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INHIBITION BY CLORGYLINE AND DEPRENYL OF THE DIFFERENT FORMS OF MONOAMINE OXIDASE IN RAT LIVER MITOCHONDRIA

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Abstract—The type of inhibition of the different forms of monoamine oxidase by clorgyline (preferentially 'A' form-inhibiting) and deprenyl (preferentially 'B' form-inhibiting) has been investigated. For both inhibitors a reversible phase of inhibition was found to precede the irreversible reaction. When incubated at 25° for 20 min, clorgyline inhibited the 'A' form in an irreversible fashion, while 4 hr at 37° was needed to inhibit the 'B' form irreversibly. In contrast, deprenyl inhibited the 'A' form reversibly even when incubated at 37° for 4 hr, whereas the 'B' form was irreversibly inhibited under these conditions. The selectivity of both inhibitors was considerably lower with longer incubation times. The implications of the results for the interpretation of previous findings on multiple forms of monoamine oxidase (an 'A' and a 'B' form) and for chronic treatment with the inhibitors *in vivo* is discussed.

There are many reports postulating the existence of multiple forms of monoamine oxidase either directly by partial separation or indirectly by kinetic studies (for a review see [1]). In particular, many studies of the latter kind have been performed since Johnston [2] introduced the inhibitor clorgyline and found that one part of monoamine oxidase activity in rat brain was highly sensitive to that inhibitor (the 'A' form of the enzyme) and that another part was considerably less sensitive (the 'B' form of the enzyme). At about the same time Knoll *et al.* [3] found that deprenyl inhibited the 'B' form of rat liver monoamine oxidase at low concentrations and the 'A' form only at considerably higher concentrations. These two forms of the enzyme have been found in many other tissues from various species, [4–7]. Moreover, it has been shown that in, for example, liver and brain from rat and man, serotonin and norepinephrine are mainly oxidized by the 'A' form of monoamine oxidase and that benzylamine and β-phenylethylamine are preferred substrates for the 'B' form, while tyramine and dopamine are substrates for both forms of the enzyme [8].

It is of interest whether the two functional forms of monoamine oxidase are due to two different enzyme species or to one single species with allotropic properties [8, 9]. When interpreting the results obtained from inhibition by clorgyline and deprenyl, the point arises whether the inhibition was irreversible or not. If the inhibition was irreversible, the results seem clearly to demonstrate the presence of two different monoamine oxidase active sites. If the inhibition was reversible, however, the results might as well be explained by one active site which might bind different substrates in different ways as proposed by Severina [9].

Knowing that both clorgyline and deprenyl belong to the pargyline-type of monoamine oxidase inhibitors in which a reversible phase is known to precede the irreversible "suicide reaction" [10], we have, in this communication, studied in detail the nature of the inhibition with the two inhibitors under the conditions generally used.

MATERIAL AND METHODS

Chemicals

Preparation of mitochondria. Male Sprague–Dawley rats, weighing 200–250 g, were killed by a blow on the head and the livers were quickly removed and chilled. Mitochondria were prepared in 0.25 M sucrose as described earlier [11], with the exception that a Potter–Elvehjem homogenizer was used. The volume was adjusted to contain 50 mg of protein per ml.

Assay of monoamine oxidase. Monoamine oxidase activity was estimated with [^{14}C]serotonin (0.5 mM), [^{14}C]tyramine (0.5 mM), or β-[^{14}C]phenylethylamine (0.05 mM) as substrate, as described earlier [6].

Estimation of protein. The protein content was estimated according to Lowry *et al.* [12] with human serum albumin as a standard.

RESULTS

The effect of increasing concentrations of clorgyline (preferentially 'A' form inhibitor) on the monoamine oxidase activity in rat liver mitochondria is shown in Fig. 1. After incubation at 25° for 20 min with the inhibitor, the monoamine oxidase activity with serotonin as substrate (substrate for the 'A' form of the enzyme) was highly sensitive and the activity towards β-phenylethylamine (substrate for the 'B' form) was less sensitive to the inhibitor, while a plateau-shaped curve was obtained with tyramine as substrate, indicating that this substrate was oxidized by both the 'A' and the 'B' form of the enzyme. These results are in agreement with the findings previously reported by several groups [2–4, 7, 13, 14]. After incubation of the mitochondrial preparation with clorgyline at 37° for 4 hr no significant difference was obtained with serotonin as substrate as compared to incubation at 25° for 20 min, whereas considerably lower concentrations of clorgyline now were needed

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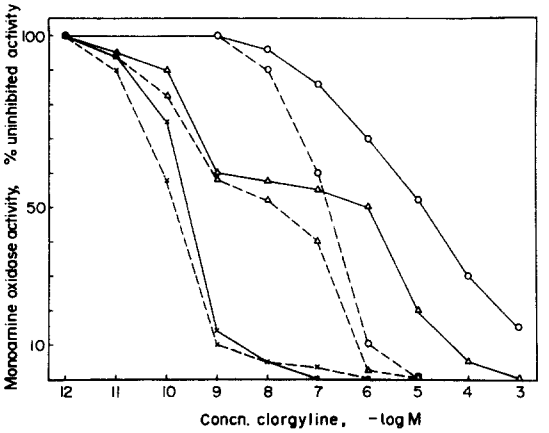

Fig. 1. Inhibition of monoamine oxidase activity towards serotonin, tyramine and β-phenylethylamine by various concentrations of clorgyline at different times and temperatures. Rat liver mitochondria (60 μg of protein) were incubated at 25° for 20 min or at 37° for 4 hr in the presence of various concentrations of clorgyline in a total volume of 275 μl of potassium phosphate (0.01 M, pH 7.4). Labelled substrate (25 μl) was then added to estimate the monoamine oxidase activity. ×—×—× incubation at 25° for 20 min with clorgyline prior to estimation of monoamine oxidase activity with serotonin as substrate; ×---×---× 37°, 4 hr, serotonin; Δ—Δ—Δ 25°, 20 min, tyramine; Δ---Δ---Δ 37°, 4 hr, tyramine; O—O—O 25°, 20 min, β-phenylethylamine; O---O---O 37°, 4 hr, β-phenylethylamine.

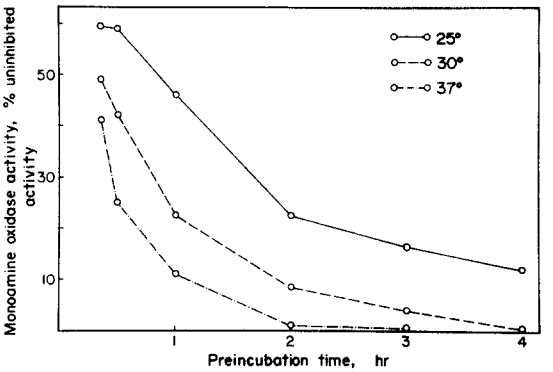

Fig. 2. The effect of incubation time and temperature on the inhibition by clorgyline of monoamine oxidase activity towards β-phenylethylamine. Rat liver mitochondria were incubated for the times indicated at different temperatures in the presence of 10^{-5} M clorgyline in a total volume of 275 μl of potassium phosphate (0.01 M, pH 7.4). β-[^{14}C]Phenylethylamine was then added to estimate the monoamine oxidase activity.

to inhibit the activity towards β-phenylethylamine. The result obtained with tyramine as substrate is in agreement with the findings for the other two substrates, i.e. incubation time and temperature was only of importance for the effect of clorgyline on the 'B' form of the enzyme.

The effect of incubation time and temperature on the inhibition by clorgyline of the 'B' form of monoamine oxidase was further studied with β-phenylethylamine as substrate. As shown in Fig. 2, 10^{-5} M clorgyline inhibited about 40 per cent of the activity after 20 min of incubation at 25° and with increasing incubation time and temperature the degree of inhibition approached 100 per cent.

In order to investigate whether the inhibition by clorgyline was reversible or irreversible, the mitochondria were repeatedly washed after the incubation with the inhibitor (Table 1). Even at a low concentration of clorgyline (10^{-10} M), no significant increase of the activity towards serotonin was obtained by washing, while even at a high concentration of clorgyline (10^{-5} M) there was a marked reactivation when β-phenylethylamine was used as substrate (from 54 to 86 per cent). With tyramine as substrate there was a reactivation by washing only at high concentration of clorgyline. These results support the conclusion that the inhibition of the 'A' form of monoamine oxidase by clorgyline was irreversible, while that of the 'B' form, to a great extent, was reversible.

The effect of the incubation time with the inhibitor, clorgyline is further demonstrated in Fig. 3, in which it can be seen that the effect of washing on inhibition decreased with the length of the incubation time.

The degree of reversibility of the inhibition of the monoamine oxidase activity by clorgyline was further studied by means of the titration technique devised by Ackerman and Potter [15] and previously applied to monoamine oxidase with inhibitors of the pargyline-type [10, 16, 17]. In these experiments various amounts of mitochondria were incubated with a fixed

Table 1. Effect of washing on the inhibition of monoamine oxidase activity after incubation of mitochondria with clorgyline*

Substrate	Clorgyline	Remaining activity (%)†	
		Before washing	After washing
Serotonin	10^{-10}	45	50
Tyramine	10^{-10}	75	73
	10^{-8}	60	68
	10^{-5}	12	57
β-Phenylethylamine	10^{-5}	54	86

* Samples of the mitochondrial preparation (625 μg protein) were incubated with the indicated concentrations of clorgyline in a total volume of 2.75 ml of 0.01 M potassium phosphate (pH 7.4) for 20 min at 25°. Ten ml of potassium phosphate (pH 7.4) was then added and the mitochondria were spun down at 100,000 *g* for 20 min. The pellet was washed once again and then suspended in 2.75 ml of the same buffer. Samples were taken for estimation of monoamine oxidase activity.

† The remaining activity is expressed as per cent of the activity in a control experiment without clorgyline.

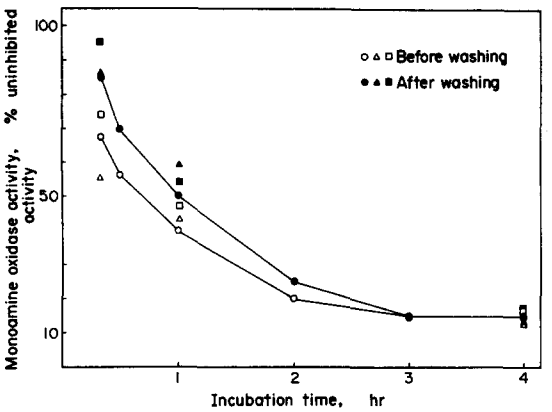

Fig. 3. Effect of washing on the inhibition of monoamine oxidase activity after incubation with clorgyline for different times. Samples of the mitochondrial preparation were incubated with 10^{-6} M clorgyline at various times and then washed as described in Table 1. The activity was estimated with β-phenylethylamine as substrate. The result from one representative experiment is demonstrated as well as data from two additional experiments at 20 min, 1 and 4 hr.

concentration of clorgyline prior to estimation of activity. If the inhibition is reversible, the slope of the activity curve will be decreased in the presence of the inhibitor, but the curve will still originate from the origin. If the inhibition is irreversible, on the other hand, the curve will be parallel to the activity curve without inhibitor and originate from a point on the abscissa to the right of the origin. As shown in Fig. 4, a curve indicating at least partially reversible inhibition was obtained with β-phenylethylamine as substrate after incubation of the mitochondrial preparation with 10^{-5} M clorgyline at 25° for 20 min. When the preparation was incubated for 4 hr at 37°, irreversible inhibition of the activity towards β-phenylethylamine was obtained, although the concentration of clorgyline was lower (10^{-6} M). With serotonin as substrate incubation with 10^{-9} M clorgyline at 25° for 20 min was sufficient to cause irreversible inhibition.

When corresponding experiments were performed with deprenyl instead of clorgyline as the inhibitor, opposite results were obtained (Figs. 5 and 6). Now, β-phenylethylamine oxidation was most sensitive and the serotonin oxidation least sensitive to the inhibitor (Fig. 5). The increase in the degree of inhibition by deprenyl upon an increase in time and temperature of the incubation was most pronounced for the oxidation of serotonin and the 'A' form oxidation of tyramine, while the effect on β-phenylethylamine oxidation was relatively small.

The titration experiment performed with deprenyl (10^{-7} M) (Fig. 6) showed a curve typical for reversible inhibition with β-phenylethylamine as substrate after 20 min of incubation at 25°. When the incubation time was increased to 4 hr and the temperature to 37°, irreversible inhibition was obtained with β-phenylethylamine, although the concentration of the inhibitor was lower (10^{-8} M). With serotonin as substrate, however, reversible inhibition was obtained even after 4 hr of incubation at 37° with 5×10^{-7} M deprenyl.

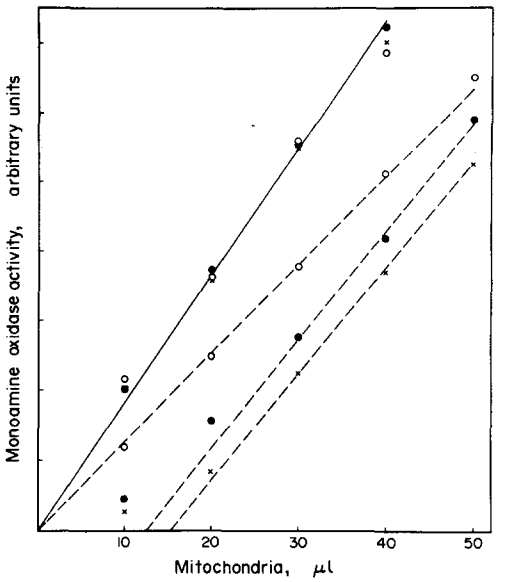

Fig. 4. Effect of time and temperature on the inhibition by clorgyline of monoamine oxidase activity towards β-phenylethylamine and serotonin. Various amounts of rat liver mitochondria were incubated at 25° for 20 min or at 37° for 4 hr with clorgyline in a total volume of 0.275 ml of potassium phosphate (0.01 M, pH 7.4). In the control experiments no clorgyline was present. After the incubation 25 μl of labelled substrate was added to estimate the monoamine oxidase activity, as described in the text. The units of the monoamine oxidase activity were chosen so as to give a common curve for β-phenylethylamine and serotonin in the absence of clorgyline irrespective of incubation time and temperature. O——O incubation without clorgyline at 25° for 20 min prior to estimation of monoamine oxidase activity with β-phenylethylamine as substrate; O---O incubation in the presence of 10^{-5} M clorgyline at 25° for 20 min, β-phenylethylamine; ●——● without clorgyline at 37° for 4 hr, β-phenylethylamine; ●---● 10^{-6} M clorgyline at 37° for 4 hr, β-phenylethylamine; ×——× without clorgyline at 25° for 20 min, serotonin; ×---× 10^{-9} M clorgyline at 25° for 20 min, serotonin.

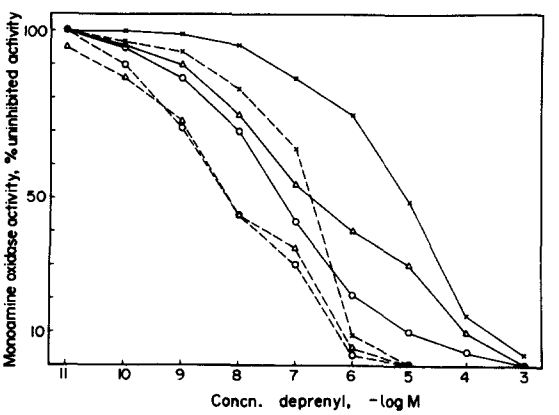

Fig. 5. Inhibition of monoamine oxidase activity towards serotonin, tyramine and β-phenylethylamine by various concentrations of deprenyl at different times and temperatures. The experiments were carried out as described in the legend to Fig. 1 but with deprenyl instead of clorgyline as inhibitor. The symbols represent the same temperatures, incubation times and substrates as described in the legend to Fig. 1.

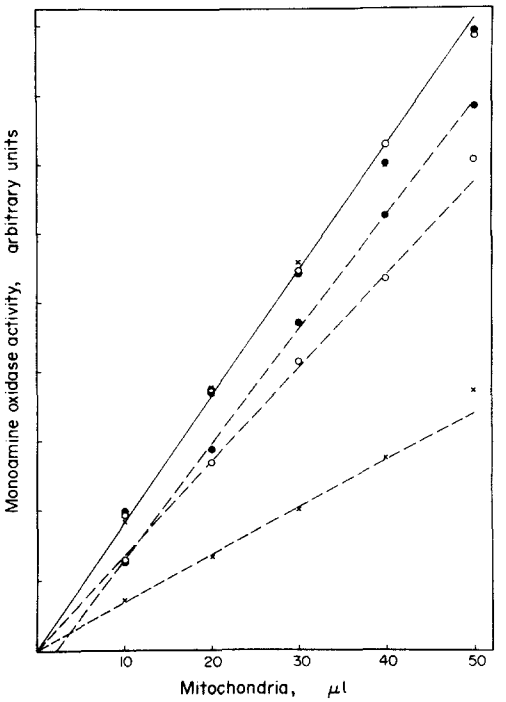

Fig. 6. Effect of time and temperature on the inhibition by deprenyl of monoamine oxidase activity towards β-phenylethylamine and serotonin. The experiments were carried out as described in the legend to Fig. 4, but with deprenyl instead of clorgyline. O——O incubation without deprenyl at 25° for 20 min prior to estimation of monoamine oxidase activity with β-phenylethylamine as substrate; O---O incubation in the presence of 10^{-7} M deprenyl at 25° for 20 min, β-phenylethylamine; ●——● wthout deprenyl at 37° for 4 hr, β-phenylethylamine; ●---● 10^{-8} M deprenyl at 37° for 4 hr, β-phenylethylamine; ×——× without deprenyl at 37° for 4 hr, serotonin; ×---× in the presence of 5×10^{-7} M deprenyl at 37° for 4 hr, serotonin.

DISCUSSION

The concept of two different functional forms of monoamine oxidase, the 'A' and 'B' form, is based on the selective inhibition of the 'A' form by low concentrations of clorgyline [2]. At high concentrations of clorgyline, however, the 'B' form is also inhibited. Compared to the inhibition of the 'A' form, an approximately 1000 times higher concentration of the inhibitor was needed to inhibit the 'B' form activity when the inhibitor and enzyme were incubated for 20 min at 25° prior to estimation of the activity [14]. Correspondingly, the 'B' form is about 1 000 times more sensitive to inhibition by deprenyl than the 'A' form [14].

In the present investigation clorgyline and deprenyl were incubated with rat liver mitochondria prior to estimation of monoamine oxidase activity in order to study the effect of incubation time and temperature on the inhibition of the two forms of the enzyme. The results demonstrate that a reversible phase of inhibition preceded the irreversible phase. This reversible phase was most pronounced when the 'A' form was to be inhibited by deprenyl (Fig. 6) and the 'B' form by clorgyline (Fig. 4). It can be concluded that at least the major part of the 'A' form of the enzyme ought to have been irreversibly inhibited by clorgyline with the incubation conditions normally used [2, 7, 13, 14, 18]. Since irreversible inhibition implies that the flavine part of the active site of the enzyme has been inactivated by the formation of a covalent flavin-inhibitor adduct [19, 20] it seems impossible to interpret the results shown in, for example, Fig. 1 in other than by the existence of two different active sites. If reversible inhibition had occurred the results would have been compatible also with one active site, which according to Severina [9] contains at least two different substrate binding areas with one common flavin group. Whether the active sites are chemically different or are due to allotropic properties cannot be stated with the present experiments. In a previous communication, however, we have put forward some evidence for the existence of chemically different forms of the enzyme [14]. When the incubation time was prolonged and the temperature elevated the inhibition both by clorgyline and deprenyl tended to be less selective (Figs. 1 and 5). This may be of importance when these inhibitors are given chronically *in vivo*, although it has been shown that the selectivity was retained in acute experiments [7, 18, 21, 22].

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