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OXIDATION OF PHENYLETHANOLAMINE AND OCTOPAMINE BY TYPE A AND TYPE B MONOAMINE OXIDASE

EFFECT OF SUBSTRATE CONCENTRATION

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Abstract—Phenylethanolamine (PEOA) and octopamine (OA) were characterized as substrates for type A and type B monoamine oxidase (MAO) at various substrate concentrations, using rat brain mitochondria. The experiments on sensitivity to clorgyline and deprenyl showed that the inhibition patterns with PEOA as substrate differed markedly at different substrate concentrations: at 12.5 μM, PEOA acted as a specific substrate for type B MAO, but at 125 and 1250 μM it became a common substrate for both types of MAO. However, when OA was used as substrate, there were only slight or no differences in the inhibition patterns among the various concentrations tested; OA was found to be a common substrate for both types of MAO. Benzylamine was also examined for comparison and confirmed to be highly specific for type B MAO over a wide concentration range of the substrate. Kinetic analyses were carried out for PEOA and OA. High and low affinities for MAO were identified for PEOA: K_m values were 22.7 and 465 μM, and V_{max} values were 6.90 and 19.2 nmoles/mg of protein/30 min respectively. Pretreatment of the enzyme with 10^{-6} M clorgyline resulted in the disappearance of the low affinity component, and pretreatment with 10^{-6} M deprenyl resulted in the disappearance of the high affinity component. Therefore, the high affinity corresponded to that for type B MAO and the low one to that for type A MAO. For OA, however, the double reciprocal plots were linear with a single affinity component showing K_m and V_{max} values of 455 μM and 90.9 nmoles/mg of protein/30 min respectively. From the present study, it can be concluded that, when sensitivity of MAO to clorgyline or deprenyl is studied, it is necessary to check the effect of substrate concentration for each substrate and enzyme preparation, suspecting the different affinities of the substrate for type A and type B MAO.

Mitochondrial monoamine oxidase [amine: oxygen oxidoreductase (deaminating, flavin-containing); EC 1.4.3.4] (MAO) is believed to exist in many animal tissues in two functional forms called type A and type B MAO [1–3]. Type A MAO has been shown to be active with 5-hydroxytryptamine and norepinephrine as substrates, and sensitive to inhibition by a low concentration of clorgyline. Type B MAO has been shown to be active with β-phenylethylamine and benzylamine (BA), and sensitive to inhibition by a low concentration of deprenyl.

In most inhibition studies on type A and type B MAO, however, there has been no consideration of substrate concentration. We reported previously that the substrate specificity of β-phenylethylamine was highly dependent on its concentration [4]. In the present paper, we have extended the study to phenylethanolamine (PEOA) and octopamine (OA), the β-hydroxylated analogs, which are of growing interest as trace amines in the central nervous system [5]. The data with BA are also presented for comparison.

MATERIALS AND METHODS

Enzyme. Male albino rats of the Sprague–Dawley breed, weighing 100–150 g, were decapitated and the brains were removed rapidly. They were homogenized with 9 vol. of 0.25 M sucrose in a Potter–Elvehjem homogenizer fitted with a Teflon pestle, being cooled in an ice bath and centrifuged at 1,500 *g* for 5 min to remove cellular debris. The resulting supernatant fraction was centrifuged at 18,000 *g* for 20 min and the crude mitochondrial pellet was suspended in the sucrose solution. The suspension was recentrifuged at 18,000 *g* for 20 min and the pellet was resuspended in 0.1 M sodium phosphate buffer (pH 7.4). This was used as an enzyme source.

Chemicals. D,L-PEOA, D,L-OA–HCl and horseradish peroxidase (type II) were obtained from the Sigma Chemical Co., St. Louis, MO; homovanillic acid, pargyline–HCl and BA–HCl from Nakarai Chemicals, Ltd., Kyoto; and hydrogen peroxide from Mitsubishi-Gasukagaku, Ltd., Tokyo. Clorgyline, a selective inhib-

itor of type A MAO [1], was generously supplied by May & Baker Ltd., Dagenham, England. Deprenyl, a selective inhibitor of type B MAO [6], was kindly donated by Prof. J. Knoll, Department of Pharmacology, Semmelweis University of Medicine, Budapest, Hungary.

MAO assays. MAO activities were determined by a slight modification of the method of Guilbault *et al.* [7] and Snyder and Hendley [8]. In this method, hydrogen peroxide formed in the MAO reaction is measured fluorometrically, by converting homovanillic acid to the highly fluorescent compound in the presence of peroxidase. The assay mixture consisted of 0.1 M sodium phosphate buffer (pH 7.4), enzyme solution (0.08 to 1.41 mg of protein), peroxidase solution (0.2 mg), homovanillic acid solution (0.5 mg), substrate solution and water, 0.5 ml of each, to give a final volume of 3.0 ml. After incubation at 37° for 30 or 60 min, the enzyme reaction was terminated by adding 0.1 ml of pargyline solution (0.2 mg), and the mixture was centrifuged at 1,500 *g* for 10 min. The supernatant fraction was subjected to fluorescence measurement with excitation at 315 nm and with emission at 425 nm. Blank assays differed from controls only in the respect that the substrate solution was added with pargyline after incubation. Under these conditions, the assays were linear during incubation at 37° for at least 60 min. Care was taken not to convert more than 20 percent of the substrate to the reaction product. Standards were taken by adding 0.5 ml of hydrogen peroxide solution (4.42 to 22.1 nmoles) to the assay mixture in place of the enzyme solution. The fluorometric measurements were made in a Shimadzu corrected spectrofluorophotometer RF-502 at 20°.

Inhibition studies. Clorgyline and deprenyl were dissolved in distilled water, added to the assay mixture without substrate, and preincubated at 37° for 10 min to ensure maximal enzyme inhibition. Seven different concentrations for each inhibitor were employed over the range of 10^{-10} to 10^{-4} M. It was confirmed that each inhibitor neither interferred with the formation of the fluorescent compound nor quenched its fluorescence when hydrogen peroxide was added directly.

Protein determinations. Protein was determined by a slight modification of the conventional biuret method, using bovine serum albumin as a standard [9].

Kinetic studies. MAO activities were measured over the range of substrate concentrations of 20.0–5000 μM for PEOA, 150–3000 μM for OA and 150–500 μM for BA. The K_m and V_{max} values were determined graphically from Lineweaver–Burk plots.

For kinetic studies with selective inhibitors, the enzyme was incubated with 10^{-6} M clorgyline or 10^{-6} M deprenyl at 37° for 30 min and centrifuged at 18,000 *g* for 20 min. The resulting pellet was suspended in 30 ml of 0.25 M sucrose solution and recentrifuged at 18,000 *g* for 20 min. This procedure was repeated twice to wash the enzyme completely. The resulting pellet was suspended in 20 ml of water and subjected to MAO assays as described above.

RESULTS

Inhibition by clorgyline and deprenyl. Figure 1 shows MAO inhibition by clorgyline and deprenyl,

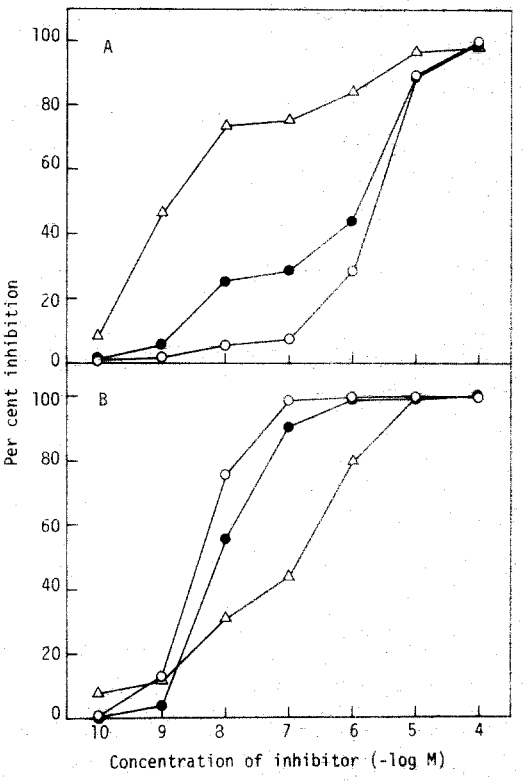

Fig. 1. Inhibition of MAO in rat brain mitochondria by clorgyline (A) and deprenyl (B) using various concentrations of PEOA as substrate. The concentrations of the substrate were 12.5 (○——○), 125 (●——●) and 1250 μM (△——△) with specific activities of 1.69, 4.42 and 11.6 nmoles/mg of protein/30 min respectively. Each point represents the mean obtained from duplicate determinations.

using various concentrations of PEOA as substrate. At 12.5 μM, the inhibition curves obtained with both inhibitors were almost single sigmoidal; clorgyline, when present in the incubation medium at a concentration of 10^{-7} M, hardly affected the PEOA deamination, but deprenyl almost completely blocked the deamination at the same concentration. These results clearly indicate that PEOA is highly specific for type B MAO at this concentration. At 125 μM, however, the patterns were changed dramatically: a clear plateau appeared in the curve at 10^{-8}–10^{-7} M clorgyline. The susceptibility of PEOA deamination to clorgyline was increased significantly, while that to deprenyl was decreased. When PEOA concentration was increased up to 1250 μM, the susceptibility to clorgyline was increased even more, showing about 75 per cent inhibition with 10^{-8}–10^{-7} M clorgyline; the susceptibility to deprenyl was decreased further, showing a plateau at 10^{-8}–10^{-7} M deprenyl. These data clearly show that PEOA loses its substrate specificity for type B MAO at concentrations of 125–1250 μM.

The inhibition of MAO by both inhibitors using 12.5, 250 and 2500 μM OA as substrate is illustrated in Fig. 2. In the curves with clorgyline, clear plateaus appeared at 10^{-8}–10^{-7} M. The sensitivity to clorgyline with 12.5 μM OA was slightly lower than that with 2500 μM. In the curves with deprenyl, plateaus also

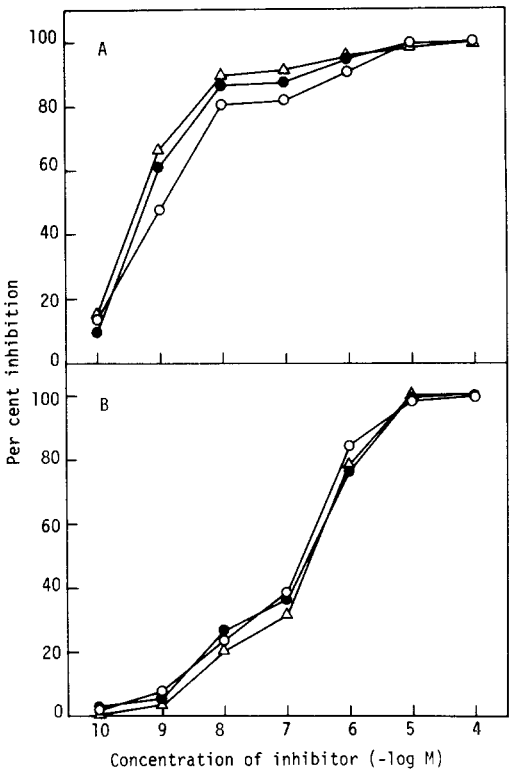

Fig. 2. Inhibition of MAO in rat brain mitochondria by clorgyline (A) and deprenyl (B) using various concentrations of OA as substrate. The concentrations of the substrate were 12.5 (○——○), 250 (●——●) and 2500 μM (△——△). Each point represents the mean obtained from duplicate determinations.

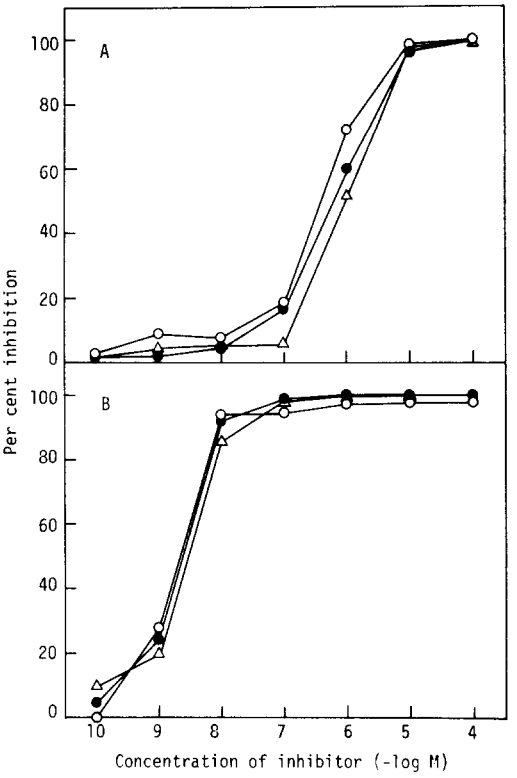

Fig. 3. Inhibition of MAO in rat brain mitochondria by clorgyline (A) and deprenyl (B) using various concentrations of BA as substrate. The concentrations of the substrate were 12.5 (○——○), 1250 (●——●) and 5000 μM (△——△). Each point represents the mean obtained from duplicate determinations.

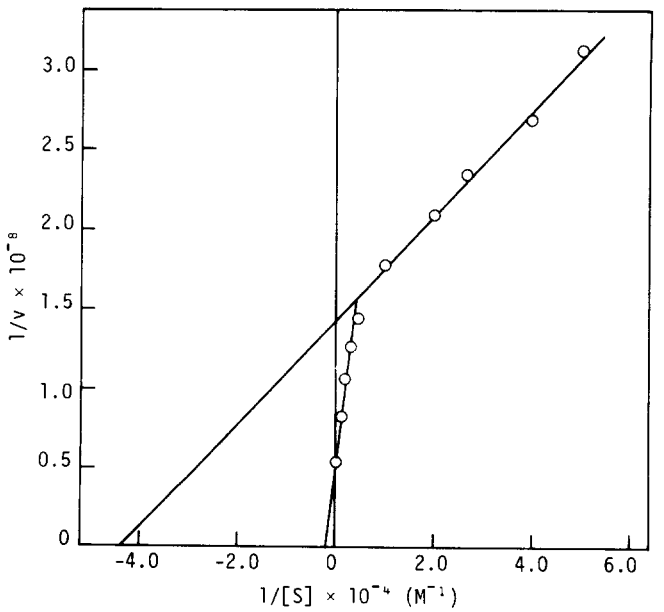

Fig. 4. Lineweaver–Burk plots for MAO in rat brain mitochondria with PEOA as substrate. Each point represents the mean obtained from duplicate determinations; v: moles H_2O_2 formed/mg protein/30 min.

Table 1. Kinetic constants for MAO activities toward PEOA, OA and BA in rat brain mitochondria

Substrate	K_m (μM)	V_{max} (nmoles/mg protein/30 min)
PEOA		
High affinity	22.7	6.90
Low affinity	465	19.2
Clorgyline-treated *	11.4	6.28
Deprenyl-treated *	1490	5.56
OA	455	90.9
BA	161	63.7

* The procedure of the treatment is given in the text.

appeared at 10^{-8}–10^{-7} M; no significant differences in the inhibition pattern were observed with deprenyl among the three concentrations of OA. These results show that OA is deaminated by both types of MAO over a wide concentration range of the substrate, though a major part of the activity is due to the type A enzyme.

In Fig. 3, the inhibition patterns using various concentrations of BA as substrate are presented for comparison. All the curves with both inhibitors were almost single sigmoidal; clorgyline, at a concentration of 10^{-8} M, hardly inhibited the BA deamination, but deprenyl at the same concentration almost completely inhibited the activity. It appeared that the deamination with 5000 μM BA was slightly more resistant to clorgyline than that with the lower concentrations of BA, but this was not true for the curves with deprenyl. These results show that BA is highly specific for type B MAO over a wide concentration range of the substrate.

Kinetic studies with uninhibited MAO. PEOA and OA as substrates for MAO were characterized further by determining the Michaelis–Menten kinetic constants from the Lineweaver–Burk plots (Figs. 4 and 5). The results with BA were also presented in Table 1. As can be seen from Fig. 4, the double reciprocal plots with PEOA revealed a notable downward deflection at higher concentrations of the substrate, which probably shows another affinity component. The K_m and V_{max} values for the low affinity component were also calculated from the four plots of the highest concentrations as listed in Table 1.

Figure 5 shows the plots for OA as substrate, revealing a linear line over a wide concentration range except for slight substrate inhibition at the highest concentrations. The K_m and V_{max} values are also presented in Table 1.

Kinetic studies on MAO pretreated with selective inhibitors. To further investigate the nature of the double components observed with PEOA, rat brain mitochondrial MAO was pretreated with clorgyline or deprenyl at a concentration of 10^{-6} M. At this concentration, type A MAO was completely inhibited by clorgyline and type B enzyme completely by deprenyl. As can be seen in Fig. 6, the downward deflection (the

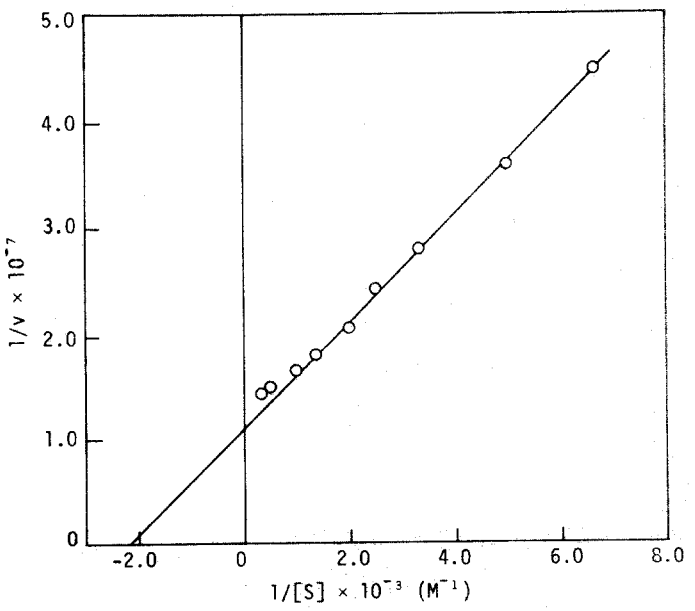

Fig. 5. Lineweaver–Burk plots for MAO in rat brain mitochondria with OA as substrate. Each point represents the mean obtained from duplicate determinations; v: moles H_2O_2 formed/mg of protein/30 min.

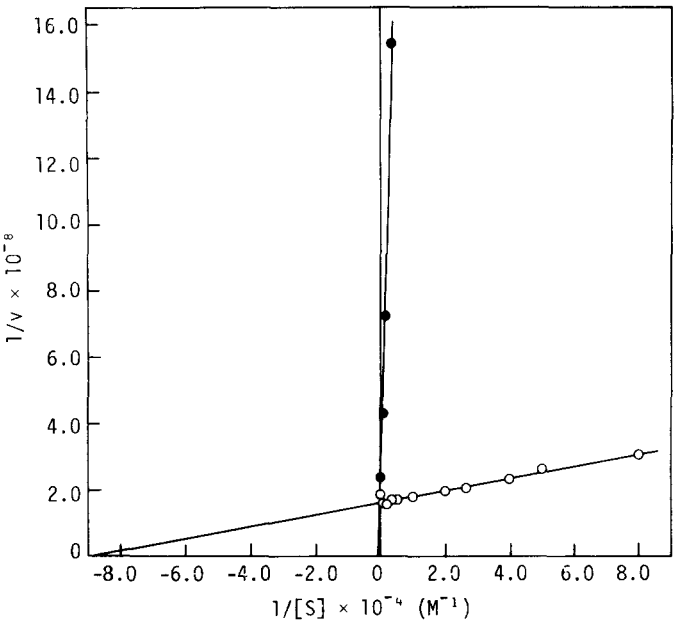

Fig. 6. Lineweaver–Burk plots for MAO in rat brain mitochondria pretreated with 10^{-6} M clorgyline (○——○) or 10^{-6} M deprenyl (●——●) using PEOA as substrate. Each point represents the mean obtained from duplicate determinations: v: moles H_2O_2 formed/mg of protein/30 min.

low affinity component) completely disappeared after treatment with clorgyline; the high affinity component also disappeared after treatment with deprenyl. The respective K_m and V_{max} values are presented in Table 1. The K_m value of deprenyl-treated enzyme was much higher than that of the low affinity component of the uninhibited enzyme, and the value of clorgyline-treated enzyme was lower than that of the high affinity component.

DISCUSSION

The present experiments show that the MAO inhibition patterns with PEOA as substrate differ markedly at different substrate concentrations (Fig. 1); at 12.5 μM, PEOA acted as a specific substrate for type B MAO, but at 125 μM, it became a common substrate for both types of MAO. At 1250 μM, a major part of MAO activity toward PEOA was found to be due to type A MAO. This phenomenon was not found for OA and BA (Figs. 2 and 3), but we observed a similar phenomenon for β-phenylethylamine [4]. During the preparation of this manuscript, Edwards [10] reported that PEOA was a specific substrate for type B MAO, but he did not notice the change in the substrate specificity of PEOA at different concentrations.

The Lineweaver–Burk plots with PEOA using the uninhibited MAO preparation revealed a characteristic feature in the curve (Fig. 4), showing two different affinity components of MAO for PEOA in rat brain mitochondria. The plots of MAO pretreated with selective inhibitors showed that the low affinity component disappeared with the clorgyline treatment and the high affinity component with the deprenyl treatment (Fig. 6). Therefore, it can be concluded that the low and the high affinity components correspond to that of type A MAO and to that of type B MAO respectively. This is also apparent from the observation that, at a high concentration of PEOA, a major part of MAO activity was due to type A MAO (Fig. 1). These results support the idea that the active site of type A MAO is independent of that of type B MAO [11]. To our knowledge, this characteristic curve obtained with PEOA has not been easily observed with other substrates. In the plots with OA (Fig. 5), we could not observe such double components. This is probably because the K_m value of type A MAO for OA may be similar to that of type B MAO.

The specific type of MAO involved in the deamination of OA has been controversial. Houslay and Tipton [12] reported that OA is deaminated exclusively by type A MAO in rat liver mitochondria using an oxygen electrode assay method. In contrast, Lyles [13] has concluded, by a radiochemical method, that OA is a common substrate for both types of MAO. Our present results with a fluorometric assay agree well with those of Lyles. Presumably, Houslay and Tipton [12] could not identify type B MAO owing to the low sensitivity of their method.

In recent years, trace amines such as PEOA and OA have been the objects of growing interest [5]. PEOA and OA have been shown to be present normally in mammalian brain in extremely small quantities [14–17]. Even though their physiological roles have not been well defined, it is possible that they may serve as neurotransmitters or neuromodulators. In view of the extremely low levels of PEOA and OA in the brain, it may be that PEOA is oxidized exclusively by type B MAO while OA is oxidized by both forms of MAO in the normal brain. There is some evidence suggesting that these β-hydroxylated amines exist in sympathetic neurons [14, 16]. It has also been suggested that only type A MAO may exist inside the sympathetic neurons [18]. It is tempting, therefore, to speculate that PEOA is metabolized exclusively outside the sympathetic neu-

rons while OA is metabolized both inside and outside them.

In the present paper, we have demonstrated that substrate concentration does affect sensitivity to clorgyline or deprenyl, especially when PEOA is used as substrate. Since many workers pay no attention to substrate concentration in MAO inhibition studies, it seems necessary to check the effects of substrate concentration over a wide range for each substrate and enzyme preparation, while suspecting the different affinities of the substrate for type A and type B MAO.

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