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The Inhibition of Rat Heart Type A Monoamine Oxidase by Clorgyline as a Method for the Estimation of Enzyme Active Centers

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SUMMARY

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The interaction between clorgyline and the Type-A monoamine oxidase (MAO-A) in homogenates and mitochondrial fractions of the rat heart appears to take place almost entirely at specific binding sites. After an initial reversible interaction, the inhibition of enzyme activity becomes irreversible in the presence of concentrations of clorgyline of the same order as that of the enzyme. Use has been made of this relationship (1) to determine the molecular turnover numbers of the enzyme toward three substrates for the A-form: serotonin, tyramine and β-phenethylamine and (2) to determine the concentration of enzyme active centers in homogenates and mitochondrial fractions of rat hearts. As the rats grew older, both the specific activity of MAO-A, and the concentration of clorgyline/(mg protein) required to cause inhibition of the enzyme activity increased in a parallel fashion. It was concluded that the concentration of active centers of the MAO-A also increased with age. The usefulness of this method of determining the concentration of MAO-A is discussed.

INTRODUCTION

The flavoprotein enzyme, monoamine oxidase (monoamine: O_2 oxidoreductase, EC 1.4.3.4; MAO), appears to exist in more than one catalytically active form (1, 2). Johnston (3) defined two of these forms as type-A and type-B MAO (MAO-A, MAO-B), by the use of the selective inhibitor, clorgyline, and a variety of substrates. MAO-A was inhibited by significantly lower concentrations of clorgyline than was MAO-B. In the rat heart, tyramine, serotonin and β-phenethylamine are metabolized by MAO-A alone, while benzylamine is metabolized by both MAO-A and MAO-B (4-6). In addition, a small percentage of the activity that metabolizes benzylamine and β-phenethylamine is due to a clorgyline-resistant, but semicarbazide-sensitive enzyme (4-6). The substrate specificities of the rat heart MAO-A and MAO-B differ from those seen in many other mammalian tissues, and are exceptional in that no substrate has been found for MAO-B alone (2).

Clorgyline belongs to a class of acetylenic inhibitors of MAO that appear to inhibit the enzyme by a reversible association with the active center of the enzyme, followed

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by a "suicide" reaction with the flavin moiety (7, 8). The kinetics of the inhibition by clorgyline of the deamination of serotonin by rat liver MAO-A are consistent with such a model (9). However, in this tissue, clorgyline is such a potent inhibitor that it is effective in concentrations of the same order as that of the enzyme (9). If the concentration of MAO in the reaction mixture is increased, more clorgyline is then needed to produce the same degree of inhibition (9, 10).

In many tissues the MAO activity appears to be under some sort of hormonal control (11). In the rat heart, for instance, the specific activity of the MAO increases as the animal grows older (12), to give proportionately more MAO-A (13). Although this increase in specific activity could be explained by a decrease in the rate constant of degradation of the enzyme (14), there is no direct evidence to distinguish between an increase in the number of active centers and an increase in molecular turnover number as the cause.

If it could be shown that clorgyline interacts with the active center of the rat heart MAO-A to form an irreversible complex, without appreciable nonspecific binding, some estimate of the number of active centers of this enzyme form should be possible. Any change in the number of active centers produced by growth of the animal could then be detected.

MATERIALS AND METHODS

Twelve rats (of body weight 541 ± 20 g, heart weight 1.35 ± 0.04 g) were killed by a blow to the head, and their hearts removed and divided into four groups, each three hearts. After blotting and trimming away fat and blood vessels, the hearts were homogenized 1:8 (w/v) in "sucrose buffer" (0.25 M sucrose, buffered with 10 mM potassium phosphate buffer, pH 7.8) in a conical glass homogenizer. This buffer was chosen because the best recovery of MAO was seen with buffers of low ionic strength, isotonicity being maintained with sucrose (15). The homogenates were centrifuged at 600 × *g* for 15 min to remove nuclei and cell debris, and the supernatants (crude homogenate fractions) further centrifuged at 6,500 × *g* for 20 min. The pellets were resuspended in sucrose buffer and recentrifuged at 6,500 × *g* for 20 min to yield mitochondrial fractions. The crude homogenates and mitochondrial fractions were stored frozen until used for assay. Control experiments showed that the MAO in the homogenates and mitochondrial fractions had the same K_m toward the three substrates used in this study. When rats of different body weights were used, homogenates and mitochondrial fractions were prepared in the same way, and the body weight shown in the tables.

The MAO activity of the various fractions was assayed by a method modified from that of McCaman *et al.* (16, 17). [^{3}H]serotonin, [^{3}H]tyramine and [^{14}C]β-phenethylamine were used as substrates. Unless otherwise stated, assays were carried out at 37° in an atmosphere of oxygen. Preliminary experiments were undertaken to establish the linearity of the reaction with the three substrates. Progress curves were drawn from incubation periods of 1.5–15 min. There was no significant deviation from linearity in any case up to 3.0 min of incubation. In consequence, all incubation periods used subsequently were within this time.

The efficiency of extraction of the deaminated metabolites into the organic phase of the reaction mixture was estimated as follows: homogenates and mitochondrial fractions were incubated with 0.25 mM of substrate in an atmosphere of either air or oxygen. After extraction of products with water-saturated benzene/ethyl acetate (1:1 v/v), half was counted for radioactivity and the other half back-extracted into an acid-inactivated reaction mixture with only the radiolabel missing. The layers were vortex mixed, separated by centrifugation, and extracted in the normal way (see 17). The extracted organic phase was counted, divided by the count for the initial extraction procedure, and converted into a percentage. The extraction efficiencies were similar for MAO assayed in crude homogenates and mitochondria whether under atmospheres of oxygen or air, and were: serotonin, 93%; tyramine, 90% and β-phenethylamine, 97%. It should be stressed, however, that these values represent an upper limit to the effi-

ciencies of extraction, as the method will bias against metabolites that are poorly extracted into the organic layer. However, similar values for the efficiencies of extraction of the metabolites of these substrates into a toluene/ethyl acetate (1:1 v/v, saturated with water) mixture have been found by use of either the method described above, or by a repeated extraction method (C. J. Fowler and L. Oreland, unpublished observations). These extraction efficiencies, which were unaffected by any of the procedures described here, were used to correct all data in this paper.

When clorgyline was used as the inhibitor, it was preincubated at 37° with the homogenates for various times before addition of substrate for the assay of MAO activity. The time of this incubation period with substrate was limited to 90 sec to minimize further interaction of the clorgyline with the enzyme. As 50 μl of substrate solution was added to 50 μl of the enzyme-clorgyline mixture, the concentration at incubation of the free inhibitor fell to half that at preincubation. 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 *et al.* (18) with bovine serum albumin as standard. MAO activities are expressed in terms of nmol (of substrate metabolized)/(mg protein)/min unless otherwise stated.

Statistical significance between groups was determined by Student's *t*-test on the absolute values, or by 95% confidence limits of a ratio (19) where appropriate.

The radioactive substrates for MAO [^{3}H]tyramine (2 Ci/mmole) and [^{3}H]serotonin (0.5 Ci/mmole) were obtained from the Radiochemical Centre, Amersham, England and [^{14}C]β-phenylethylamine (50 mCi/mmole) from New England Nuclear GMBH, Dreieichenchain, Germany. Clorgyline hydrochloride (M & B 9302) was a gift from May & Baker Ltd., Dagenham, England. All other materials used were standard laboratory reagents of analytical grade where possible. Male Wistar rats were obtained from A. J. Tuck & Son, Rayleigh, England.

RESULTS

When rat heart mitochondria were incubated with tyramine as substrate and clorgyline simultaneously, the MAO activity appeared to be inhibited in a competitive manner (Fig. 1A). Preincubation of enzyme with clorgyline for 1 hour prior to the ad-

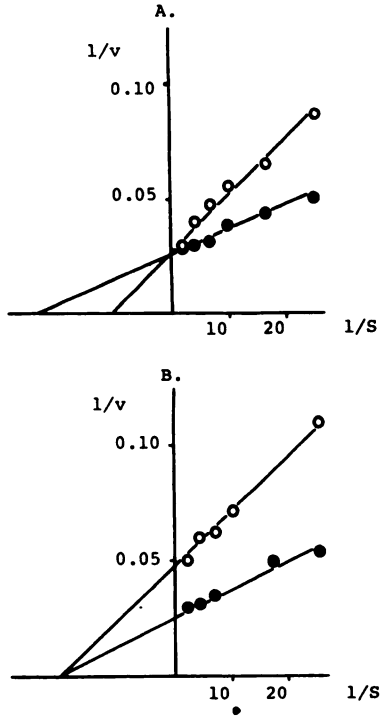

FIG. 1. *Lineweaver-Burk plots of the inhibition of rat heart mitochondrial MAO by clorgyline*

Ordinates: 1/(initial velocity in nmol/mg protein/min); abscissae 1/(substrate concentration in mM). Panel A: Various concentrations of [^{3}H]tyramine were incubated in the absence (●) and presence (○) of clorgyline (1.5 nM) without preincubation of inhibitor with enzyme. Panel B: As for A except: absence (●) and presence (○) of clorgyline (0.15 nM at preincubation) with preincubation of inhibitor with the enzyme for 60 min prior to the addition of substrate. The enzyme in the absence of inhibitor was also preincubated for 60 min with an appropriate volume of distilled water. Each point represents the mean of triplicate determinations in four groups of mitochondrial fractions, each derived from the hearts of three rats. Protein concentrations at preincubation were set to 0.3 mg/ml.

dition of substrate produced an apparent noncompetitive inhibition with concentrations of inhibitor 10 times less than those used initially (Fig. 1B).

Time courses of the increase in inhibition of MAO activity toward three MAO-A substrates, serotonin, tyramine and β-phenethylamine, by three concentrations of clorgyline were measured in mitochondrial fractions of the rat heart (Fig. 2). With 3 nM clorgyline, inhibition was complete by about 60 min. However, with 1.25 and 2 nM clorgyline, complete inhibition of MAO activity was never reached. With these concentrations, the inhibition of MAO activity reached a plateau within 10 min of preincubation, which did not increase even when the period of preincubation was lengthened to 240 min.

When mitochondrial fractions of rat heart were preincubated for 20 min with increasing concentrations of clorgyline, plots of percent inhibition of MAO activity against clorgyline concentration yielded straight lines, passing through the origin (Fig. 3). The concentration of clorgyline required to produce 100% inhibition is equivalent to the enzyme concentration (see DISCUSSION). When the homogenates were diluted 1:4 (v/v) with "sucrose buffer" to reduce the protein and MAO content by 75%, there was a fourfold decrease in the concentration of clorgyline required to produce 100% inhibition of activity. Addition of bovine serum albumin to increase the concentration of nonenzyme protein had no effect on the concentration of clorgyline needed to produce inhibition (Fig. 3).

The amount of clorgyline needed to inhibit the MAO of homogenates was determined in a similar way, straight lines passing through the origin on a plot of percent inhibition against clorgyline concentration again being found. The amount of clorgyline causing 100% inhibition/(mg protein) was determined for both homogenates and mitochondria. The ratios of these values were closely similar to the ratios of the

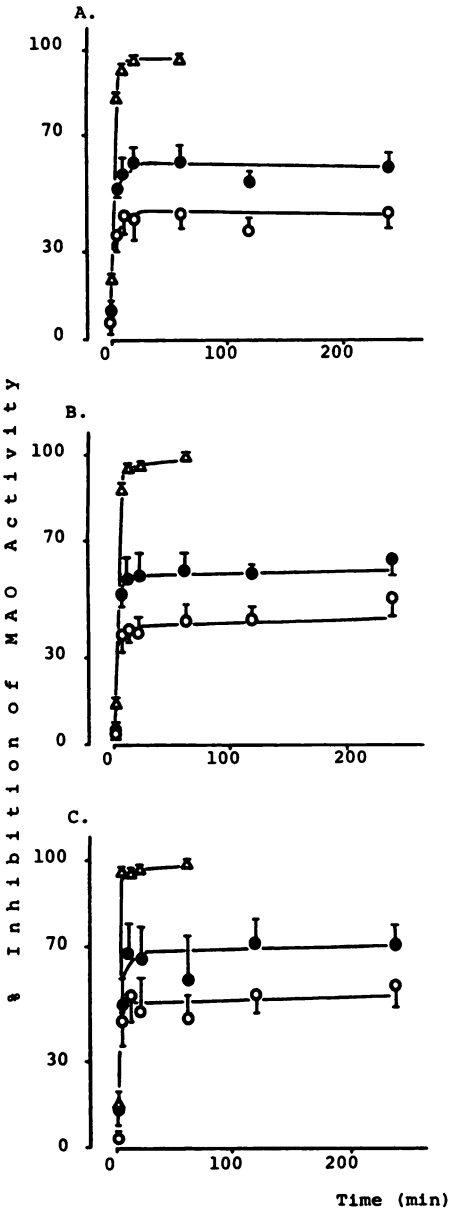

FIG. 2. *Time courses of the increase in the inhibition of rat heart mitochondrial MAO by clorgyline*

Each point represents the mean (± S.E.R.) of duplicate determinations in four groups of mitochondrial fractions, each derived from the hearts of three rats, of the percentage inhibition of MAO activity by clorgyline with respect to mitochondrial fractions preincubated for the same time with distilled water. Substrates used were: A, serotonin (0.25 mM); B, tyramine (0.25 mM); C, β-phenethylamine (0.25 mM). Clorgyline concentrations at preincubation were 1.25 nM (○), 2.0 nM (●) and 3.0 nM (△). There was no significant change in MAO activity of the fractions preincubated with distilled water. Protein concentrations at preincubation were set to 2.0 mg/ml.

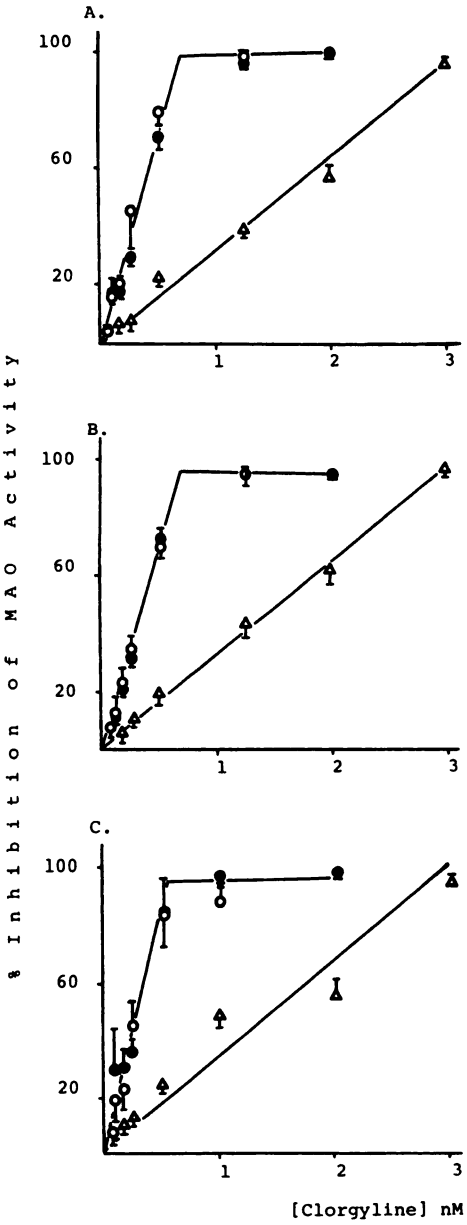

FIG. 3. *The effect of dilution of the total protein content of rat heart mitochondrial fractions on the inhibition of MAO by clorgyline*

Each point represents the mean activity (± S.E.R.) of duplicate determinations in four groups of mitochondrial fractions, each derived from the hearts of three rats, and preincubated with clorgyline for 20 min prior to assay, expressed as percent inhibition of the activity in the absence of inhibitor. Protein contents of the fractions at preincubation were set to: 2.0 mg/ml (△), 0.5 mg/ml (○) and 0.5 mg/ml made up to 2.0 mg/ml with bovine serum albumin (●). Substrates used were: A, serotonin (0.25 mM); B, tyramine (0.25 mM); C, β-phenethylamine (0.25 mM). There was no significant difference between the inhibition produced by the same concentrations of clorgyline in the two fractions that contained 0.5 mg/ml of mitochondrial protein (95% confidence limits of a ratio, 19).

MAO specific activities in these fractions with all three substrates when assayed under an atmosphere of oxygen (Table 1). A similar result was obtained when the MAO activities were assayed in air. Furthermore, when the MAO activities of these two fractions were set to the same level by suitable dilution with sucrose buffer, in spite of their different protein contents, the percent inhibition caused by 0.1 nM clorgyline was approximately the same. For example, with serotonin as substrate, the values were 46% and 41% for mitochondrial fractions and homogenates respectively.

From these data, molecular turnover numbers could be calculated for the MAO toward the three substrates assayed under an atmosphere of air or oxygen. The values in oxygen were in each case significantly higher than those in air, and the values for respective determinations in homogenates and mitochondrial fractions were closely similar. These results are shown in Table 2.

The MAO specific activity and the amount of clorgyline causing 100% inhibition/(mg protein) were determined in homogenates and mitochondrial fractions of hearts from rats of 140, 304 and 487 g mean body weight. In both fractions, as the animals grew older, the specific activity toward serotonin and tyramine increased, with no change in the K_m of the enzyme toward these substrates (Table 3). Plots of percent inhibition against clorgyline concentration were found to be linear, passing through the origin (Fig. 4A & B). These data were used to calculate the amount of clorgyline causing 100% inhibition/(mg protein) for each age group in both homogenates and mitochondrial fractions. The increase in specific activity of MAO toward serotonin and tyramine was accompanied by an increase in amount of clorgyline causing 100% inhibition/(mg protein) (Table 3).

DISCUSSION

Acetylenic inhibitors of MAO react with the enzyme first through a reversible association with the active center, followed by covalent bond formation with the MAO flavin prosthetic group (7, 8). A similar mechanism would account for the inhibition of rat heart MAO-A by clorgyline where initially competitive inhibition is converted to a noncompetitive interaction with time (Fig. 1). The kinetics of this interaction can therefore be described as:

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

TABLE 1

The MAO specific activity and enzyme content of crude homogenates and mitochondrial fractions of rat heart

V_{max} values were calculated from triplicate determinations of MAO activity with six substrate concentrations, incubated at times which were linear with both time of incubation and enzyme concentration, and expressed as nmol (of substrate metabolized)/(mg protein)/min, calculated by the method of Wilkinson (26), and corrected for the efficiency of extraction of the deaminated metabolites. The enzyme content was calculated from the amount of clorgyline needed to inhibit the activity of MAO by 100%, divided by the protein concentration, and expressed as fmol/mg protein, calculated from the lines of best fit (19). Four groups, each derived from the hearts of three rats of body weight 541 ± 20 g were used. "Ratio" describes the ratio of the mean specific activities or enzyme contents of the mitochondrial fractions with respect to those in the homogenates. Values are expressed in terms of means (±S.E.M.). For the determination of enzyme contents, all concentrations of substrate were 0.25 mM. The K_m values for the individual substrates were not different for the two fractions, when assayed in atmospheres of either air or oxygen. The V_{max} values given here are for the activity in oxygen.

Substrate	Crude homogenate	Crude mitochondria	Ratio
Serotonin			
Specific activity	22.78 ± 2.40	45.26 ± 6.74	1.99
Enzyme content	745 ± 45	1605 ± 45	2.15
Tyramine			
Specific activity	25.83 ± 2.16	53.43 ± 5.26	2.07
Enzyme content	680 ± 30	1540 ± 50	2.26
β-Phenethylamine			
Specific activity	2.87 ± 0.33	6.59 ± 1.19	2.30
Enzyme content	695 ± 90	1615 ± 65	2.32

TABLE 2

Molecular turnover numbers of MAO for the same homogenates and mitochondrial fractions as described in TABLE *1*

The values were determined from the V_{max} values divided by the enzyme contents, and expressed as moles (of substrate deaminated)/mole of MAO/min. All assays were performed in triplicate and expressed as means (±S.E.M.). No significant differences were found between the molecular turnover numbers of mitochondrial fractions and homogenates (95% confidence limits of a ratio, 19).

	Molecular turnover numbers moles ((of substrate deaminated)/mole MAO/min)	
	Assayed in oxygen	Assayed in air
Serotonin		
Crude homogenates	29,600 ± 2,400	20,500 ± 2,000*
Crude mitochondria	29,300 ± 6,400	19,500 ± 4,500*
Tyramine		
Crude homogenates	36,100 ± 2,000	24,600 ± 1,100*
Crude mitochondria	34,200 ± 4,800	24,500 ± 3,100*
β-Phenethylamine		
Crude homogenates	5,100 ± 900	3,400 ± 500*
Crude mitochondria	4,300 ± 1,200	2,500 ± 600

* Significantly different from the corresponding values obtained in oxygen (95% confidence limits of a ratio, 19).

TABLE 3

The effect of age upon the specific activity (V_{max}) and K_m of MAO and upon the enzyme content of homogenates and mitochondrial fractions of rat heart

V_{max} (corrected for the efficiency of extraction of the deaminated metabolites) and K_m values were determined by the method of Wilkinson (26) from duplicate determinations at six substrate concentrations. Enzyme contents were determined by line of best fit to data from at least six concentrations of clorgyline by the method described in TABLE 1, with 0.25 mM substrate. Results are expressed as means ± S.E.M. Statistical significance was measured by Student's *t*-test upon the original values. Significant values refer to comparisons of all groups with the Group A values.

Group A: 4 groups, each of 6 rats, body weight 140 ± 2 g, heart weight 468 ± 8 mg; Group B: 4 groups, each of 6 rats, body weight 304 ± 4 g, heart weight 865 ± 17 mg; Group C: 4 groups, each of 6 rats, body weight 487 ± 15 g, heart weight 1231 ± 34 mg. Figures in brackets represent the absolute values as a percentage of the values from Group A.

	Group	K_m (μM)	V_{max} (nmol/mg protein/min)	Enzyme content (fmol/mg protein)
Crude homogenates				
Serotonin	A	134 ± 31	5.23 ± 0.48 (100)	261 ± 10 (100)
	B	92 ± 12 (NS)	7.59 ± 0.33** (145)	340 ± 23* (130)
	C	112 ± 30 (NS)	25.01 ± 2.61** (479)	1147 ± 37** (439)
Tyramine	A	39 ± 7	4.66 ± 0.25 (100)	267 ± 11 (100)
	B	40 ± 13 (NS)	8.04 ± 0.78* (172)	368 ± 16* (138)
	C	51 ± 12 (NS)	26.36 ± 2.22** (565)	1154 ± 40** (432)
Crude mitochondria				
Serotonin	A	68 ± 11	11.18 ± 0.57 (100)	654 ± 37 (100)
	B	69 ± 10 (NS)	16.25 ± 0.69** (145)	820 ± 57* (125)
	C	100 ± 20 (NS)	47.81 ± 3.40** (428)	3302 ± 201** (505)
Tyramine	A	44 ± 8	16.90 ± 0.99 (100)	666 ± 37 (100)
	B	47 ± 17 (NS)	25.53 ± 2.83* (151)	838 ± 46* (126)
	C	68 ± 13 (NS)	75.74 ± 5.43** (448)	3164 ± 146** (475)

NS, not significant.
* $p < 0.05$.
** $p < 0.01$.

where E = enzyme, I = clorgyline, EI = reversible complex and EI^* = irreversible enzyme-inhibitor adduct.

The rate equations can be written:

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

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

These equations have been considered in detail in the case where both enzyme and inhibitor concentrations are reduced by the formation of EI and EI^* (9). The present discussion is restricted to the boundary conditions of these equations.

At infinite time, it can be assumed that all reactions have stopped, and therefore $d[EI]/dt$ and $d[EI^*]/dt = 0$. From Eq. 3, $[EI]$ is also 0. Therefore, at infinite time, $k_1[E][I] = 0$. In the general case, where the concentrations of free enzyme and inhibitor are both reduced by the formation of enzyme-inhibitor complexes, $[E]$ and $[I]$ can be expressed as:

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

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

Where $[E_o]$ and $[I_o]$ are initial concentrations of enzyme and inhibitor, respectively. Thus at infinite time,

$$k_1([E_o] - [EI^*])([I_o] - [EI^*]) = 0 \quad (4)$$

i.e., two solutions are found. When $[I_o] < [E_o]$, $[EI^*] = [I_o]$; and when $[I_o] > [E_o]$, $[EI^*] = [E_o]$. Thus, at infinite time, a plot of percent inhibition against concentration of inhibitor should give a straight line passing through the origin. The concentration

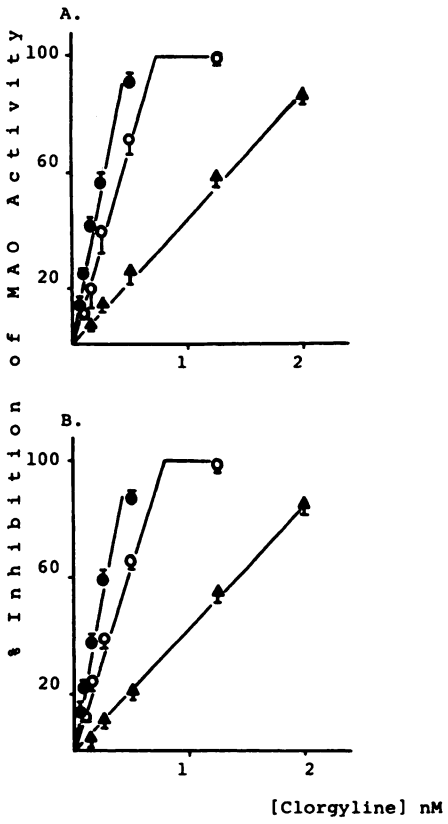

FIG. 4. *The effect of age on the inhibition of rat heart MAO-A by clorgyline*

Each point represents the mean activity (± S.E.R.) of duplicate determinations in four groups of homogenates each derived from the hearts of six rats and preincubated with clorgyline for 20 min prior to assay, expressed as percent inhibition of the activity in the absence of inhibitor. Body weights of the rats were 140 ± 2 g (●); 304 ± 4 g (○) and 487 ± 15 g (▲). Protein concentrations of the homogenates at preincubation were set to 2 mg/ml. Substrates used were: A, serotonin (0.25 mM); B, tyramine (0.25 mM).

of inhibitor required to give exactly 100% inhibition equals the concentration of enzyme in the reaction mixture, provided that there are no other factors such as nonspecific binding of the inhibitor to sites other than the active center of the enzyme.

The inhibition of rat heart MAO-A by clorgyline follows a time-dependent reaction, with the inhibition of enzyme activity reaching a plateau by about 10 min (Fig. 2). In the rat liver, about 1 hr is required for the inhibition to reach the plateau value (9). The inhibition of MAO-B activity in the rat liver by clorgyline with β-phenethylamine as substrate needed 4 hr (20). No assessment of the time course for the inhibition of activity of MAO-B in the rat heart has been possible as no substrate for this form alone has been found (2).

The value for k_1 (rate constant of association of the reversible component) can be calculated from the data in Figure 2 by the method described previously (9) to give a value of about 10^8/m/min. In the rat liver, the corresponding value is about 50 times less (2×10^6/m/min) (9). From theoretical considerations (see 9) the maximum concentration of the reversible complex (*EI*) appeared at about 2 min after the addition of clorgyline to the enzyme, but no accurate experimental measure was possible due to the speed of the reaction.

For the MAO-A of rat heart, the plateau value represents the situation at time infinity, when all reactions have stopped. Thus a plot of percent inhibition against the concentration of clorgyline will be a straight line passing through the origin (Fig. 3). Furthermore, when the mitochondrial fractions are diluted fourfold, there is a fourfold decrease in the concentration of clorgyline required to produce 100% inhibition (Fig. 3). Addition of bovine serum albumin to increase the nonenzyme protein content does not change the amount of clorgyline required for inhibition (Fig. 3), which would suggest that the clorgyline does not bind irreversibly to this particular protein. In fact there seems to be no detectable nonspecific binding after 20 min of preincubation, as the molecular turnover numbers for homogenate and mitochondrial fractions appear to be the same (Table 2). Moreover, when the fractions are set to the same MAO activity, the degree of inhibition by clorgyline is the same. This is in contrast to the liver, where a considerable amount of nonspecific binding has been found (9).

Another complication in the rat liver is the presence of clorgyline metabolizing enzymes. Preincubation of rat liver homogenates for 1 hr with 50 μM SKF 525A, a concentration that inhibits the microsomal enzymes (21) did not itself cause any inhibition of MAO, but slightly decreased the concentration of clorgyline needed to in-

hibit the MAO (unpublished observations). In the rat heart, this concentration of SKF 525A, which caused a substantial inhibition of MAO activity by itself, did not change the concentration of clorgyline needed to produce the same degree of inhibition as that seen in the absence of SKF 525A. Furthermore, the molecular turnover numbers for MAO from homogenates, which have a large amount of microsomal content, are the same as those for the mitochondrial fraction, in which the microsomal contamination is much smaller (Table 2). This would also suggest that the microsomal enzymes do not play a significant part in the reaction of clorgyline with rat heart MAO.

The deamination of β-phenethylamine and benzylamine in the rat heart is brought about not only by MAO, but also by a clorgyline-resistant, semicarbazide-sensitive enzyme found largely in the supernatant fraction (4–6). However, in the present results, this activity accounted for less than 5% of the total enzyme activity toward β-phenethylamine and did not contribute at all to the deamination of either serotonin or tyramine. Since the proportion of the clorgyline-resistant component declines with age (13), the homogenates were obtained from old rats (541 ± 20 g) when β-phenethylamine was used as substrate to minimize any contribution from this enzyme.

The very low level of nonspecific binding makes it possible to use the inhibition of MAO activity in rat heart homogenates by clorgyline as an assay for the number of MAO active centers/(mg protein), and an estimate to be made of the molecular turnover numbers for rat heart MAO toward the different substrates for MAO-A (Table 2). The values reported here are much higher than those reported by Egashira *et al.* (22) who used preincubation times of 4 hr duration and pargyline as the inhibitor, which is MAO-B selective in most tissues (2). At present, the reason for this discrepancy is not clear but may be associated with the different inhibitors used.

The values for the molecular turnover numbers when the assay was done in air were about two-thirds of those obtained when the assay was done in oxygen (Table 2). This is to be expected, as the deamination of substrates follows a ping-pong, or double displacement, reaction in most tissues (see 23, 24), but it is important to specify the oxygen concentration in the assays when the molecular turnover numbers are calculated.

The inhibition of the activity of MAO-A by clorgyline described above was used to determine the effects of growth upon the number of active centers of this enzyme form in the rat heart. Horita (12) has shown that there is an increase in MAO specific activity as the rat grows older. Turnover studies after irreversible inhibition of MAO with pargyline *in vivo* indicated that this increase is due to a decreased rate of apparent degradation of the enzyme, rather than an increase in the rate of synthesis (14, 25). Furthermore, there appears to be a selective increase in the specific activity of MAO-A (13).

The results in Table 3 and Figures 4A and B show that, as the rats grew older, there was an increase in the amount of clorgyline needed to produce the same degree of MAO inhibition. This would suggest that the increase in the specific activity of MAO toward the substrates tyramine and serotonin is accompanied by a concomitant increase in the number of enzyme active centers responsible for the deamination of these substrates. Thus it would seem that the increase in MAO specific activity with age is due to an increased number of available active centers, rather than to an increased molecular turnover number of the enzyme. At present, although other evidence indicated a reduction in the rate of enzyme degradation as the cause, which would lead to an increased number of enzyme active centers, there is still uncertainty concerning the mechanism responsible for this increase in the number of active centers of MAO in the rat heart as the animal grows older.

This approach toward an estimate of the number of the active centers of MAO-A was possible due to the exceptional properties of the rat heart: a high rate of association (k_1) between clorgyline and the enzyme leading to a fast reaction; a very low amount of nonspecific binding; and no sign

of drug metabolism *in vitro*. In view of the less satisfactory situation found in the rat liver (9), the use of this assay in tissues other than the rat heart must be evaluated.

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