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SHORT COMMUNICATIONS

Inhibition of monoamine oxidase by clorgyline and deprenyl in circumventricular structures of rat brain

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Since Johnston [1] and Knoll and Magyar [2] reported specific monoamine oxidase (MAO) (EC 1.4.3.4) inhibitors, such as clorgyline, a preferential inhibitor for MAO-A_2 and deprenyl, a preferential inhibitor for MAO-B, these two types of MAO have been found in many tissues from various species [3–5]. Some tissues, however, contain only one type, e.g. human placenta [6] contains only MAO-A and platelets [7, 8] contain only MAO-B, and the proportions of the two types of MAO have been shown to vary widely from tissue to tissue [3, 4]. Recently, Williams *et al.* [9], from studies using a histochemical technique [10], suggested the possible existence of an additional type of MAO, MAO-C, distinct from MAO-A and MAO-B, in circumventricular structures of rat brain. This suggestion was based on the finding that, with serotonin (5-hydroxytryptamine) as substrate, the enzyme activity could not be inhibited completely by concentrations of clorgyline usually sufficient to inhibit both MAO-A and MAO-B [9].

Previously [11], we reported histochemical results suggesting that evidence for the existence of MAO-C in rat brain was due to too short a preincubation time with clorgyline and too low a preincubation temperature. In the present work, we carried out biochemical studies, using three different types of substrate, on the possible existence of MAO-C, and on inhibition of MAO in circumventricular structures of rat brain by selective inhibitors, since the previous histochemical technique cannot be used with substrates other than serotonin.

(−)Deprenyl and clorgyline were gifts from Dr. K. Magyar, Department of Pharmacology, Semmelweis University of Medicine, Budapest, Hungary, and from May & Baker Ltd., Dagenham, U.K., respectively. Radiochemical substrates, [2-^{14}C]-5-hydroxytryptamine binoxalate (serotonin, 44 mCi/mmole), [1-^{14}C]tyramine hydrochloride (56 mCi/mmole), and [1-^{14}C]-β-phenylethylamine hydrochloride (PEA, 50.98 mCi/mmole), were purchased from New England Nuclear, Boston, MA, U.S.A. All other chemicals used were of the highest commercial grade available.

Male Wistar rats, weighing about 200 g, were decapitated; their brains were quickly removed, and the circumventricular structures were carefully isolated using the atlas of König and Klippel [12]. The tissues from several rats were pooled and homogenized in a small volume of 10 mM phosphate buffer (pH 7.6), containing 320 mM sucrose, in a glass homogenizer. Protein was determined by the modified biuret method [13], with bovine serum albumin as a standard. MAO activity was determined radiochemically by the method described earlier [14] using [^{14}C]tyramine (0.5 mM), [^{14}C]serotonin (0.5 mM) and [^{14}C]PEA (0.3 mM, unless otherwise stated) as substrates.

The inhibitory effects of clorgyline and deprenyl on MAO in homogenates of circumventricular structures of rat brain were studied. On preincubation with the inhibitor for 5 min at room temperature (about 20°), as in the work of Williams *et al.* [9], the MAO activity toward serotonin was found to be most sensitive to clorgyline, while activity toward tyramine and more particularly toward PEA was less sensitive to this inhibitor. MAO activity toward serotonin was found not to be inhibited completely by 10^{-4} M clorgyline (about 10 per cent of the activity remained), in accordance with our previous observations using a histochemical technique [11]. With tyramine and PEA as substrates, about 20 and 35 per cent of the activity remained after preincubation with 10^{-4} M clorgyline. Plots of the degrees of inhibition of tyramine and PEA oxidation against the concentration of clorgyline gave double-sigmoidal curves, indicating that tyramine and PEA may be oxidized by at least two types of MAO. When the substrate PEA was used at a low concentration (0.01 mM), however, the plot for inhibition by clorgyline gave a single-sigmoidal curve and activity was less sensitive to this inhibitor, indicating oxidation by MAO-B only. The pI_{50} values with clorgyline toward serotonin, tyramine and PEA were 10^{-8} M, 5.4×10^{-6} M and 1.6×10^{-5} M, respectively. The opposite inhibition patterns were obtained on repeating the experiments with deprenyl as inhibitor. Preincubation with 10^{-4} M deprenyl caused 95, 84 and 68 per cent inhibition of the activities toward PEA, tyramine and serotonin, respectively. The pI_{50} values toward these substrates were 2.5×10^{-7} M, 2.1×10^{-5} M and 4×10^{-5} M, respectively, under these experimental conditions. After preincubation of the preparation with either of these inhibitors at 37° for 30 min, the inhibition curves with all three substrates shifted to lower concentrations of the inhibitors. Furthermore, this long preincubation at higher temperature resulted in complete inhibition of the enzyme activity by both clorgyline and deprenyl at 10^{-4} M, irrespective of whether serotonin, tyramine or PEA was used as substrate (Fig. 1). These results are in accord with our previous histochemical observations [11]. The difference in the modes of inhibition under these two different conditions of preincubation is consistent with the current view that propargyl-type inhibitors bind covalently and irreversibly to a flavin moiety in the active site of the enzyme [15–18], after a reversible phase of inhibition [19–21].

The presence of MAO-A in the preparation was confirmed by selective inhibition of MAO-B with deprenyl (5×10^{-7} M) after which about 60 and 30 per cent of the activity toward tyramine and PEA remained. On subsequent increase in the clorgyline concentration, all the remaining activity toward tyramine and PEA was highly sensitive to clorgyline, and single-sigmoidal inhibition curves shifted to lower concentrations of clorgyline were obtained (Fig. 2). Similar results were obtained when deprenyl was used as inhibitor on a preparation preincubated with 10^{-7} M clorgyline. Both results indicate that the remaining activity in each preparation was due to a single MAO, presumably MAO-A in the former case and MAO-B in the latter. The present results indicate that both substrates are oxidized only by MAO-A and MAO-B. The results are consistent with our previous finding that PEA is oxidized by both MAO-A and MAO-B in rat brain, the relative activities of the two types depending mainly on the PEA concentration used [22].

After preincubation of various amounts of the homogenate with clorgyline at room temperature for 5 min, straight lines passing through the origin were obtained with

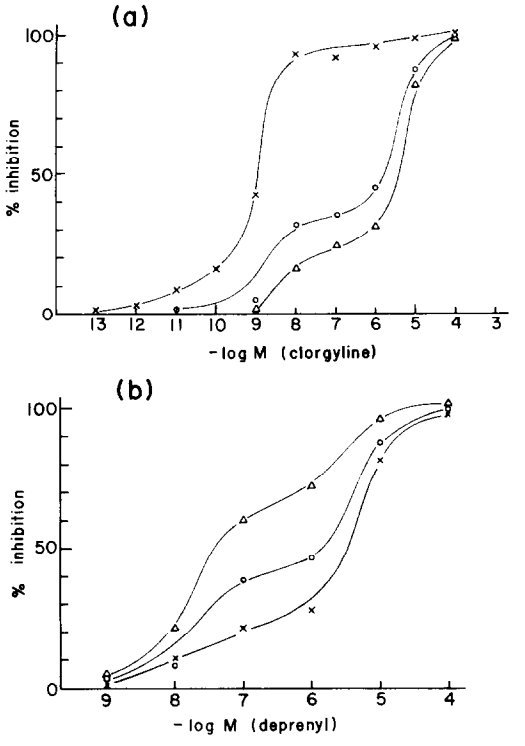

Fig. 1. Inhibition of MAO activity toward serotonin, tyramine and β-phenylethylamine by various concentrations of clorgyline and deprenyl. The enzyme preparation (1 mg protein) from circumventricular structures of rat brain was preincubated at 37° and pH 7.6 for 30 min with various concentrations of (a) clorgyline, or (b) deprenyl. After preincubation, the remaining activity was estimated by the radiometric method described in the text at 37° and pH 7.6, using 0.5 mM serotonin (—×—), 0.5 mM tyramine (—○—) or 0.3 mM β-phenylethylamine (—△—) as substrate. Each point represents the mean of the activities in duplicate samples from two groups of five to six rats and is expressed as the percent inhibition of the uninhibited control. Values are plotted against the negative logarithm of the molar concentration of the inhibitor (a) clorgyline, or (b) deprenyl. The specific control MAO activities with 0.5 mM serotonin, 0.5 mM tyramine and 0.3 mM β-phenylethylamine were 0.68, 1.28 and 0.22 nmoles product formed·mg protein^{-1}·min^{-1} at 37° and pH 7.6, respectively.

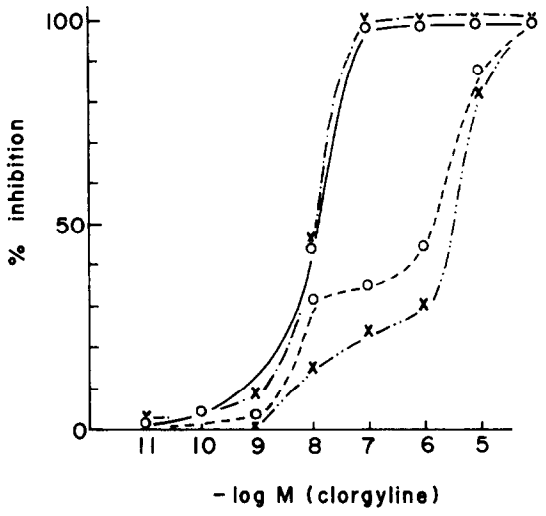

Fig. 2. Effect of clorgyline on MAO activity in untreated and deprenyl-treated preparations from circumventricular structures of rat brain. For the deprenyl-treated preparation, the homogenate (1 mg protein) was first preincubated with 5×10^{-7} M deprenyl at 37° and pH 7.6 for 30 min, and then the samples were further preincubated with different concentrations of clorgyline at 37° for 30 min. The untreated preparation was first preincubated without inhibitor at 37° and pH 7.6 for 30 min, and then the samples were further preincubated with various concentrations of clorgyline at 37° and pH 7.6 for 30 min. MAO activity was estimated as described in the legend to Fig. 1, with 0.5 mM tyramine and 0.3 mM β-phenylethylamine as substrate. Each point represents the mean of the activities in duplicate samples from groups of five to six rats, and activity is expressed as the percent inhibition of the activity in the absence of clorgyline (deprenyl-treated preparation) or deprenyl (untreated preparation). Key: (– –○– –) untreated preparation with tyramine; (—○—) deprenyl-treated preparation with tyramine; (···×···) untreated preparation with β-phenylethylamine; and (·–×–·) deprenyl-treated preparation with β-phenylethylamine.

serotonin and PEA as substrates (Fig. 3A). Thus, by the criteria of Ackermann and Potter [23], clorgyline is a reversible inhibitor of the oxidations of these two substrates under these experimental conditions. On the other hand, on preincubation for 30 min at 37°, the lines obtained with clorgyline, serotonin, and PEA as substrates all intersected the abscissa to the right of the origin (Fig. 3B). Together with findings in experiments on 'washing' the preparations with the buffer, these results indicate that the inhibition by clorgyline was reversible on preincubation at room temperature for 5 min, but irreversible on preincubation at 37° for 30 min. On preincubation with 10^{-5} M deprenyl at room temperature for 5 min, the line for enzyme inhibition passed through the origin with serotonin or PEA as substrate. On preincubation with 5×10^{-9} M and 2×10^{-8} M deprenyl for 30 min at 37°, the lines obtained with serotonin and PEA also passed through the origin, indicating reversible inhibitions, but that with serotonin and deprenyl at a higher concentration (10^{-6} M) intersected the abscissa to the right of the origin (data not shown), indicating irreversible inhibition. These results are in accordance with findings that irreversible inhibition of the MAO-A by deprenyl requires a longer incubation time and a higher temperature than that by clorgyline [20]. These findings also confirm the reports of others that the extent and irreversibility of MAO inhibition by these inhibitors depend on the ratio of the concentrations of inhibitor and enzyme protein [21], and that irreversible inactivation is more rapid when the ratio is high. The MAO activity in the preparation of circumventricular structures of rat brain was not affected by potassium cyanide or semicarbazide (10^{-3} M). This result; together with the finding that the MAO activity in the preparation could be completely inhibited by clorgyline and deprenyl, excludes the possibility that the preparation used in this study contained any other amine oxidase, such as a soluble amine oxidase [24], a blood vessel or connective tissue amine oxidase [25, 26], or a benzylamine oxidase [27, 28]. Thus, the present biochemical results support the conclusion presented earlier from histological studies [11] that, like preparations of whole rat brain, the circumventricular structures of rat brain contain only MAO-A and MAO-B activities in a ratio of about 3:7, defined as 'clorgyline-sensitive' and 'less clorgyline-sensitive' activity toward tyramine.

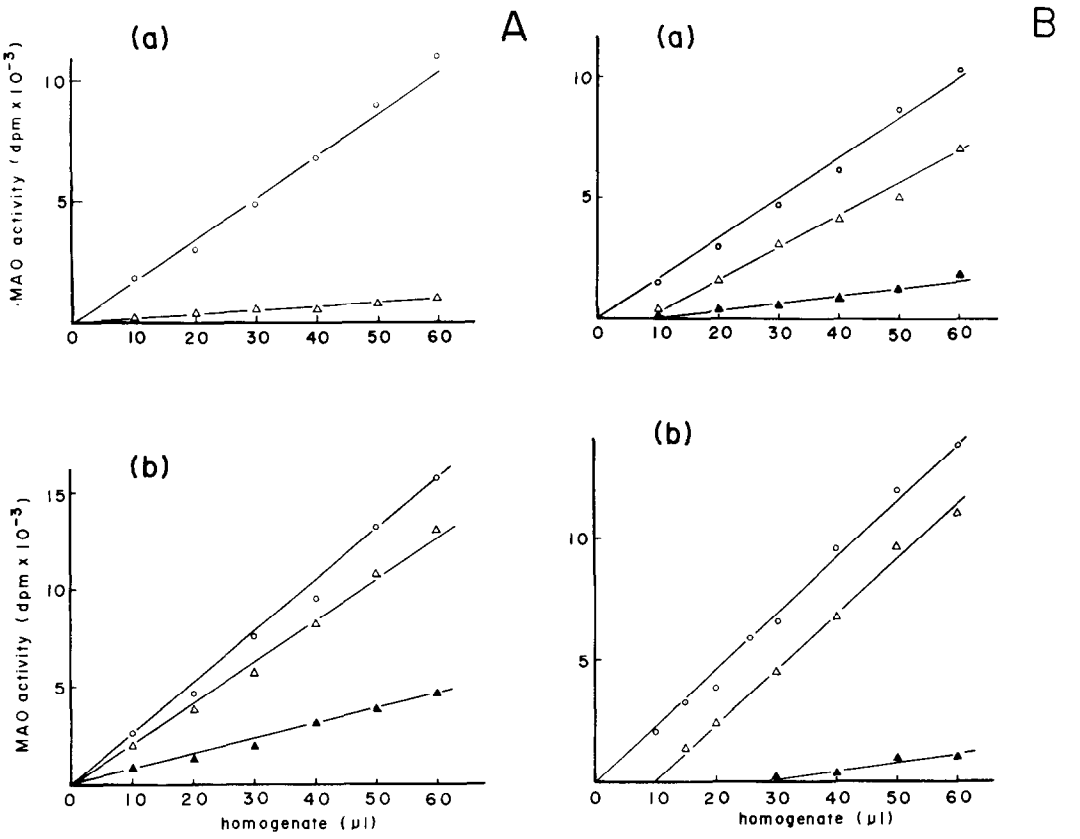

Fig. 3. Ackermann–Potter plots for MAO in the circumventricular structures of rat brain. The enzyme concentration was varied by altering the amount (μl) of the homogenate (20 mg/ml of protein) in the assay. The enzyme preparation was preincubated (A) at room temperature and pH 7.6 for 5 min with (a) clorgyline [5×10^{-8} M (—△—) or (b) clorgyline [10^{-8} M (—△—); 10^{-4} M (—▲—), or (B) at 37° and pH 7.6 for 30 min with (a) clorgyline [5×10^{-9} M (—△—); 2×10^{-8} M (—▲—) or (b) clorgyline [10^{-8} M (—△—); 10^{-5} M (—▲—)]. After preincubation, MAO activity was estimated by the radiometric method described in the text with (a) 0.5 mM serotonin, or (b) 0.3 mM β-phenylethylamine as substrate. Points are mean MAO activities for duplicate samples. The open circles (—○—) in (a) and (b) represent control values without inhibitor.

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Effect of imipramine on hepatic gamma-glutamyltransferase in female rats. Interaction with contraceptives

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The increase of gamma-glutamyltransferase (GGT, EC 2.3.2.2) in the plasma of treated subjects has been reported for numerous drugs, as described in a recent review [1]. Besides the measurement of GGT activity in clinical chemistry in cases of liver injuries, particularly in cholestasis, carcinoma, and after alcohol consumption, the use of this enzyme as an indicator of induction by certain drugs, especially those of the phenobarbital group, has been proposed [2]. Correlations between induction of liver drug-metabolizing enzymes and GGT in the liver have not been verified for all inducers.

We have recently shown that liver GGT is localized mainly in plasma membranes [3] and that its passage into the plasma after phenobarbital treatment is related to membrane disturbances due to induction and to solubilizing effect of bile salts [4, 5]. We have also found elevated GGT in the plasma of subjects treated long-term with imipramine. This elevation was particularly important in women who were also taking oral contraceptives [6].

In order to clarify the role of membrane alterations in the phenomenon of the release of GGT into the plasma after administration of such drugs, this paper reports a study of variations of GGT in the rat under the influence of imipramine and steroid contraceptives. These two drugs are known to have effects on liver membranes and, in some cases, their hepatic toxicity can lead to intrahepatic cholestasis [7, 8]. Furthermore, interactions of imipramine and oral contraceptives, which were manifested in some cases by mental disturbances, were extensively studied in females of different species [9]. It is not yet clearly established how these phenomena could be related to interaction in hepatic metabolism of the two drugs. We have studied, therefore, the variations of GGT and cytochrome P-450 (cyt. P-450) in the liver and plasma of female rats treated with imipramine, Mestranol–Norethynodrel mixture (MNm), or both administered simultaneously.

The effect of imipramine and MNm (obtained from Sigma Chemical Co, St. Louis, MO, U.S.A.) on the hepatic GGT activity was determined in liver subcellular fractions of female Sprague–Dawley rats (160–180 g body wt). The animals were divided into four groups: a control group received daily 0.3 ml of corn oil; the imipramine group was injected with a saline solution of imipramine (20 mg·kg^{-1}·day^{-1}); the contraceptive-treated group was injected with 0.3 ml of a corn oil solution containing Mestranol (0.07 mg·kg^{-1}·day^{-1}) and Norethynodrel (4 mg·kg^{-1}·day^{-1}); and the last group received injections of both imipramine solution and Mestranol–Norethynodrel mixture. The drugs were injected intraperitoneally over a period of 15 days.

The rats were fasted 16 hr before being killed. Plasma membranes were prepared from livers as described by Neville [10]. When complete subfractionation was performed, we used the method of Amar-Costesec *et al.* [11]. Proteins were assayed by the method of Lowry *et al.* [12] and GGT according to the method of Szasz [13] at 37° using L-gamma-glutamyl-3-carboxy-4-nitroanilide as the substrate. The concentration of cyt. P-450 was determined in microsomes by the method of Matsubara *et al.* [14]. Serum alkaline phosphatase (ALP, EC 3.1.3.1) was assayed at 30° by the method of Morgenstern *et al.* [15] and alanine aminotransferase (ALT, EC 2.6.1.2) by a kinetic optimized method at 30° using a reagent kit from Boehringer. Statistical differences were compared between control and treated groups using comparison of variances test.

Since it is known that GGT is localized in plasma membranes, in the first part of the experiments we investigated its variations under different treatments only in this fraction. But it was very surprising that the activities found in the plasma membranes did not reflect those measured in the homogenate when the rats received imipramine either alone or associated with MNm (Table 1). Indeed, the ratio of specific activity of GGT in plasma membranes to that in homogenate was 26 in controls and 23 in MNm-treated rats, but when imipramine or a combination of imipramine and MNm were administered, this ratio decreased to 4.4 or 4.6, respectively. These variations were due to the loss of GGT in plasma membranes under the effect of imipramine. Indeed, administration of imipramine did not alter significantly total activity in the liver homogenate (607 mU/g wet liver against 672 mU/g in controls) but resulted in an important decrease in the enzyme activity in the plasma membranes. In the same way, the combined treatment of rats with imipramine and MNm led to a 5-fold increase of GGT in the liver homogenate but did not change the plasma membrane activity. Conversely, there was a 2-fold increase in GGT activity in both homogenate and plasma membranes when the rats received MNm only. These data indicate that when imipramine was administered either alone or combined with MNm the subcellular distribution of GGT was altered. To understand how this phenomenon occurred, we studied the variations of the