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TIME-DEPENDENT INHIBITION OF MONOAMINE OXIDASE BY β-PHENETHYLAMINE

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Abstract—Several reports have suggested that monoamine oxidase activity towards β-phenethylamine is inhibited by high concentrations of that substrate. This inhibition is not found if initial velocities are measured, but there is a slower time-dependent inhibition at higher β-phenethylamine concentrations. Such time-dependent inhibition is not found with tyramine as substrate or upon incubation of the enzyme with the reversible inhibitor amphetamine. The inhibition is not due to the accumulation of phenacetaldehyde, phenylethanol or phenacetic acid, or to a reaction of any of these three products either with each other or with β-phenethylamine. Although the inhibition is time-dependent, the inactivated enzyme slowly regains activity upon removal of the β-phenethylamine. A model is proposed to explain the observed inhibition.

It has been reported from several laboratories, that the substrate β-phenethylamine appears to inhibit the activity of monoamine oxidase (MAO, monoamine O_2: oxidoreductase, EC 1.4.3.4) at high concentrations [1–4]. Such an inhibition has been shown not to be due to impurity of either the non-radioactive or the radioactive substrate used [5]. However, other groups [6–10] have not found any inhibition at high β-phenethylamine concentrations. The reasons for this discrepancy have not previously been clarified and this paper reports the results of a series of experiments on the inhibition of the enzyme by β-phenethylamine demonstrating that time-dependent inhibition does occur at high concentrations of this substrate, but that no high-substrate inhibition is seen when initial rates are measured. Such time-dependent inhibition is not seen with the substrate tyramine nor with the reversible inhibitor amphetamine.

MATERIALS AND METHODS

Male Wistar rats of body weight 250–300 g were killed by a blow to the head and the livers rapidly removed, blotted on filter paper, weighed and homogenised 1:5 (w/v) in 0.25 M sucrose, 10 mM potassium phosphate, pH 7.2, with a Dounce homogeniser. Homogenates derived from pairs of rats were pooled and centrifuged at 600 *g* for 10 min to remove unbroke cells, nuclei and cell debris. The supernatants were centrifuged at 15,000 *g* for 10 min and the mitochondrial pellets obtained were resuspended in the sucrose–phosphate buffer and recentrifuged for 10 min at 15,000 *g* before resuspension in the sucrose–phosphate buffer. The mitochondrial fractions were adjusted to a protein concentration of 5 mg/ml and stored frozen at −20° until used for assay.

Homogenates were also made of four human liver samples, obtained at autopsy. The homogenates were made as described above, and aliquots set to a protein concentration of 2 mg/ml with the sucrose–phosphate buffer at both pH 7.2 and 7.8 and stored frozen until used for assay.

The rat liver samples were assayed for MAO activity by the method of Otsuka and Kobayashi [11] as modified by Fowler and Tipton [12]. The human liver samples were assayed by the method of Callingham and Laverty [13] as modified by Fowler *et al.* [14]. All assay, unless otherwise specified, were carried out under an atmosphere of air and at 37°. The MAO activities were compensated for the efficiencies of extraction of the deaminated metabolites [15] and expressed as nmoles of substrate metabolised/mg protein/min.

Protein concentrations were determined by the method of Markwell *et al.* [16]. Tyramine-[7-^{14}C]hydrochloride and β-phenethylamine-[ethyl-2-^{14}C]hydrochloride were obtained from the Radiochemical Centre (Amersham, U.K.). D-Amphetamine sulphate was a gift from Smith, Kline and French Ltd. (Welwyn Garden City, U.K.). Phenacetaldehyde, distilled before use, was obtained from British Drug Houses (Poole, U.K.). Phenylethanol and phenacetic acid were obtained from Koch-Light (Colnbrook, U.K.), and May and Baker Ltd. (Dagenham, U.K.), respectively. Ox liver aldehyde dehydrogenase was purified as described by Houslay and Tipton [17], NAD^+ was obtained from Boehringer (Mannheim, West Germany). All other reagents were standard laboratory reagents of analytical grade wherever possible.

RESULTS AND DISCUSSION

Time courses of the deamination of tyramine by rat liver mitochondria are shown in Fig. 1A and B. At low (60–120 μM) concentrations of tyramine, the

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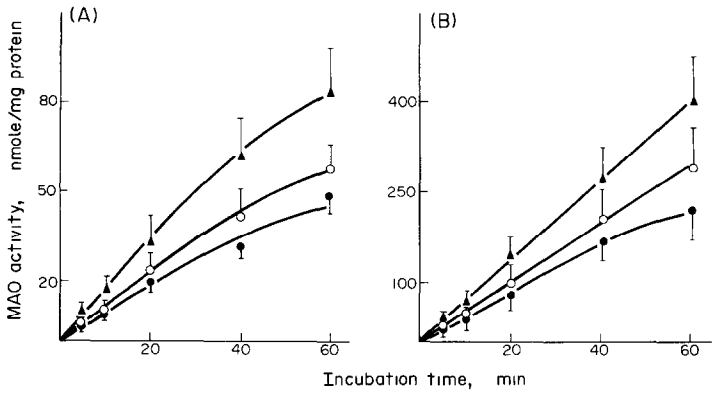


Fig. 1. Time courses of the deamination of tyramine by rat liver monoamine oxidase. Ordinate, MAO activity expressed as nmoles/mg protein; abscissa, incubation time in minutes. All points represent duplicate determinations in three mitochondrial preparations, and are expressed as means ± S.E.M. Tyramine concentrations were: (A) ●——●, 60 μM; ○——○, 80 μM; ▲——▲, 120 μM; and (B) ●——●, 600 μM; ○——○, 1200 μM; ▲——▲, 4000 μM.

deamination is linear with time up to about 20 min, but then deviates from linearity for longer incubation periods (Fig. 1A). Such a deviation is not found with higher substrate concentrations (1200 and 4000 μM), even after 60 min of incubation (Fig. 1B). It can be calculated that for the 60 μM substrate concentration, 51 ± 7% (mean ± S.E.M.) of available tyramine had been deaminated after 60 min of preincubation, whereas at 4000 μM, only 6 ± 1% (mean ± S.E.M.) of the available substrate had been utilised. The K_m for rat liver MAO towards tyramine under these conditions is about 300 μM [7, 12], and thus a deviation from linearity would be expected at low substrate concentrations due to substrate depletion and perhaps the accumulation of inhibitory products, whereas no significant deviation would be expected at high substrate concentrations. Thus, the interaction between tyramine and rat liver MAO appears to follow the expected time course.

With β-phenethylamine as substrate, and either rat or human liver as enzyme source, the time courses are rather different (Fig. 2A and B). In both cases, there is a deviation from linearity at low substrate concentrations, due to depletion of substrate. In the rat liver, for example, 82 ± 6% (mean ± S.E.M.) of the β-phenethylamine (initial concentration 15 μM) had been utilised after 20 min of incubation. At high substrate concentrations (600 μM and 1100 μM for rat and human liver, respectively), the time courses are also non-linear with time, although only 5 ± 0.3% (rat liver) and 1.4 ± 0.3% (human

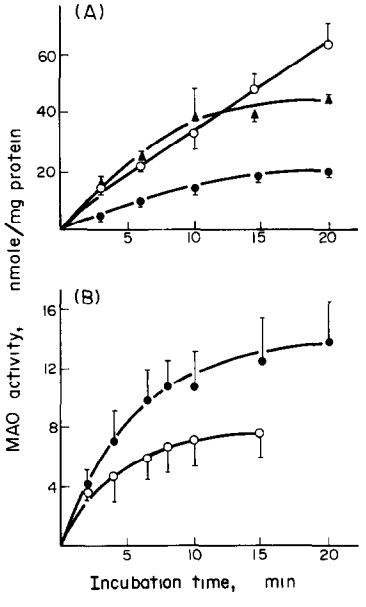


Fig. 2. Time courses of the deamination of β-phenethylamine. Ordinate, MAO activity expressed as nmoles/mg protein; abscissa, incubation time in minutes. All points are given as means ± S.E.M. All assays were performed in duplicate. Enzyme sources and β-phenethylamine concentrations were: (A) rat liver mitochondria (n = 3) with β-phenethylamine concentrations of 15 μM (●), 150 μM (○) and 600 μM (▲); and (B) human liver homogenates (n = 4) at pH 7.2, with β-phenthylamine concentrations of 10 μM (○) and 1100 μM (●). A similar result was found at pH 7.8.

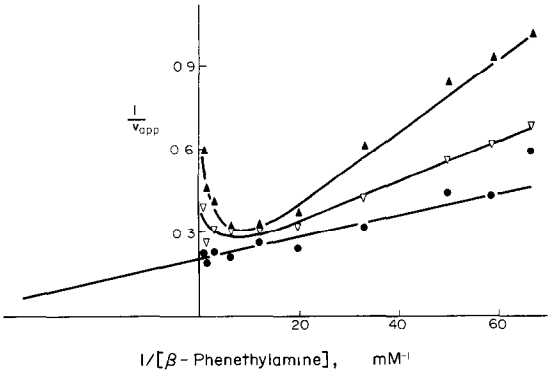


Fig. 3. Double reciprocal plot of the deamination of β-phenethylamine by rat liver mitochondrial MAO. Ordinate, 1/apparent velocity (in nmoles/mg protein per min); abscissae, 1/β-phenethylamine concentrations in mM. The apparent velocity was defined as the total product found/time. Each pair represents the mean of duplicate determinations for three mitochondrial preparations. Each assay sample contained 250 μg protein. The incubation times used to assay the activities were 3 min (●), 10 min (▽) and 20 min (▲). Incubation for 6 and 15 min gave the appropriate intermediate curves.

liver) (mean ± S.E.M.) of the substrates had been utilised. At an intermediate β-phenethylamine concentration (150 μM) in the rat liver, there is little deviation from linearity with time up to 20 min of preincubation, by which time 28 ± 3% (mean ± S.E.M.) of the substrate had been utilised (Fig. 2A and B). The results for human liver at pH 7.2, shown in Fig. 2B, were also found to be similar at pH 7.8.

When the data for each time point were plotted as 1/apparent velocity (calculated as the total product formed/incubation time) against 1/β-phenethylamine concentration, a linear plot was obtained after 3 min of incubation, but an apparent high-substrate inhibition was found with longer incubation times (Fig. 3). Such a result was not found with tyramine as substrate. Thus, the apparent high-substrate inhibition by β-phenethylamine reported earlier [1–4] is due to a time-dependent inhibition and failure to determine the true initial rates of reaction.

Several explanations of the apparent hysteretic behaviour of β-phenethylamine can be put forward, and these are discussed below:

1. *β-Phenethylamine is unstable and forms (or is converted by an enzyme other than MAO to) an inhibitor during long incubation periods at* 37°. It is possible that β-phenethylamine could be converted to an MAO inhibitior as a result of long incubation periods at 37°. However, the interaction between β-phenethylamine (at concentrations of either 100, 500 or 1000 μM) and MAO from either rat or human liver was not affected after prior preincubation of the substrate for up to 40 min with either distilled water, high-speed supernatant fractions from human liver (obtained by centrifugation of homogenates at 70,000 *g* for 120 min), human platelet-poor plasma (prepared as described elsewhere [18]), or for up to 30 min with a pH inactivated mitochondrial fraction (made by setting a mitochondrial fraction to pH 2 for 30 min, then pH 11 for 30 min, before finally resetting to pH 7.2). Since none of these preparations contained detectable MAO activity, it seems reasonable to assume that β-phenethylamine is stable during long incubation periods, and does not react with any pH-stable or cytoplasmic component to form an inhibitor. Contamination of either radioactive or non-radioactive β-phenethylamine has been discounted as the source of the inhibition from the results of both isotope-dilution experiments and experiments with recrystallised non-radioactive β-phenethylamine [5].

In the rat liver, β-phenethylamine is predominantly metabolised by MAO-B, although a small (about 10%) proportion of the substrate is metabolised by MAO-A at high substrate concentrations [19, 20]. Incubation of rat liver mitochondrial fractions with concentrations of up to 1.5 mM *d*-amphetamine for periods up to 90 min at 37° did not produce any time-dependent inhibition. This compound is a reversible MAO-A selective inhibitor of the enzyme [21], although significant inhibition of MAO-B is observed at the concentrations used (about 63% for 1.5 mM). Thus the failure to find any time-dependent inhibition of MAO-B with this compound suggests that structural considerations, similar to those that prevent amphetamine acting as a substrate for the enzyme to any significant extent, may be involved in the time-dependent inhibition of MAO-B by β-phenethylamine.

2. *β-Phenethylamine interacts with its deaminated metabolites to form an inhibitor of MAO.* The inhibition of MAO shown in Fig. 3 is not consistent with simple product inhibition, since this type of inhibition would not give a rapid onset of inhibition at high substrate concentrations (see [22]). This was confirmed experimentally, since neither 10, 40 nor 100 μM phenylethanol or phenacetic acid produced significant inhibition of rat liver MAO, even after 30 min of preincubation. These concentrations are greater than the maximum concentrations of the products that would be found in the mitochondrial fractions after incubation with β-phenethylamine.

Phenacetaldehyde, the aldehyde produced by the interaction between MAO and β-phenethylamine, was found to be inhibitory at concentrations higher than 30 μM, but this inhibition was not found after preincubation between the aldehyde and the mitochondria for either 20 min or 40 min, indicating that sufficient aldehyde dehydrogenase activity is present in the mitochondrial fractions to metabolise the phenacetaldehyde to phenacetic acid. Furthermore, addition of an excess of NAD^+ and purified ox liver aldehyde dehydrogenase did not affect the pattern of the time-dependent inhibition of MAO activity by high concentrations of β-phenethylamine. Thus build-up of an inhibitory aldehyde can be discounted as an explanation for the time-dependent inhibition.

Simple substrate inhibition of MAO cannot be responsible for the inhibition, since no such inhibition is found after short incubation times (Fig. 3). One possibility is, however, that high concentrations of β-phenethylamine may react with one of the products to produce an inhibitory compound. Such a reaction would explain the time-dependence of the inhibition. For example, a simple condensation reaction between β-phenethylamine and phenacetaldehyde to produce a Schiff base, which then might cyclise (see e.g. [23]) can be postulated. However, preincubation for 60 min at 37° of β-phenethylamine (500 μM) with phenacetaldehyde (10 μM), phenylethanol (40 μM) or phenacetic acid (40 μM), either singly or in combination, were not found to be inhibitory towards rat liver MAO. Thus, the inhibition observed in Fig. 3 is not due to any reaction of β-phenethylamine with its products.

3. *β-Phenethylamine is a 'suicide substrate'.* Recently, Waley [24] has published a kinetic treatment for a substrate that also acts as a 'suicide inhibitor' of an enzyme. In order to test whether such a model can be applied to the interaction between β-phenethylamine and MAO, the reversibility of the time-dependent inhibition was determined by two different methods. Firstly, 250 μl of rat liver mitochondria (protein concentration 5 mg/ml) were preincubated with 250 μl of 2 mM β-phenethylamine for 60 min at 37°. The samples were then put on ice and centrifuged at 10,000 *g* for 10 min and washed (by resuspension in 1 ml sucrose–phosphate buffer followed by recentrifugation at 10,000 *g* for 10 min) twice, and finally resuspended in 1 ml sucrose–phosphate buffer. The activity recovered in the samples was the same as that found in either samples preincubated with distilled water instead of

β-phenethylamine or samples to which the β-phenethylamine had been added after the preincubation period. This method would therefore suggest that the inhibition is reversible in nature.

Secondly, non-radiolabelled β-phenethylamine and mitochondria were preincubated at 37°, and aliquots taken and added to an assay mixture containing radioactively labelled β-phenethylamine, resulting in a twenty-fold dilution of both enzyme and non-radiolabelled β-phenethylamine. Any inhibition found (with respect to samples not preincubated) must be irreversible in nature (see [9] for discussion). With 1 mM β-phenethylamine, no irreversible inhibition was found after 3 min of preincubation, but inhibition was found after 10 min, which was not increased even when the preincubation period was lengthened to 30 min (Fig. 4). The simplest explanation of the results obtained by the two methods for testing reversibility is that the β-phenethylamine inhibits the enzyme in a time-dependent manner, but there is a slower reactivation of inhibited enzyme. Since a relatively long incubation period was necessary to assay for activity of the twenty-fold diluted samples, the 40% irreversible inhibition found in Fig. 4 presumably represents a more complete inhibition coupled with considerable enzyme recovery during the assay period. This suggestion is supported by the 'dose–response' curve shown in Fig. 5, where no irreversible inhibition is found with preincubation concentrations of 100 and 200 μM β-phenethylamine, but maximum (~40%) inhibition is found with 500, 800 and 1250 μM β-phenethylamine (Fig. 5). When the preincubations were performed under an atmosphere of nitrogen instead of air, effectively inhibiting the MAO activity since the enzyme has a relatively high K_m value towards oxygen [15, 25, 26], similar irreversible inhibition was found as when the samples were preincubated under air. The data would suggest that complete irreversible inhibition by β-phenethylamine is found at concentrations higher than 500 μM, but no significant inhibition is detectable at concentrations lower than 100 μM.

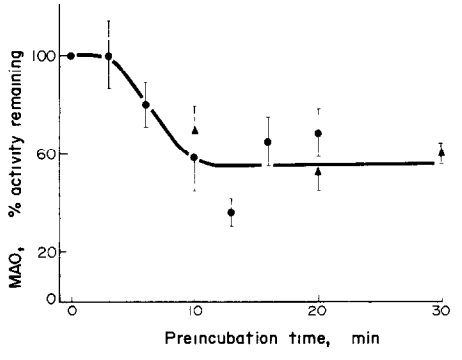


Fig. 4. The irreversible inhibition of monoamine oxidase by preincubation with β-phenethylamine. Mitochondrial preparations were preincubated at 37° with 1 mM non-radiolabelled β-phenethylamine for the times shown and then 20 μl aliquots (containing 50 μg protein) added to 380 μl assay mix (containing 80 μM radiolabelled β-phenethylamine). Thus, since little or no β-phenethylamine was utilised during the preincubation period, the final assay concentration of β-phenethylamine was 100 μM. At 15 min incubation time was necessary due to the low concentration of mitochondria in the assay mixture. All points represent means ± S.E.R. of duplicate determinations in three mitochondrial preparations of the %MAO activity remaining with respect to samples where no preincubation had taken place.

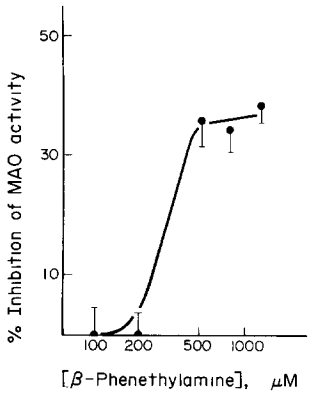
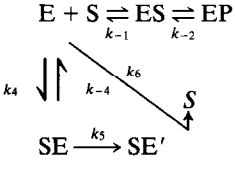


Fig. 5. Irreversible inhibition of monoamine oxidase by preincubation with β-phenethylamine. Mitochondrial preparations were preincubated with different concentrations of non-radiolabelled β-phenethylamine at 37° under an atmosphere of air for 20 min. After preincubation, 20 μl aliquots (containing 90 μg protein) were added to 380 μl assay mix containing a fixed amount of radiolabelled β-phenethylamine and the appropriate amount of non-labelled β-phenethylamine so that the final assay concentration of substrate was 100 μM. A 10 min incubation time was necessary due to the low concentration of mitochondria in the assay mixture. All points represent means ± S.E.R. of duplicate determinations in three mitochondrial preparations of the %MAO activity remaining with respect to samples preincubated the same length of time with distilled water.

If β-phenethylamine were acting as a 'suicide inhibitor' it would be expected that the inhibition would not occur under nitrogen, analogous to the situation found for the inhibition of MAO activity by phenylhydrazine [27], dimethylpropynylamine [28] and histamine [29]. In the case of β-phenethylamine, however, the inhibition is not affected by the lack of oxygen. In addition, if β-phenethylamine were acting as a 'suicide substrate' of the type described by Waley [24], it would be expected that the affinity of the substrate as inhibitor would be similar to its affinity as a substrate. In the present case, the K_m for MAO towards β-phenethylamine is about 16 μM (Fig. 3), consistent with other studies under the same conditions [7, 20], whereas the inhibition is not apparent until much higher concentrations are reached (see Figs. 3 and 5). Thus, β-phenethylamine does not appear to be acting as a 'suicide substrate'.

One model consistent with the data is shown below:



where E = enzyme, S = substrate, P = deaminated product, and E′ = inactivated enzyme. Such a model would explain both the time-dependent nature of the inhibition, the recovery of enzyme activity, the different affinities of β-phenethylamine as inhibitor and as substrate, and the failure to observe high-substrate inhibition when initial rates are determined. The model is similar to that postulated by Frère *et al.* [30] to explain the time-dependent inhibition of the exocellular DD-carboxypeptidase-transpeptidase from Streptomyces R61 by β-lactam antibiotics. It is possible that the actual inhibition is more complicated than shown above, but the model represents the simplest kinetic system consistent with the data.

If it is assumed that the formation of SE is a slow process, the thermodynamic equilibrium $S + E \rightleftharpoons SE$ will not appreciably be disturbed and can be represented by a simple dissociation constant (K_i). If P_m is defined as the amount of product formed at time t in the absence of an inhibitory action, and P is the product formed at time t in the presence of inhibitory action, then it can be calculated that:

$$\frac{P}{P_m} = \frac{k_4}{b(k_4 + k_a)} + \frac{k_a}{b(k_4 + k_a)^2 t}[1 - e^{-(k_4 + k_a)t}]$$

where

$$k_a = k_3/(1 + K_i/[S] + K_i/K_m)$$

$$b = k_1 + K_m/[S] + K_m/K_i$$

and

$$K_m + (k_{-1} + k_2)/k_1$$

(see [30]).

Such a solution adequately describes the time-courses for β-phenethylamine deamination shown in Figs. 2A and B, and hence the time-dependent inhibition at high substrate concentrations (Fig. 3). It should be stressed, however, that this model is postulated for the interaction between β-phenethylamine and MAO-B, since about 90% of the metabolism of this substrate, even at high concentrations, is by this enzyme form in the rat liver [19, 20]. Further studies would be necessary in a tissue where the A form of the enzyme plays a more predominant role, such as the rat heart [19], to determine whether or not a similar interaction between β-phenethylamine and MAO-A is found.

In both human liver and rat brain, MAO has been shown preferentially to oxidise the unionised form of the substrate [1, 31, 32]. It was therefore possible that, while the unionised form of the substrate is metabolised by the enzyme, the ionised form of the substrate forms the inhibitory (SE) complex. Although such a suggestion might explain the pH-dependence of the apparent high-substrate inhibition by β-phenethylamine that has been reported for human brain MAO [33], this seems unlikely in view of the observation that the inhibition increases with increasing pH [5].

The failure to observe any time-dependent inhibition by tyramine and amphetamine could be due either to their failure to react in such a way that the inhibited species (E′) is formed at a significant rate or to the rate of reactivation of this species to the native enzyme (E) occurring so much more rapidly that the steady-state concentration of E′ is not significant. Further work would be required to investigate whether such an explanation for the action of these compounds is correct.

In conclusion, the data presented here demonstrate, once again, the importance of ensuring that initial rates are determined in studies of enzyme kinetics and that in this case it is not adequate simply to restrict the incubation time to one in which less than 5% of the substrate has been consumed.

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