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THE DEAMINATION OF ISOAMYLAMINE BY MONOAMINE OXIDASE IN MITOCHONDRIAL PREPARATIONS FROM RAT LIVER AND HEART : A COMPARISON WITH PHENYLETHYLAMINE

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Abstract—Isoamylamine (IAA) and phenylethylamine (PEA) have been studied as substrates for MAO activity *in vitro* in rat liver and heart mitochondrial fractions. The metabolism of both IAA and PEA in the liver was brought about by low- and high-K_m activities which, by the use of the inhibitor clorgyline, were found to correspond to MAO-B and MAO-A, respectively. Similar conclusions were reached for IAA metabolism in the rat heart. In contrast, only a single enzyme component of PEA metabolism in the rat heart was detected by Lineweaver–Burk analysis, although inhibition studies revealed that there was a small proportional contribution of MAO-B activity towards this amine. It was concluded from these results that the relative importance of MAO-A and MAO-B for metabolism of these amines depends upon the substrate concentration used. In addition, the possible physiological significance of IAA as a biogenic amine is discussed.

Monoamine oxidase (MAO : EC 1.4.3.4) is a flavoprotein enzyme, located predominantly in the outer mitochondrial membrane, and distributed widely throughout tissues of many different animal species (see ref. 1, for review). Some of the more important physiological substrates for this enzyme include the biogenic amines noradrenaline, dopamine and 5-hydroxytryptamine. Although much effort continues to be directed towards clarifying the role of MAO in controlling the intracellular concentrations of these putative neurotransmitter amines, recent interest has also focused upon the properties, as MAO substrates, of various trace amines whose physiological function is less clearly defined, and which are normally found only at very low endogenous concentrations in mammalian tissues [2]. In particular, the demonstration that two types of enzyme activity, called MAO-A and MAO-B, can be distinguished on the basis of their relative sensitivities towards inhibition by the drugs clorgyline [3] and deprenyl [4] has prompted most contemporary investigations to be carried out within the context of providing information about the possible functional significance *in vivo* of this binary classification for MAO activity (see ref. 5, for review).

To date, a number of research papers have described the properties, as substrates of MAO-A and MAO-B, of a variety of aromatic trace amines, among which are phenylethylamine (PEA) [6–8], phenylethanolamine [9,10], *p*-octopamine [10–12], *m*-octopamine [13], *p*-tyramine [3], *m*- and *o*-tyramine [14] and *p*-synephrine [15]. In contrast, relatively little is known about the ability of MAO-A and MAO-B to deaminate various aliphatic monoamines, which may gain access to the mammalian body either as a result of dietary influences or by bacterial action in the gut (see Discussion).

The present paper describes the results of our investigations into the deamination of one such compound, namely isoamylamine (IAA, 3-methyl butylamine), by mitochondrial fractions from rat liver and heart. In this work we have confirmed and extended previous reports that IAA is, indeed, metabolized *in vitro* by MAO [16–18] and by the use of clorgyline we have provided evidence for the involvement of both MAO-A and MAO-B in this action. In view of certain structural similarities between IAA and PEA, the latter amine has been included in this study for comparative purposes.

MATERIALS AND METHODS

Male albino Sprague–Dawley rats weighing 150–200g were supplied by A. J. Tuck and Son, Rayleigh, Essex, U.K. Clorgyline hydrochloride was a gift from May & Baker Ltd., Dagenham, Essex, U.K. Leuco-2′,7′-dichlorofluorescein diacetate (DCF) was purchased from Eastman Kodak, Kirkby, Liverpool, U.K., horseradish peroxidase (POD), IAA (free base) and PEA hydrochloride from Sigma, London, U.K. IAA was converted to its hydrochloride salt for use in the studies described here.

Preparation of mitochondria. Mitochondrial fractions from liver and heart were prepared from the pooled tissues of 6 rats. Animals were killed by a blow to the head and their hearts and livers were removed, blotted and weighed. The tissues were homogenized by the use of a Sorvall Omnimixer in 10 vol. of a buffer containing 0.25 M sucrose and 0.01 M potassium phosphate, pH 7.8. The homogenate was centrifuged, first at 800 *g* for 10 min to remove debris, unbroken cells etc., and then the supernatant fraction was decanted and centrifuged at 12,000 *g* for 20 min in an MSE Prepspin centrifuge.

The mitochondrial pellets obtained in this way were resuspended in an appropriate volume of homogenization buffer, and the total suspension was divided into a number of 3 ml portions which were stored at −20° until used separately for subsequent experiments. All results reported in this paper were obtained with mitochondria which had thus been frozen and thawed once only. The protein concentrations of these suspensions, measured by the method of Goa [19] were 40.1 and 7.1 mg/ml for liver and heart, respectively.

Spectrophotometric assay of MAO. The assay described by Köchli and von Wartburg [20], which follows the oxidation of leuco-DCF by H_2O_2 produced as a result of amine metabolism by MAO, was used to measure the deamination of IAA and PEA.

Assay mixtures contained 0.6 ml leuco-DCF solution (2.5×10^{-4} M in 0.01N NaOH), 1.8 ml POD-buffer (8.3 mg POD dissolved in 100 ml of 0.1 M potassium phosphate buffer, pH 7.8), made up to a total cuvette volume of 3 ml with appropriate volumes of mitochondrial suspension and amine (dissolved in distilled water to give the required final amine concentrations in the assay). As previously reported [20], it was found necessary to preincubate reaction mixtures (minus enzyme) for a short period (5 min) in the dark, to allow a small background rate of leuco-DCF oxidation to subside. The enzyme assay was then started by the addition of mitochondria, and the change in absorbance at 502 nm was monitored in air at room temperature for up to 4 min (liver) or 5 min (heart), over which time the rate of reaction remained linear with respect to both protein concentration in the cuvette and the assay time. Actual protein concentrations chosen for the various experiments reported here are described in the figure legends. Blank assays represented the change in absorbance produced in these periods by reaction mixtures lacking only amine substrate, and all results were corrected for this tissue blank.

Reaction rates were converted to nmoles amine metabolized/hr/mg protein, assuming a stoichiometry of 5.3 moles DCF formed/mole H_2O_2 generated in the MAO reaction, and also using the molar extinction coefficient for DCF of 91,000 $M^{-1}cm^{-1}$ quoted by Köchli and von Wartburg [20]. In these experiments, where the effects of various concentrations of the inhibitor clorgyline upon amine metabolism were studied, mixtures containing sufficient volumes were prepared containing liver or heart mitochondria (at final concentrations of 8.02 and 3.55 mg/ml, respectively) and the appropriate concentration of inhibitor (from 5×10^{-10} to 5×10^{-4} M), such that after incubation for 20 min at 37°, aliquots of 50 μl (liver) and 100 μl (heart) could be removed from these mixtures and assayed for MAO activity as described above. It should be noted that all concentrations of clorgyline quoted in the text represent preincubation concentrations. Thus, the addition of samples from the preincubation mixtures to make the final 3 ml volume for spectrophotometric assay will result in some slight dilution of these inhibitor concentrations.

RESULTS

Rat liver MAO. Initial reaction velocities for deamination of IAA in rat liver mitochondria were determined at concentrations of IAA ranging from 10 μM to 5 mM. The resulting Lineweaver–Burk plot of this data (Fig. 1) was biphasic, and from the linear regions of this plot, it was possible to estimate values of approximately 16 and 95 μM, for a low- and high-K_m enzyme component, respectively. However, when two enzyme components with different kinetic constants may each be contributing to the total metabolism of a single substrate, a more accurate estimation of these individual parameters may be obtained by applying the iterative fitting method described by Spears *et al.* [21] to the data. By using

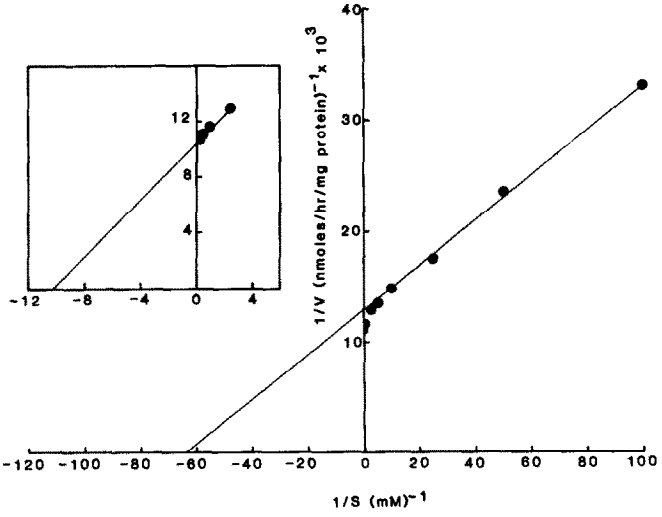

Fig. 1. Lineweaver–Burk plot for IAA metabolism in rat liver. Initial reaction velocities (V) were determined at different IAA concentrations (S), ranging from 10 μM to 5 mM (inset : 500 μM to 5 mM). Each point is the mean of duplicate determinations using 0.20 mg mitochondrial protein in each assay.

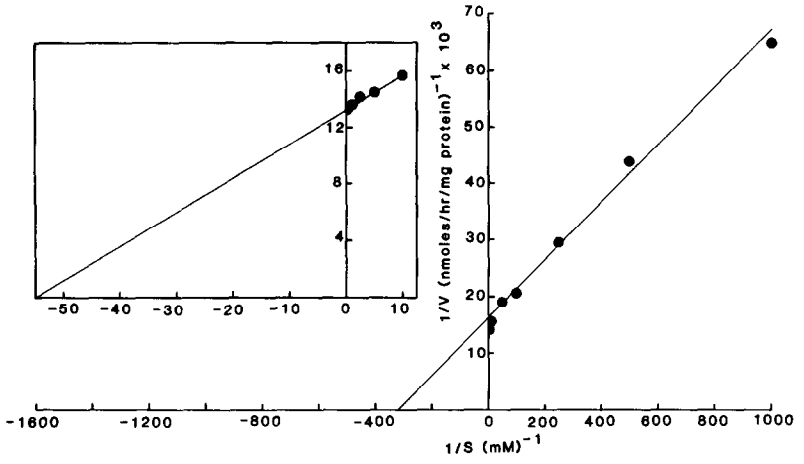

Fig. 2. Lineweaver–Burk plot for PEA metabolism in rat liver. Initial reaction velocities (V) were determined at different PEA concentrations (S), ranging from 1 μM to 4 mM (inset : 1 mM to 4 mM). Each point is the mean of duplicate determinations using 0.40 mg mitochondrial protein in each assay.

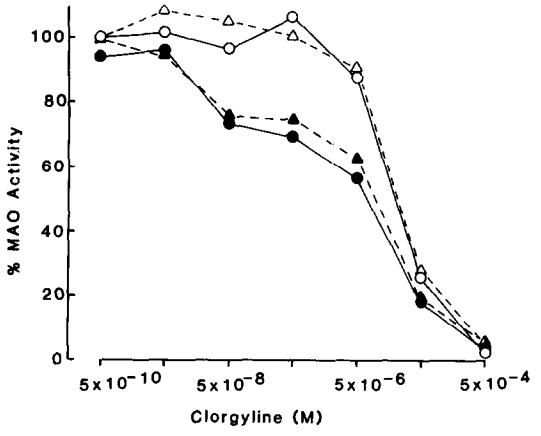

Fig. 3. Inhibition of rat liver MAO activity by clorgyline. Substrates used were IAA at 20 μM (○), 5 mM (●); PEA at 5 μM (△), 5 mM (▲). Each point is the mean of duplicate determinations, using 0.40 mg mitochondrial protein in each assay.

this method, the experimental data was best described by two components with K_m values of 13.1 and 679 μM and V_{max} values of 68.4 and 29.8 nmoles/hr/mg protein, respectively.

The deamination of PEA (between 1 μM and 4 mM) similarly gave rise to a Lineweaver–Burk plot showing two components with approximate K_m values of 3 and 19 μM (Fig. 2). By means of the iterative fitting procedure, these values were estimated to be 2.8 and 101 μM with corresponding V_{max} values of 52.2 and 20.5 nmoles/hr/mg protein, resepectively.

The inhibition of rat liver MAO activity towards these substrates is shown in Fig. 3. In these studies, two different concentrations of IAA (20 μM and 5 mM) and PEA (5 μM and 5 mM) were used to estimate the remaining enzyme activity after preincubation with different concentrations of clorgyline. At the lower concentrations of the two amines, the inhibitor plots were single-sigmoid, with little inhibition being observed below the concentration of 5 $\times$ 10^{-6} M clorgyline. From Johnston's [3] definition

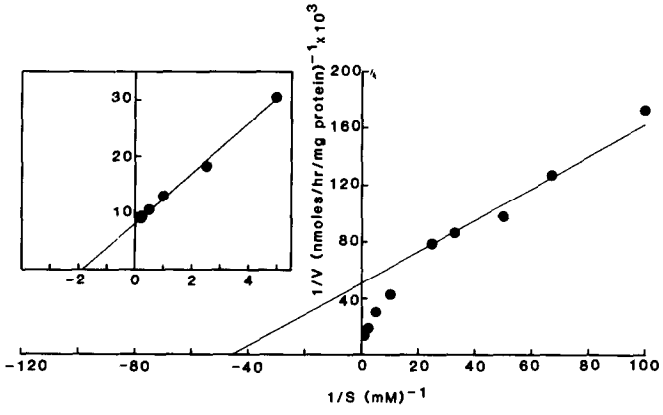

Fig. 4. Lineweaver–Burk plot for IAA metabolism in rat heart. Initial reaction velocities (V) were determined at different IAA concentrations (S), ranging from 10 μM to 5 mM (inset : 200 μM to 5 mM). Each point is the mean of duplicate determinations using 0.36 mg mitochondrial protein in each assay.

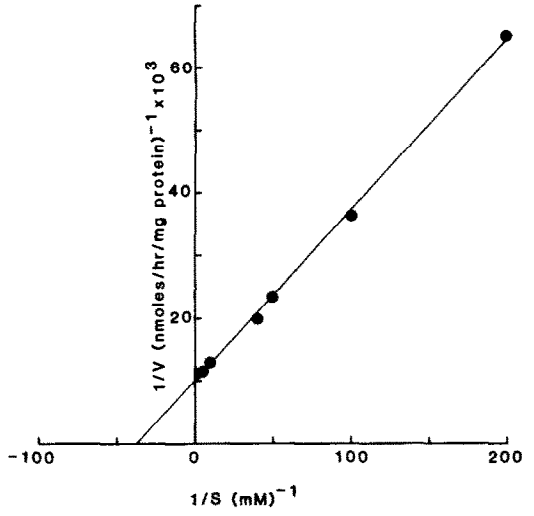

Fig. 5. Lineweaver–Burk plot for PEA metabolism in rat heart. Initial reaction velocities (V) were determined at different PEA concentrations (S), ranging from 5 μM to 1 mM. Each point is the mean of duplicate determinations, using 0.36 mg mitochondrial protein in each assay.

of MAO-A and MAO-B on the basis of their relative sensitivities towards clorgyline, the present results indicate that MAO-B alone is responsible for the metabolism of these amines at these particular substrate concentrations. In contrast, the corresponding plots using 5 mM IAA and PEA are biphasic, indicating a contribution by MAO-A of approximately 30 and 25% towards the total metabolism of IAA and PEA, respectively, at this substrate concentration.

Rat heart MAO. Similar experiments to those described above for the rat liver were performed for comparative purposes with rat heart mitochondrial preparations. The Lineweaver–Burk plot from the data for metabolism of IAA (10 μM to 5 mM) again showed two components with approximate K_m values of 22 and 526 μM (Fig. 4). The iterative fitting procedure for estimating the kinetic constants gave values of 13.9 and 739 μM for the low and high K_m components, with corresponding V_{max} values of 10.5 and 113.9 nmoles/hr/mg protein.

In contrast, a linear plot for PEA metabolism was obtained over the whole range of concentrations studied (5 μM to 1 mM), and linear regression analysis of this data indicated a K_m of 26 μM and a V_{max} of 97.0 nmoles/hr/mg protein (Fig. 5).

Inhibition by clorgyline of the deamination of IAA and PEA was studied at two different concentrations of each of these amines (Fig. 6). The double-sigmoid inhibitor plot for IAA indicated that MAO-A was responsible for approximately 68 and 96% of total IAA deamination at 100 μM and 5 mM, respectively. In contrast, these proportions were approximately 92 and 96% for the contribution of MAO-A towards the deamination of PEA at concentrations of 10 μM and 5 mM, respectively.

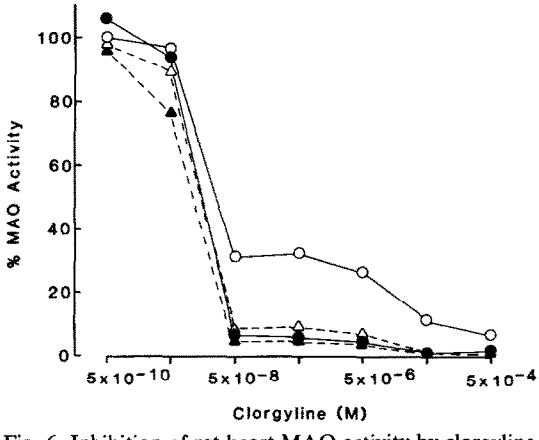

Fig. 6. Inhibition of rat heart MAO activity by clorgyline. Substrates used were IAA at 100 μM (○), 5 mM (●); PEA at 10 μM (△), 5 mM (▲). Each point is the mean of duplicate determinations, using 0.36 mg mitochondrial protein in each assay.

DISCUSSION

In this paper, a comparison has been made between the kinetic properties and sensitivity towards inhibition by clorgyline of the deamination of both IAA and PEA in mitochondrial fractions from rat liver and heart. These two compounds show a fair degree of structural similarity, with the aminoethyl side-chain moiety of PEA and C–1, –2,–6 of its aromatic ring bearing a strong resemblance to the overall structure of IAA. Thus, it is perhaps not surprising that the biochemical properties of these amines as substrates for MAO-A and MAO-B also show several similar features.

In the rat liver, Lineweaver–Burk plots indicated that two kinetically-distinguishable enzyme components were responsible for the metabolism of these amines, although the values for the low and high-K_m components of PEA metabolism were several times lower than the corresponding constants for IAA. Presumably, this indicates that the aromatic ring of PEA influences the binding of this amine to both of the enzyme components. The use of clorgyline to inhibit IAA and PEA deamination showed that both MAO-A and MAO-B can metabolize these amines, although the MAO-A component was only apparent at the higher concentrations of these amines used for the inhibition studies. These results suggested that the low- and high-K_m components of the Lineweaver–Burk plots may, in fact, be MAO-B and MAO-A, respectively.

Further support for this supposition was obtained by the following approach. If two independent enzyme activities (A and B) are acting upon a single amine substrate, the total metabolism (v_{total}) at a given concentration [S] of the substrate should be given by the sum of the individual Michaelis–Menten equations:

$$v_{total} = v_A + v_B = \frac{V_{max_A}[S]}{K_{m_A} + [S]} + \frac{V_{max_B}[S]}{K_{m_B} + [S]}.$$

In this theoretical approach, possible effects of changes in the concentration of oxygen, the second substrate in the MAO reaction, upon the kinetic constants for metabolism of the amines have been

ignored, since all reactions were performed at the fixed oxygen concentration of air.

Thus, substitution into this equation of the appropriate apparent kinetic constants (K_m and V_{max}) for the low- and high-affinity components derived by the iterative-fitting procedure [21] from the Lineweaver–Burk plots enables the calculation to be made that the high-K_m component is responsible for approximately 2 and 28% of total IAA metabolism at 20 μM and 5 mM, respectively, and 2 and 27% of total PEA metabolism at 5 μM and 5 mM, respectively. These values were extremely close to the proportions of MAO-A which were found by the appropriate inhibitor plots. Our observations that the ability to reveal metabolism of PEA by MAO-A is dependent upon the use of a suitably high substrate concentration are in excellent agreement with conclusions reached previously by others [7, 8], and the present results show that similar considerations also apply to IAA.

In rat heart mitochondria, there were some differences in the kinetic behaviour of IAA and PEA as substrates. Two components for IAA metabolism were again obtained by double-reciprocal plot analysis, and the values for the low- and high-K_m components were very similar to the corresponding values in the rat liver. Again, the results of inhibition experiments, which showed a greater proportion of MAO-A when the higher IAA concentration was employed, are consistent with the low- and high-K_m components representing MAO-B and MAO-A, respectively. Calculations carried out as above predict that MAO-A should be responsible for approximately 60 and 90% of total IAA metabolism at concentrations of 100 μM and 5 mM, respectively, again in good agreement with the subsequent inhibition data. In contrast to IAA, the Lineweaver–Burk plot for PEA metabolism indicated only a single kinetic component with a K_m of 26 μM, intermediate between the two components for PEA metabolism in the liver. We originally reported that PEA metabolism (at 1 mM concentration) was brought about predominantly by MAO-A in adult rat hearts [22]. The present inhibition data also indicated that almost all of the enzyme activity was represented by MAO-A, although small proportions of MAO-B (approximately 8 and 4% at 10 μM and 5 mM PEA, respectively) could be detected. The inability to distinguish two components for PEA deamination in the double-reciprocal plots over the range of PEA concentrations studied may well be due to the preponderance of MAO-A activity in this tissue, disguising the presence in kinetic studies of a very small low-K_m MAO-B component acting upon PEA. It was not possible to measure PEA deamination reliably below 5 μM, due to the sensitivity limits of the assay, and this may therefore be an additional factor. However, our findings were similar to those of Edwards *et al.* [23], who were also unable to find biphasic Lineweaver–Burk plots for PEA metabolism in the hearts of adult (15 week) rats, but could demonstrate two components in young (4 week) rats. It appears that the selective increase in MAO-A activity which occurs with age in the rat heart may provide an explanation for all these observations with PEA [23, 24].

The present studies with IAA may have some physiological importance, besides providing comparative information about the ability of different amine structures to be bound and metabolized by MAO-A and MAO-B. IAA was found as long ago as 1909 by Barger and Walpole [25] to be a pressor agent which could be extracted from putrefied meat, and it is now clear that its formation is brought about by the decarboxylation of the amino acid leucine. Although mammals have not been found to decarboxylate leucine in this manner, some bacteria appear to possess the necessary specific decarboxylase enzymes, among which are the lactobacillus, *Str. lactis* [26], and a micro-organism found in the human gut, *Proteus vulgaris* [27]. In addition, IAA and other aliphatic amines have been described as constituents of several plant species, and of particular interest is their presence in apple fruits [28]. Despite long-standing reports in the literature of the pressor effects of IAA and other aliphatic amines [29], probably by an indirectly sympathomimetic action [30], relatively little attention appears to have been paid to the potential importance of these compounds in mammalian physiology. Since their access to the human body seems feasible as a result of either bacterial production in the gut, or by introduction in the diet, it would appear prudent that these compounds should also be kept in mind as potential triggers of untoward sympathomimetic effects, along with the more commonly studied aromatic amines such as tyramine and phenylethylamine which may also be derived from dietary factors. It needs little emphasis to stress that such considerations may be of particular consequence after the administration of MAO inhibitors in clinical practice.

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