J. Pharm. Pharmac., 1978, 30, 304–309 Received January 24, 1978

The inhibition by clorgyline of 5-hydroxytryptamine deamination by the rat liver

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The inhibition of the monoamine oxidase activity in the rat liver by the substrate selective inhibitor clorgyline has been investigated with 5-hydroxytryptamine as substrate. The results obtained are consistent with a theoretical model whereby the inhibition of enzyme activity by clorgyline follows a reversible association phase leading to an irreversible 'suicide' reaction. The relative concentrations of enzyme and inhibitor are of the same order, and can account both for the failure of the reaction to go to completion, and for the differences in the apparent sensitivity of enzyme preparations to inhibition by clorgyline. The possible value of this type of inhibition as a means of assay for monoamine oxidase active centres is discussed.

There is now much evidence to show that the activity of the enzyme monoamine oxidase (MAO, EC 1.4.3.4) can exist in more than one form (see Tipton, 1975; Houslay, Tipton & Youdim, 1976). Use of the inhibitor clorgyline and a variety of substrates, enabled Johnston (1968) to classify the enzyme activities into two forms, MAO-A and MAO-B, where the MAO-A was much more sensitive to the inhibitor. According to this classification, 5-hydroxytryptamine (5-HT) is metabolized by MAO-A alone, and tyramine by both forms in the rat liver (Hall, Logan & Parsons, 1969).

Clorgyline is generally considered to inhibit MAO through a reversible association with the active centre of the enzyme, followed by a 'suicide' reaction involving covalent interaction with the FAD prosthetic group (see Rando, 1974). Clorgyline is such a potent inhibitor of MAO when 5-HT is used as the substrate, that it seems likely that measurable inhibition is seen with concentrations of clorgyline of the same order as those of the enzyme itself. The onset of inhibition with 5-HT as substrate is rapid reaching a steady state within 5 min. If a substrate for MAO-B such as benzylamine or β-phenethylamine is used, the time course of the reaction is greatly prolonged, taking up to 4 h to reach maximum inhibition in some instances (Egashira, Ekstedt & Oreland, 1976; Lyles & Greenawalt, 1977).

The observed differences in the time to maximal inhibition with the different substrates may possibly be due to differences in the rates of association of the inhibitor with the active centres of the enzyme forms. Such differences could stem from the presence of some sort of diffusional or conformational barrier.

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In an attempt to see if this explanation could be valid, a theoretical model has been developed in which the inhibitor concentration is of the same order as that of the enzyme. The value of this model in predicting actual experimental observations has been tested in the rat liver with 5-HT as substrate.

MATERIALS AND METHODS

Materials

The radioactive substrates for MAO, [^{3}H]5-hydroxytryptamine (^{3}H-5-HT) and [^{3}H]tyramine were obtained from the Radiochemical Centre, Amersham, U.K. Clorgyline hydrochloride was a gift from May & Baker, Ltd, Dagenham, U.K. All other reagents were standard laboratory reagents of analytical grade wherever possible. Male Wistar rats were obtained from A. J. Tuck & Son, Rayleigh, U.K.

Preparation of crude homogenates

Five rats, 350–400 g, were killed by a blow to the head and the livers excised, blotted, and homogenized in 1 mM ice-cold potassium phosphate buffer, pH 7·8 (1:10 w/v) in a conical glass homogenizer. Nuclei and cell debris were removed by centrifugation at 600 *g* for 15 min, and the supernatant fractions used for assay.

Preparation of MAO-depleted homogenates

Six rats, 350–400 g, were killed and the livers removed as described above. Three groups of two livers were blotted and homogenized in 'sucrose buffer' (0·25 M sucrose, 10 mM potassium phosphate buffer, pH 7·8), 1:8 (w/v) in an MSE Atomix blender. Nuclei and cell debris were removed as described above. Part of the supernatant fractions (S1) was used for assay and the remainder further centrifuged at 6500 *g* for 20

min, to sediment the mitochondria. These final supernatant fractions (S2), depleted of the bulk of their content of MAO by the removal of the mitochondria, were also used for assay.

The MAO activity of the various fractions was assayed by the method of McCaman, McCaman & others (1965), as modified by Callingham & Laverty (1973). ^{3}H-5-HT and [^{3}H]tyramine were used as substrates. When clorgyline was used as an inhibitor, it was preincubated with the homogenate for various times before the addition of substrate. The time of the incubation period with substrate was limited to 90 s to minimize further interaction of the clorgyline with the enzyme. At the time when the substrate was added to the enzyme-clorgyline mixture, the concentration of the inhibitor fell by half. Extra clorgyline was not added to the substrate solution to reduce the chance of reversible interactions taking place after the end of the preincubation period. Thus the clorgyline concentrations given throughout are those at preincubation.

Protein determination was by the method of Lowry, Rosebrough & others (1951). MAO activities are expressed in terms of nmol (of substrate metabolized) (mg protein)$^{-1}$h^{-1}.

RESULTS

The time course of the inhibition of the deamination of 5-HT by three concentrations of clorgyline was measured in homogenates of rat liver (Fig. 1). With $1{\cdot}25 \times 10^{-7}$ M clorgyline, inhibition was complete by about 60 min from the time of addition of the inhibitor to the enzyme preparation. However, with $7{\cdot}5$ and 5×10^{-8} M clorgyline, complete inhibition of enzyme activity had not been reached even after 240 min of preincubation before the addition of substrate. Control experiments showed that no decomposition of the clorgyline occurred during the period of preincubation.

The data from Fig. 1 are replotted in the form of t/(% inhibition) against t in Fig. 2A. With this plot the data points appeared to lie on straight lines. From the slopes of these lines divided by inhibitor concentrations, a value for k_1 (the rate constant for association of enzyme and inhibitor) of about 2×10^6 M^{-1} min^{-1} was found. Fig. 2B shows a family of theoretical plots with the same axes derived from this value of k_1 and several inhibitor concentrations at a fixed enzyme concentration of 10^{-7} M. This enzyme concentration was chosen from the plateau values of the inhibition in Fig. 1 (see Discussion).

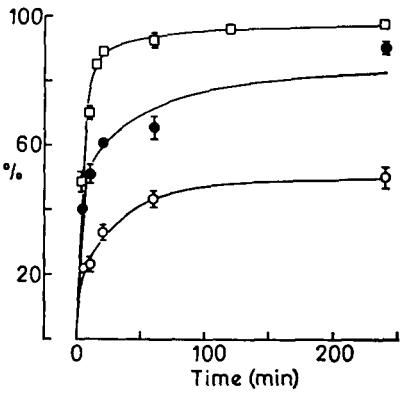

FIG. 1. The time (min) course of the increase in the inhibition of rat liver MAO (%) by clorgyline. Each point represents the mean ± s.e. of a ratio of duplicate determinations from 4 homogenates with 0·25 mM 5-HT as substrate. The standard errors of a ratio are only shown when larger than the size of the points. Clorgyline concentrations were: ○—○, 5×10^{-8}; ●—●, $7{\cdot}5 \times 10^{-8}$; □—□, $1{\cdot}25 \times 10^{-7}$ M.

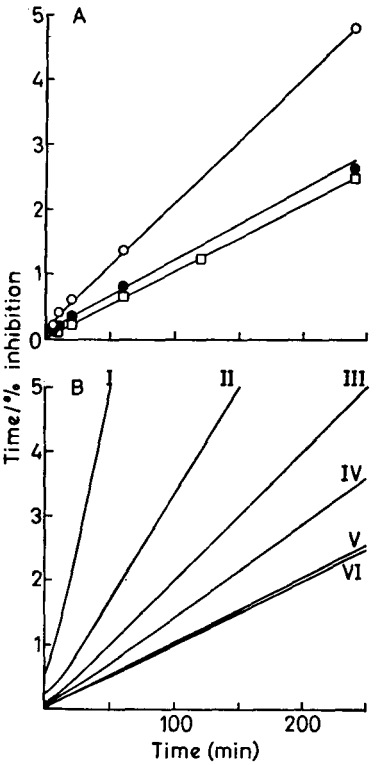

FIG. 2. A. Replot of the data shown in Fig. 1 as time/% inhibition against time. Clorgyline concentrations were: ○—○, 5×10^{-8}; ●—●, $7{\cdot}5 \times 10^{-8}$; □—□, $1{\cdot}25 \times 10^{-7}$ M. B. Theoretical plots of time/% inhibition against time, derived from eqns 7 and 8 where $[E_0] = 10^{-7}$ M and $k_1 = 2 \times 10^6$ M^{-1} min^{-1}. Clorgyline concentrations were chosen as: I, 10^{-8}; II, 3×10^{-8}; III, 5×10^{-8}; IV, 7×10^{-8}; V, 10^{-7}; VI, $1{\cdot}5 \times 10^{-7}$ M.

If the liver homogenates were diluted 1:4 (v/v) with 1 mM phosphate buffer, there was a considerable increase in the apparent sensitivity of the MAO activity to inhibition by clorgyline (Fig. 3).

Homogenates were prepared in which the MAO activity had been depleted by removal of most of the mitochondria. The properties of the MAO that remained in the 'MAO-depleted' homogenates (S2 fractions) were not significantly different from those of the MAO in the original homogenates (S1 fractions) (Table 1). When the protein contents of the MAO-depleted and original homogenates were set to the same value (by dilution of the original homogenates with buffer), the depleted homogenates appeared to be more sensitive to inhibition by clorgyline (Fig. 4A). Further dilution of the original homogenates (S1 fractions) to set the enzyme contents to the same value as those of the S2 fractions, yielded mean protein concentrations of 6·0 mg ml^{-1} for the MAO-depleted, and 1·4 mg ml^{-1} for the original homogenates. In this instance, the original homogenates were inhibited by smaller concentrations of clorgyline (Fig. 4B).

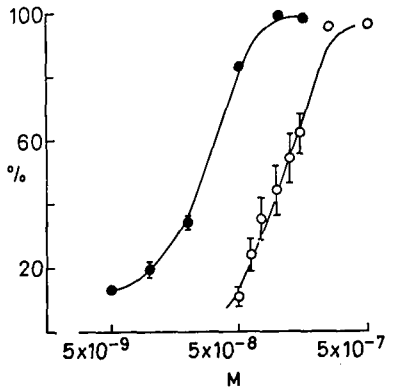

FIG. 3. The effect of dilution of the total protein content of rat liver homogenates on the inhibition of MAO activity (%) by clorgyline (M). Each point represents the mean (± s.e. of a ratio) of duplicate determinations from 5 homogenates with 0·25 mM 5-HT as substrate. Clorgyline preincubation time, 20 min. ○—○, crude homogenates (1:10 w/v); ●—●, crude homogenates (1:40 w/v). The two curves were significantly different (95% Confidence limits of a ratio, Goldstein, 1967).

DISCUSSION

The inhibition of MAO by clorgyline is thought to proceed according to the general reaction:

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_3} EI^* \quad .. \quad .. \quad .. \quad .. \quad (1)$$

(see Rando, 1974)

Table 1. *Kinetic parameters of MAO in original* (S1) *and 'MAO-depleted'* (S2) *homogenates.* The yield of enzyme was determined with 0·25 mM 5-HT. Michaelis constants were determined from initial velocities calculated from 5 substrate concentrations (0·1, 0·2, 0·4, 0·6 and 1 mM 5-HT). The oxygen ratio was determined as activity in an atmosphere of oxygen/activity in air, with 0·25 mM 5-HT as substrate. The ratio of MAO-A: MAO-B was determined from the inhibition of 0·25 mM tyramine activity at a concentration of clorgyline ($1·5 \times 10^{-7}$ M) which inhibited MAO-A but not MAO-B. All assays were in triplicate and expressed in terms of the means ± s.e. (or s.e. of a ratio where appropriate).

	Yield (%)		Kinetic parameters			
Fraction	MAO	Protein	Km (μM)	Vmax†	Oxygen ratio	MAO-A / MAO-B
S1 homogenate	100	100	104 ±26	269·9 ±16·1	1·23 ±0·05	38:62
S2 homogenate	15·7 ±2·5*	72·5 ±1·9*	65 ±30	58·2 ±5·0*	1·38 ±0·23	42:58

* Significantly different from S1 homogenate (95% confidence limits of a ratio, Goldstein, 1967).
† nmol 5-HT metabolized (mg protein)$^{-1}h^{-1}$.

where E = enzyme, I = inhibitor, EI = reversible complex and EI* = stable complex.
Therefore the rate equations can be written:

$$\frac{d[EI]}{dt} = k_1[E][I] - (k_2 + k_3)[EI] \quad .. \quad (2)$$

$$\frac{d[EI^*]}{dt} = k_3[EI] \quad .. \quad .. \quad .. \quad .. \quad (3)$$

At infinite time it can be safely assumed that the reaction has stopped, and therefore d[EI]/dt, d[EI*]/dt = 0 and (from equation 3) [EI] = 0.
Thus from equation (2), $k_1[E][I]$ is also 0.

In the general case, where the concentrations of free inhibitor and enzyme are both reduced by the formation of [EI] and [EI*] complexes, [E] can be expressed as:

$$[E] = [E_0] - [EI] - [EI^*]$$

and I as:

$$[I] = [I_0] - [EI] - [EI^*]$$

where $[E_0]$ and $[I_0]$ are the initial concentrations of enzyme and inhibitor respectively.

Thus at $t \to \infty$,

$$k_1([E_0] - [EI^*])([I_0] - [EI^*]) = 0$$

i.e., $[EI^*] = [E_0]$, or $[EI^*] = [I_0]$.

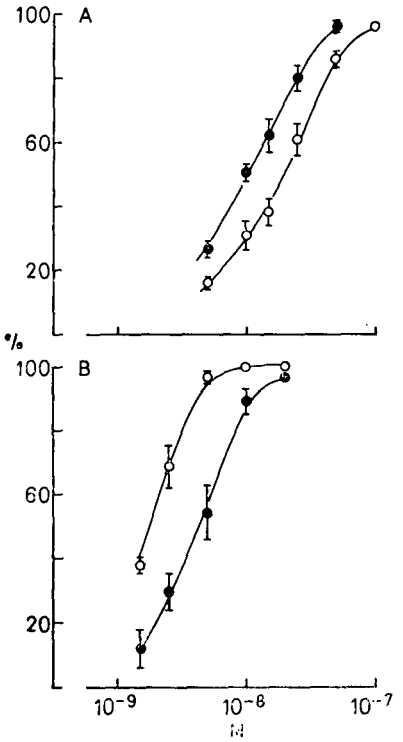

FIG. 4. A comparison between the effect of clorgyline on the MAO activities of: ○—○, S1 homogenates; ●—●, S2 homogenates. The points represent the means (±1 s.e. of a ratio) of triplicate determinations from 3 homogenates, each derived from the livers of 2 rats, with 0·25 mM 5-HT as substrate. Clorgyline preincubation time, 20 min. A. Both homogenates set to a protein content of 13 mg ml^{-1}. B. Both homogenates set to a MAO activity of 450 nmol (of substrate metabolized) ml^{-1} h^{-1}. In both A and B, the inhibition curves are significantly different (95% confidence limits of a ratio, Goldstein, 1967). Ordinate: Inhibition of MAO activity (%). Abscissa: [Clorgyline] (M).

Thus in those situations where the concentration of the inhibitor is less than that of the enzyme, complete inhibition cannot be achieved. At infinite time the degree of inhibition will depend upon the relative concentrations of enzyme and inhibitor. The data in Fig. 1 would suggest that the concentration of enzyme in these experiments is about 10^{-7} M, since when $t \to \infty$, and $[I_0] < [E_0]$, the amount of uninhibited enzyme is [E], where $[E] = [E_0] - [EI^*] = [E_0] - [I_0]$.

In those situations where the inhibitor concentration is higher than the enzyme concentration, complete inhibition can be achieved by infinite time of reaction, similar to that shown for the irreversible reaction between benzilylcholine mustard and the muscarinic receptor (Gill & Rang, 1966).

If equations (2) and (3) are summed, then:

$$\frac{d([EI] + [EI^*])}{dt} = k_1[E][I] - k_2[EI] \quad .. \quad (4)$$

at $t = 0$, both [EI] and $[EI^*] = 0$.

Therefore at $t = 0$ equation (4) becomes:

$$\frac{d([EI] + [EI^*])}{dt_0} = k_1[E_0][I_0] \quad .. \quad .. \quad (5)$$

Thus the initial rate of inhibition is directly proportional to $[E_0][I_0]$. The constant of proportionality is the rate constant of association, (k_1). From the data shown in Figs 1 and 2A, k_1 is about 2×10^6 M^{-1} min^{-1}.

From equation (4), the value of $k_2[EI]$ can be determined at different times. Fig. 5 illustrates a theoretical determination of $k_2[EI]$ taken from the line of best fit from the experimental inhibition by $1{\cdot}25 \times 10^{-7}$ M clorgyline of the deamination of 5-HT by rat liver MAO, shown in Fig. 2A. From Fig. 5 it can be seen that this component reaches a maximum at about 3 min after the addition of inhibitor to the

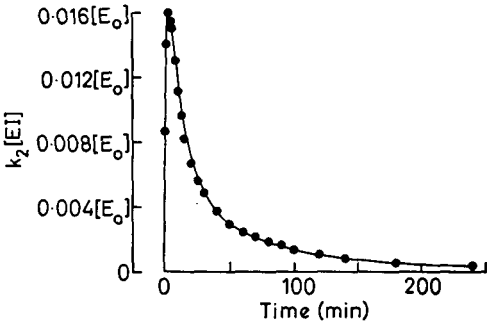

FIG. 5. Theoretical determination of the value of $k_2[EI]$ at different times of preincubation of enzyme with clorgyline. The points were derived from eqn 4 and the line of best fit for the inhibition of enzyme by $1{\cdot}25 \times 10^{-7}$ M clorgyline, shown in Fig. 2A; with $k_1 = 2 \times 10^6$ M^{-1} min^{-1}. Ordinate: $k_2[EI]$ (M min^{-1}).

enzyme, after which it declines. Even at 3 min, the value of $k_2[EI]$ only represents about 6% of the initial value of $k_1[E_0][I_0]$ and can reasonably be disregarded. Consequently, Equation 4 becomes:

$$\frac{d([EI] + [EI^*])}{dt} = k_1[E][I] \quad .. \quad .. \quad .. \quad (6)$$

This equation has two solutions:

(a) $[E_0] = [I_0]$.

$$\frac{1}{([EI] + [EI^*])} = \frac{1}{k_1 t[E_0]^2} + \frac{1}{[E_0]} =$$

$$\frac{1}{k_1 t[I_0]^2} + \frac{1}{[I_0]} \quad .. \quad .. \quad .. \quad (7)$$

(b) $[E_0] \neq [I_0]$.

$$1n\frac{[I_0] - ([EI] + [EI^*])}{[E_0] - ([EI] + [EI^*])} - 1n\frac{[I_0]}{[E_0]} = ([I_0] - [E_0])k_1t \quad . . \quad . . \quad . . \quad . . \quad (8)$$

These solutions are effectively the same as those described by Tipton (1973) for the simple irreversible interaction:

$$E + I \rightarrow EI^*$$

Equations (7) and (8) can be used to generate theoretical values for the degree of inhibition of enzyme activity at any given $[E_0]$, $[I_0]$, t and k_1, where percentage inhibition is given by $(([EI] + [EI^*])/[E_0]) \times 100$. Such curves can be seen in Fig. 2B, where k_1 is set at 2×10^6 M^{-1} min^{-1}, and $[E_0]$ at 10^{-7} M. The curves are similar to the experimentally derived curves shown in Fig. 2A. Furthermore, when $[E_0]$ is reduced, equations (7) and (8) predict that the amount of clorgyline needed to inhibit the enzyme would also be reduced. Dilution of the homogenates to reduce the enzyme concentration produces the same effect (Fig. 3).

If the concentration of clorgyline is increased until it greatly exceeds the concentration of enzyme, the solution shown in equation (8) becomes:

$$1n\frac{[E_0]}{[E_0] - ([EI] + [EI^*])} = [I_0]k_1t \quad . . \quad . . \quad (9)$$

i.e., at high concentrations of clorgyline, the reaction should follow pseudo-first order kinetics. This has been shown experimentally to be the case by Tipton (1971). He showed that the deamination of 5-HT by rat liver mitochondria was inhibited by $2{\cdot}5 \times 10^{-3}$ M clorgyline in such a manner.

The small size of the component $k_2[EI]$ with respect to $k_1[E_0][I_0]$ could be due to two possibilities. First, the rate of association (k_1) could be rate limiting, leading to a negligible accumulation of EI since any that is formed would either rapidly dissociate or be converted to EI*. Second, the rate of dissociation (k_2) is very small so that EI accumulates as a relatively stable complex. At present we cannot distinguish between the two possibilities.

It should be stressed, however, that the present experiments have been done under conditions designed to minimize any significant accumulation of the reversible inhibitor-enzyme complex, EI. If, at the time of the addition of substrate to the reaction mixture, further clorgyline is added to maintain the free inhibitor concentration at its original value, it is just possible that some EI will be generated. The picture would be confused even further by the rapid production of the irreversible complex, EI*, in addition to that already formed during the initial preincubation period.

So far no attention has been paid to the presence of non-specific binding of the clorgyline to sites other than the active centre of the MAO. Although Williams & Lawson (1975) found that [^{14}C]clorgyline did not appear to bind to proteins other than MAO in the rat liver, any significant amount of non-specific binding in situations where the clorgyline concentration was limiting, would overestimate the amount of MAO present in the reaction mixture. The effects of possible non-specific binding have been evaluated by the use of 'MAO-depleted' homogenates (S2 fractions). No significant differences were seen in kinetic properties, oxygen ratio or ratio of MAO-A: MAO-B between the two fractions (Table 1). Thus it seems likely that the MAO in the two fractions differs only in amount.

When both the normal homogenates (S1) and MAO-depleted homogenates (S2) were set to the same protein content, the curve for the inhibition by clorgyline of the MAO-depleted homogenates was found to be to the left of that for the normal homogenates (Fig. 4A), as would be expected. However, when the homogenates were set to the same MAO activity, which in the absence of non-specific binding would be expected to yield curves that were superimposable, it was found that the original homogenates were inhibited by smaller concentrations of clorgyline than the 'MAO-depleted' homogenates. In this case the difference is probably due to the presence of a greater amount of non-specific binding in the MAO-depleted homogenates since they had a much higher protein content.

Thus under the conditions used here, the inhibition of the deamination of 5-HT by homogenates of rat liver can be adequately described by a model which assumes that the amount of inhibitor needed to cause submaximal inhibition is of the same order as that of the enzyme.

It may be possible to use this approach as a crude method for estimation of the amount of MAO enzyme, i.e., the number of MAO active centres, provided that the amount of non-specific binding of clorgyline remains relatively constant.

In the rat heart adrenalectomy has been shown to cause an increase in the specific activity of MAO (Avakian & Callingham, 1968; see Della Corte & Callingham, 1977 a,b). A shift in the clorgyline inhibition curve to the right was seen in homogenates from the adrenalectomized rats when compared

with age-matched contols (Callingham & Laverty, 1973). It is tempting to suggest that if the mechanism described here also applies to the rat heart MAO, then this adds support to the earlier conclusions that adrenalectomy leads to an increase in the amount of MAO enzyme.

Acknowledgements

We wish to thank Drs M. D. L. O'Connor, A. W. Smith and J. M. Young for their help and advice. This work was supported by a generous grant from the British Heart Foundation. C. J. F. is a Medical Research Council Scholar.

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