this substrate are different [13]. However, the complete linearity of our plots for tyramine with rat liver MAO is in agreement with others who have concluded that affinities of MAO A and B for this substrate are similar in this particular tissue [14, 15]. Thus, only a single assay concentration for tyramine has been adopted in the following inhibition studies.

Briefly, appropriate concentrations of clorgyline in distilled water were pre-incubated in air at 37° with 10 $\mu$l mitochondrial samples for times indicated within the text, in a total pre-incubation volume of 50 $\mu$l. (Pre-incubation in closed, oxygen-filled assay tubes had no significant effect on the magnitude or rate of the subsequent inhibition.) All clorgyline concentrations reported within the text represent those concentrations present within the pre-incubation period. After pre-incubation, all assay tubes were cooled rapidly by immersing in an ice bath. Remaining MAO activity was measured in stoppered, oxygen-filled tubes after addition of 50 $\mu$l radioactive substrate (2 mM in 0.2 M potassium phosphate, pH 7.8), by incubating all samples for 5 min at 37°. The assay reaction was terminated by cooling in ice, followed by the rapid addition of 10 $\mu$l 3 N HCl. Deaminated metabolites were extracted into 0.5 ml water-saturated ethyl acetate–benzene (1:1 v/v). After the two phases were separated by mixing and centrifugation, 0.4 ml of the upper, organic layer was counted for radioactivity in 12 ml 0.4% butyl PBD in toluene (w/v) in a Beckman LS-230 liquid scintillation spectrometer. All counts were corrected for quench and converted to dpm for calculation of results.

Blank values for this enzyme assay are due almost exclusively to the extraction of small amounts of amine substrate along with metabolites into the organic phase. It was determined that 'denatured-enzyme' blanks were essentially identical to 'no-enzyme' blanks containing water (50 $\mu$l) and substrate (50 $\mu$l) alone. This latter method of blank assessment was used in the present studies.

## RESULTS

The effects (1) of altering enzyme-inhibitor pre-incubation times and (2) of altering the enzyme concentration (by using mitochondrial suspensions of differing protein content) in the assay on the inhibition of rat liver MAO by clorgyline, were investigated. The three substrates, tyramine, 5-HT and benzylamine were used.

*Tyramine.* The inhibition by clorgyline of rat liver MAO activity towards tyramine as substrate was measured under conditions of varying clorgyline concentrations ($2 \times 10^{-10}$ M to $2 \times 10^{-5}$ M), pre-incubation times (5 to 45 min) and different mitochondrial protein contents (1.6, 8.0 and 40 $\mu$g) within the assay. All inhibition values have been expressed as percentages of identical, uninhibited control samples measured after the appropriate pre-incubation periods. It was found, incidentally, that these control activities remained essentially unchanged during the pre-incubation periods studied, whether activity was measured with tyramine, 5-HT or benzylamine as substrates.

The following conclusions were drawn from the results obtained, which are shown in Fig. 1. The degree of MAO inhibition attained after each pre-incubation period varied with the concentration of clorgyline employed. At concentrations of $2 \times 10^{-7}$ M and lower, inhibition was essentially unchanged beyond 10 min preincubation. With higher

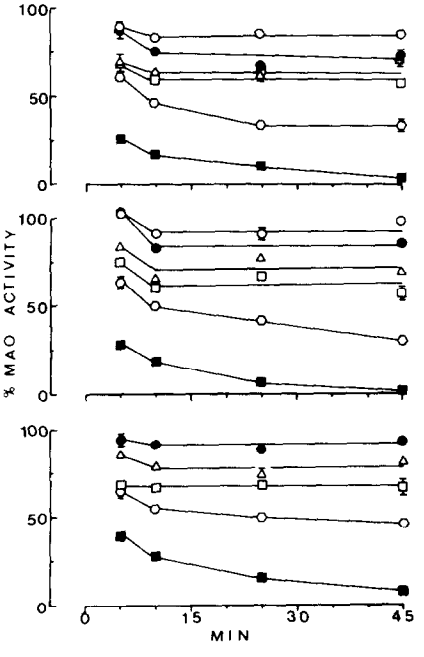


Fig. 1. Effect of pre-incubation time on inhibition of MAO in isolated rat liver mitochondria by different clorgyline concentrations. Mitochondrial protein in the assay was 1.6 $\mu$g (upper panel), 8 $\mu$g (middle panel), 40 $\mu$g (lower panel). Tyramine was used as substrate. Clorgyline concentrations were ○—○, $2 \times 10^{-10}$ M; ●—●, $2 \times 10^{-9}$ M; △—△, $2 \times 10^{-8}$ M; □—□, $2 \times 10^{-7}$ M; ⬡—⬡, $2 \times 10^{-6}$ M; ■—■, $2 \times 10^{-5}$ M. Each point is the mean of three determinations. Standard errors (±) were calculated for all points but are indicated only when they exceed the size of symbol used.

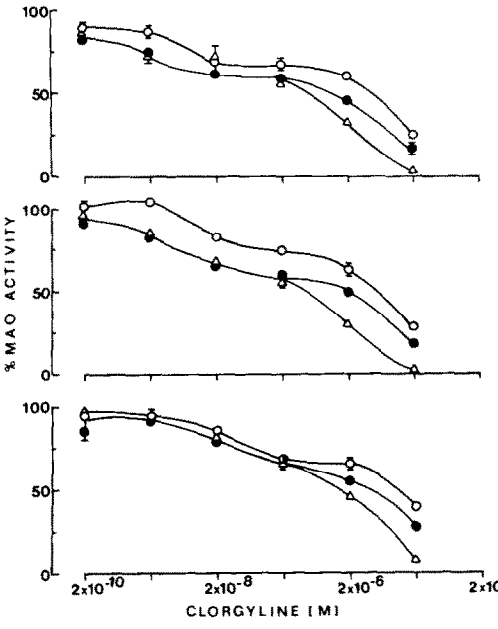


Fig. 2. Effect of pre-incubation time on inhibition curves for the inactivation of rat liver MAO by clorgyline. Mitochondrial protein used was 1.6 μg (upper panel), 8 μg (middle panel), 40 μg (lower panel). Pre-incubation times were O——O, 5 min; ●——●, 10 min; △——△, 45 min. Data is replotted from Fig. 1.

clorgyline concentrations ($2 \times 10^{-6}$ M and $2 \times 10^{-5}$ M), the degree of inhibition became progressively greater throughout the time-course of the experiment. These results were obtained with each of the three protein contents studied. However, it is apparent that the degree of MAO inhibition produced by a given drug concentration was decreased to some extent at all preincubation times, as the protein concentration was increased.

In Fig. 2, these inhibition results have been plotted against clorgyline concentrations (on a logarithmic scale). The binary nature of inhibition curves such as these has been taken as evidence that tyramine is metabolized by two forms of MAO in rat liver, one of which (MAO A) is relatively more sensitive to clorgyline than the other form (MAO B). The present results are thus in agreement with inhibition curves previously reported by other workers, describing the inhibition of tyramine metabolism by clorgyline in this tissue [3].

It is clear from these curves that the apparent sensitivity of these forms towards clorgyline can vary, depending upon differing conditions of pre-incubation time and protein content. Under the conditions used to investigate these effects in the present experiments, an increase in pre-incubation time beyond 10 min resulted in no further increase in the apparent sensitivity of MAO A towards the drug. However, the apparent sensitivity of MAO B did increase with longer pre-incubation times. Along with these observations, it is clear that the effect of increasing the protein content within the assay was to cause a decrease in the apparent sensitivities of either MAO forms towards clorgyline.

*5-HT.* Similar studies were performed using 5-HT as substrate for MAO A in rat liver. Clorgyline concentrations ranging from $2 \times 10^{-12}$ to $2 \times 10^{-7}$ M were used. Figure 3 shows the results obtained.

Clearly, the degree of inhibition of activity after each pre-incubation time depended upon the concentration of clorgyline used. With 8 or 40 μg protein within the assay, the inhibition caused by a given clorgyline concentration changed very little after a pre-incubation period of 10 min. In contrast, we did observe a time-dependent inhibition of MAO A beyond 10 min pre-incubation at certain clorgyline concentrations when only 1.6 μg protein was present in the assay.

In Fig. 4, these results are plotted to show the corresponding log dose inhibition curves obtained. When 8 or 40 μg protein were used, the resultant single sigmoid curves were typical of those previously reported describing the inhibition of MAO A by clorgyline in rat liver [3]. Furthermore, under these particular conditions of protein concentration, it was evident that the apparent sensitivity of MAO A towards clorgyline (i) was unaltered upon pre-incubation for longer than 10 min, and (ii) was decreased with increasing protein within the assay. When a smaller quantity of protein (1.6 μg) was used the apparent enzyme sensitivity changed upon pre-incubation up to 45 min. Also, the resulting curves were slightly anomalous in shape. Although no inhibition was obtained with $2 \times 10^{-12}$ M clorgyline after 10 or 45 min preincubation, there was a significant inhibition of enzyme activity with $2 \times 10^{-11}$ M clorgyline after these times.

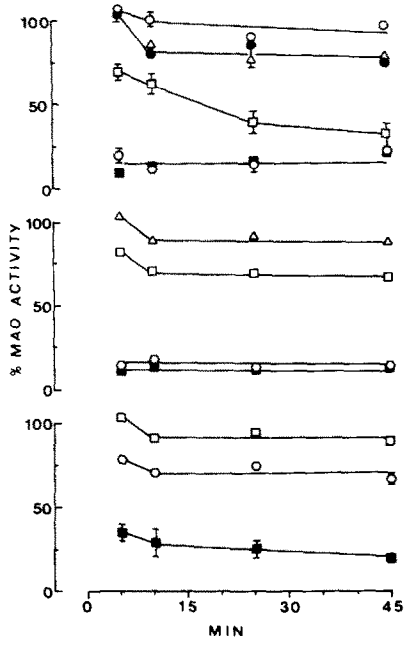


Fig. 3. Effect of pre-incubation time on inhibition of rat liver MAO by different clorgyline concentrations. Mitochondrial protein in the assay was 1.6 μg (upper panel), 8 μg (middle panel), 40 μg (lower panel). 5-HT was used as substrate. Clorgyline concentrations were O——O, $2 \times 10^{-12}$ M; ●——●, $2 \times 10^{-11}$ M; △——△, $2 \times 10^{-10}$ M; □——□, $2 \times 10^{-9}$ M; ⬡——⬡, $2 \times 10^{-8}$ M; ■——■, $2 \times 10^{-7}$ M. Each point is the mean of three determinations ± standard errors (indicated when exceeding symbol size).

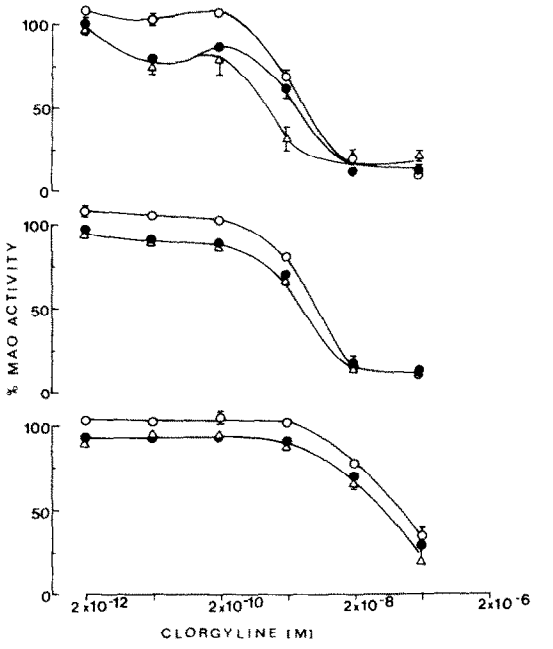


Fig. 4. Effect of pre-incubation time on inhibition curves for the inactivation of rat liver MAO by clorgyline. Mitochondrial protein used was 1.6 μg (upper panel), 8 μg (middle panel), 40 μg (lower panel). Pre-incubation times were O——O, 5 min; ●——●, 10 min; Δ——Δ, 45 min. Data is replotted from Fig. 3.

Inhibition with clorgyline at $2 \times 10^{-10}$ M appeared slightly decreased or unchanged. We have observed qualitatively similar effects at these clorgyline concentrations on a number of occasions with other rat liver mitochondrial preparations, when very small amounts of protein were used in the assay.

*Benzylamine.* The effects of pre-incubation time upon inhibition of MAO at a single mitochondrial protein content of 8 μg was investigated with benzylamine as substrate. Clorgyline concentrations of

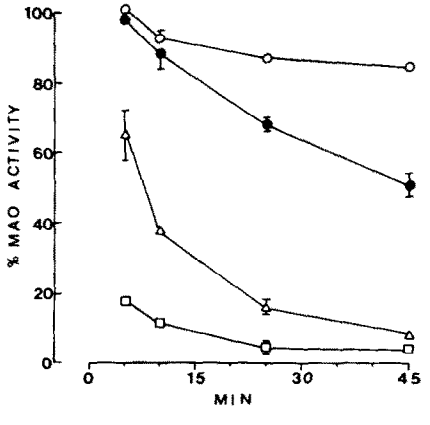


Fig. 5. Inhibition curves for inactivation of rat liver MAO by clorgyline using a mitochondrial protein content of 8 μg. Benzylamine was used as substrate. Clorgyline concentrations were O——O, $2 \times 10^{-7}$ M; ●——●, $2 \times 10^{-6}$ M; Δ——Δ, $2 \times 10^{-5}$ M; □——□, $2 \times 10^{-4}$ M. Each point is the mean of three determinations ± standard errors (indicated when exceeding symbol size).

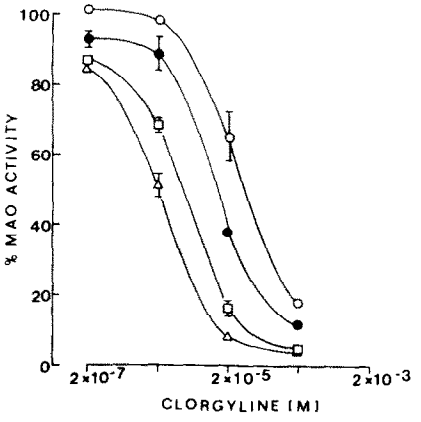


Fig. 6. Effect of pre-incubation time on inhibition curves for the inactivation of rat liver MAO by clorgyline. Pre-incubation times O——O, 5 min; ●——●, 10 min; □——□, 25 min, Δ——Δ, 45 min. Data is replotted from Fig. 5.

$2 \times 10^{-7}$ M to $2 \times 10^{-4}$ M were used. The results obtained are shown in Fig. 5.

Inhibition of MAO activity was clearly time-dependent throughout the entire time course of the experiment, although the decrease with time was only slight using $2 \times 10^{-7}$ M clorgyline.

The corresponding log dose inhibition curves plotted from this data are shown in Fig. 6. In agreement with previous workers, these curves confirmed the metabolism of benzylamine by MAO B in normal rat liver mitochondria. However, it can be seen that the apparent sensitivity of MAO B towards clorgyline increases quite markedly as pre-incubation time is increased.

## DISCUSSION

The irreversible MAO inhibitor, clorgyline, is being used increasingly to investigate the possibility of multiple forms of the enzyme within animal tissues. Many workers have described the existence of two forms of MAO, called MAO A and B, which differ in substrate specificities and also with respect to sensitivity towards clorgyline [2–5]. The particular ranges of clorgyline concentrations over which the inactivation of these two forms were originally found to occur are often used as criteria to indicate the types of MAO present within various tissues. However, relatively little attention has been paid to factors and/or assay conditions which may affect the apparent sensitivity of these enzyme forms to clorgyline.

Early studies with rat liver MAO, using tyramine as substrate, indicated that inhibition by a single concentration of clorgyline ($10^{-7}$ M) was rapid, with the maximal inhibition for that particular dose being attained after 10 min pre-incubation [8]. Tipton [9] studied the inhibitory effects of a high clorgyline concentration (2.5 mM). Under these conditions, a pseudo first-order inhibition of MAO activity was observed, which was more rapid for 5-HT as substrate (complete inhibition by 1–2 min pre-incubation), than with benzylamine (complete inhibition required 5–10 min pre-incubation). The inhibition of enzyme activity

towards tyramine occurred in two phases, corresponding to the fast and slow inhibition components shown with 5-HT and benzylamine, respectively. More recently, it has been reported that MAO from porcine brain is inhibited by clorgyline ($10^{-6}$ M) in a time-dependent manner during a pre-incubation period of 45 min [10]. Observations of time-dependent inhibition with clorgyline are similar to results obtained with pargyline, another irreversible MAO inhibitor, on human liver MAO [16]. These observations have led to the suggestion that drugs of this kind are substrate analogues and that they behave as active site-directed inhibitors by combining reversibly with the enzyme, followed by an irreversible covalent interaction with the flavin cofactor at the active site [17]. These previous studies investigating the time-dependent nature of inhibition by clorgyline were limited by the range of drug concentrations studied and also by the pre-incubation periods. The present results thus represent a more detailed investigation into the effects of varying these parameters upon the subsequent rate and magnitude of MAO inhibition by clorgyline in rat liver mitochondria. Special attention was paid to the possibility of selective effects upon MAO A and B by using substrates specific for these enzyme forms in the experiments described.

It is clear from the present results that inhibition of enzyme activity by clorgyline can be affected by both pre-incubation time and by the quantity of protein in the assay. Both MAO A and B were less sensitive towards clorgyline as mitochondrial protein (and hence amount of enzyme) was increased in the assay system. This finding is consistent with the mechanism of irreversible inhibition previously proposed for the action of the drug.

The present studies also indicate that, in general, inhibition of MAO B occurs at a slower rate despite requiring higher concentrations of clorgyline than inhibition of MAO A. These conclusions are qualitative at present. Clearly, the ranges of clorgyline concentrations used here to obtain log dose inhibition curves for MAO A and B include those concentrations which inhibit only a proportion of the total activity of a given enzyme form. These particular conditions probably involve the complete titration of available 'free' inhibitor molecules with excess enzyme. Thus, it is unlikely that pseudo first-order kinetics (when inhibitor concentration is $\gg$ enzyme concentration) for the inhibition will occur with all clorgyline concentrations. The resultant rates of enzyme inhibition and the kinetics observed are probably dependent upon a combination of factors—the concentrations of inhibitor and enzyme, and also their relative values. Under most conditions studied here, further time-dependent inhibition of MAO A (assayed with 5-HT as substrate) after 10 min pre-incubation was not observed. However, a time-dependent mechanism for MAO A did become apparent when protein content in the assay was reduced to very low levels (1.6 $\mu$g). Taken as a whole, our results suggest that previous conflicting reports by other workers concerning a prolonged time-dependent inhibition of MAO are due to the use of different single concentrations of clorgyline and to the different substrates used by them [8–10].

It still has not been determined if the activities defined as MAO A and B represent different molecular forms of the enzyme, or if they represent a single enzyme with multiple catalytic sites, perhaps, being regulated by the surrounding lipid environment in the outer mitochondrial membrane [18]. Each of these factors could account for the differences in the sensitivities of MAO A and B towards clorgyline. It has been shown recently that the potencies of a number of propargyl MAO inhibitors correlate well with their lipophilic nature [19], and thus, the ease of penetration into lipid around the membrane-bound enzyme may be a factor in determining the inhibition characteristics of these compounds.

It is difficult at this stage to explain the slightly anomalous shapes of log dose inhibition curves for MAO A obtained with 5-HT as substrate when a protein content of 1.6 $\mu$g is used in the assay. We have observed similar effects with other mitochondrial preparations when using small quantities of assay protein. The appearance of a plateau region in these particular curves may indicate the inhibition of more than one enzyme form which metabolizes 5-HT in the preparation. However, the absence of similar curves with higher protein concentrations render this explanation unlikely.

Alternatively, it is possible that clorgyline, by virtue of its lipophilic nature may partition into biological membranes and alter catalytic activity of MAO by causing some perturbation of lipid structure around the enzyme. A similar mechanism has been proposed previously for the stimulatory effects of reserpine upon guinea pig heart MAO [20]. Although direct irreversible inhibition of MAO appears to be the major action of clorgyline, it is not clear whether the drug could, to a lesser extent, cause effects similar to those of reserpine upon MAO activity. If so, the resultant enzyme activity in the presence of a given clorgyline concentration would be dependent upon the relative magnitude of these opposing effects. An indication that both effects occur may only become apparent under particular experimental conditions e.g. extremely low, relatively non-inhibitory clorgyline concentrations and at particular drug:protein ratios in the assay. Clearly, such an explanation for our unusual results with MAO A is speculative at present. Very little is known about the possible interaction of clorgyline with other cellular components. In this respect, Williams and Lawson [19] reported that radioactive clorgyline or pargyline bind specifically only to purified MAO and not several other purified proteins. However, in contrast, it is interesting that McCauley has recently reported the association of [$^{14}$C]-pargyline with rat liver outer mitochondrial membrane lipids [21].

In conclusion, our experiments indicate some of the *in vitro* characteristics of MAO inhibition by clorgyline which should be recognized by those who use the drug. Although these studies do not detract from the usefulness of clorgyline in evaluating the presence of multiple MAO forms in animal tissues, it is clear that direct comparison of inhibition studies from different workers requires considerable caution, particularly when such factors as protein content, pre-incubation time and assay time may vary considerably. Also, the source of the enzyme, the sensitivity of the assay

and the conditions required for linearity in the assay are factors which must be considered. During the final stages of revision of this paper, similar conclusions with regard to some of these factors have been reported by Egashira *et al.* [22].

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