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Some factors influencing the metabolism of benzylamine by type A and B monoamine oxidase in rat heart and liver

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The ability of MAO-A and MAO-B to metabolize benzylamine in vitro has been investigated in mitochondrial preparations from rat liver and heart. Although under normal circumstances benzylamine appeared to be metabolized exclusively by MAO-B in the rat liver, a contribution by both MAO-A and a clorgyline-resistant enzyme component was revealed when the MAO-B activity was much reduced by pretreatment of the mitochondria with appropriate concentrations of deprenyl. These three enzyme activities also contributed to benzylamine deamination in rat heart mitochondria. However, binding studies with [^{3}H]pargyline, which provided an estimate of the respective concentrations of MAO-A and MAO-B active centres in heart mitochondria, indicated a ratio between MAO-A and MAO-B, markedly different from that shown by plots of inhibition of benzylamine metabolism by various concentrations of clorgyline. The interpretation of these clorgyline plots is discussed in terms of the kinetic constants of both MAO-A and MAO-B, and the relative amounts of each enzyme. It is proposed that although the turnover rate constant for benzylamine metabolism by MAO-A is much smaller than that shown by MAO-B, in those tissues containing a large ratio of MAO-A:MAO-B content, the metabolism of benzylamine by MAO-A can be detected.

Monoamine oxidase (MAO; EC 1.4.3.4) is a flavoprotein enzyme found predominantly in the outer membrane of the mitochondrion, which catalyses the oxidative deamination of some amine substrates (see Blaschko 1974 for review).

In 1968, Johnston proposed that two forms of the enzyme may exist, based on his observations that the in vitro deamination of tyramine in rat brain mitochondria was inhibited in a biphasic manner by increasing concentrations of the acetylenic compound clorgyline. Thus, a binary classification of MAO activity, in which MAO-A shows a relatively greater sensitivity than MAO-B towards irreversible inhibition by clorgyline, has provided a useful framework for attempts to elucidate the importance of MAO activity in amine breakdown. In addition, another irreversible MAO inhibitor, deprenyl, which shows a relative selectivity for MAO-B has also been useful for this purpose (Knoll & Magyar 1972).

From early studies, particularly with rat liver and brain (Johnston 1968; Hall et al 1969), it seemed possible that the two forms of MAO would show distinct differences in their specificities for the deamination of certain amines, with for example, 5-hydroxytryptamine (5-HT) originally being described as a specific substrate for MAO-A, benzylamine for MAO-B, and tyramine as a substrate for both forms. However, some subsequent investigations with other animal tissues have contradicted this simple scheme, and there now appears to be a general consensus that the relative sensitivity towards clorgyline should provide the primary criterion for defining MAO-A and MAO-B activity (see Fowler et al 1978 for review).

It remains an interesting problem to determine why the substrate specificities of MAO-A and MAO-B should appear to differ from tissue to tissue. Our own earlier studies on the rat heart provide a pertinent example. Unlike the rat liver (Hall et al 1969), the use of clorgyline revealed that benzylamine deamination by the rat heart was brought about not only by MAO-B, but also by MAO-A (Lyles & Callingham 1974, 1975). To date, the only other similar example is the human placenta (Lewinsohn et al 1980) and it has been suggested by these investigators that these results may be explained if MAO-A is present in much greater amounts than

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MAO-B but that its ability to deaminate benzylamine is much less.

The present paper describes some studies designed to investigate further the possible importance of the relative amounts and catalytic activities of MAO-A and MAO-B in determining the final shape of inhibition curves obtained with clorgyline, for benzylamine deamination in animal tissues. Two approaches have been adopted. First, the MAO-B activity of rat liver mitochondria has been almost completely removed by irreversible inhibition with appropriate concentrations of deprenyl, in an attempt to determine whether any residual metabolism of benzylamine by MAO-A in this tissue could be detected. Second, the actual amounts of MAO-A and MAO-B have been measured directly in rat heart mitochondria by means of a recently described binding assay which uses [^{3}H]pargyline (Parkinson & Callingham 1980), and the resulting data have been compared with inhibition curves for benzylamine deamination obtained separately with clorgyline in the same heart samples.

MATERIALS AND METHODS

Materials

Radioactive substrates for MAO were [^{3}H]5-hydroxytryptamine, and [^{14}C]benzylamine from the Radiochemical Centre, Amersham, U.K., and [^{3}H]tyramine from New England Nuclear, Dreieich, West Germany.

Clorgyline hydrochloride (M&B 9302) was a gift from May & Baker Ltd., Dagenham, U.K. and (−)-deprenyl hydrochloride was a gift from Professor M. Sandler, Department of Chemical Pathology, Queen Charlotte's Hospital, London, U.K. 2-Chloropargyline was kindly supplied by Dr A. O. Geiszler, Abbott Laboratories, Chicago, U.S.A. and [^{3}H]pargyline (0·5 Ci mmol^{-1}) was prepared as previously described (Parkinson & Callingham 1980). Other reagents were of analytical grade where possible.

Male Wistar rats, about 300 g, were supplied by A. J. Tuck & Sons, Rayleigh, U.K.

Methods

Studies with rat liver mitochondria. For the preparation of liver mitochondria, the livers from six rats were pooled and homogenized in five volumes of 0·25 M sucrose–10 mM potassium phosphate buffer, pH 7·8, by the use of a Sorvall Omnimixer, and the homogenate was centrifuged for 15 min at 600 *g* to remove cell debris, nuclei etc. The supernatant was decanted and then centrifuged for 20 min at 7000 *g* to obtain a mitochondrial pellet. This was resuspended in 50 mM potassium phosphate buffer, pH 7·8 to give a protein content of 63 mg ml^{-1} by the microbiuret method of Goa (1953). This preparation was divided into several suitable aliquots which were stored deep-frozen over two weeks, during the completion of the inhibition studies. For each experiment, sufficient amounts were thawed on the day, and used only once.

To obtain liver mitochondria with greatly reduced MAO-B activity, 2 ml aliquots of the mitochondria were initially diluted to 6 ml with 50 mM potassium phosphate buffer, pH 7·8 and then incubated with 6 ml solutions of deprenyl (in distilled water) for 1 h at 37 °C in a shaking water bath. Final deprenyl concentrations in this incubation mixture ranged from 6×10^{-8} to $1{\cdot}5 \times 10^{-7}$ M. Control incubations contained all constituents except deprenyl in the mixtures. At the end of the incubation period, all mixtures were diluted to 35 ml with 50 mM potassium phosphate buffer, pH 7·8 and centrifuged for 20 min at 30 000 *g* to re-isolate mitochondrial fractions containing only irreversibly bound deprenyl. The mitochondrial pellets obtained from this step were subjected to further washing before final recovery, first by resuspension in 45 ml of 1 mM potassium phosphate buffer pH 7·8, and then by recentrifugation for 20 min at 30 000 *g*. The mitochondrial pellets obtained from this step were resuspended in 6 ml of 1 mM potassium phosphate buffer, pH 7·8, and used as the source of MAO for assays of remaining activity.

The sensitivity of this remaining activity towards inhibition by clorgyline was studied in the following manner. 25 μl samples of the mitochondrial fractions were preincubated for 20 min at 37 °C with 25 μl solutions of clorgyline in distilled water, to give final clorgyline concentrations from 10^{-9} to 10^{-3} M. MAO activity was then assayed by the addition of 50 μl [^{3}H]tyramine or [^{14}C]benzylamine (2 mM in 200 mM potassium phosphate buffer, pH 7·8) as previously described (Callingham & Laverty 1973). For the construction of inhibition plots, MAO activity in the presence of clorgyline is expressed as a percentage of control samples which contained no clorgyline during the preincubation period.

Studies with rat heart mitochondria. Individual rat hearts were homogenized in eight volumes of 0·25 M sucrose–10 mM potassium phosphate buffer, pH 7·8 in a glass homogenizer with 10 strokes of a Teflon pestle. The resulting suspension was centrifuged for 15 min at 600 *g* to remove nuclei and cell debris. To 1·5 ml of the supernatant was added either (a)

150 μl of distilled water, or (b) 150 μl of clorgyline to give a final concentration of 3×10^{-7} M. Each suspension was incubated for 20 min at 37 °C, then cooled on ice and centrifuged at 12 000 *g* for 2 min in an Eppendorf centrifuge. The pellets were resuspended in 1·2 ml of 50 mM potassium phosphate buffer, pH 7·8 by vortex mixing. They were then recentrifuged at 12 000 *g* for 2 min and the pellets resuspended in 300 μl of 50 mM potassium phosphate buffer, pH 7·8. 25 μl of each suspension were then taken for the estimation of specific [^{3}H]pargyline binding by the method of Parkinson & Callingham (1980). The total specific binding to MAO was defined as the difference between the binding of 10^{-5} M [^{3}H]pargyline after incubation with mitochondria for 1 h at 37 °C, and that obtained after prior incubation with $3{\cdot}3 \times 10^{-7}$ M 2-chloropargyline at 37 °C for 30 min. To estimate the contribution of MAO-A and MAO-B towards the total specific binding of pargyline, we compared the specific binding in samples that had or had not been pretreated with clorgyline (3×10^{-7} M), with the rationale that this concentration of clorgyline should cause virtually complete irreversible inhibition of MAO-A under these conditions, whilst leaving the MAO-B binding sites intact for interaction with pargyline. However, to check the validity of this assumption, the MAO activity towards 0·5 mM 5-HT (which is a substrate for MAO-A activity alone in the rat heart; Lyles & Callingham 1975) was measured by means of the radiochemical assay (Callingham & Laverty 1973) on identical samples to those used for the binding studies. Under these conditions, it was found that around 90% or more of 5-HT metabolizing activity was inhibited by the clorgyline, and this information enabled a correction to be made with each homogenate for its total MAO-A content, based on the assumption that the specific binding of [^{3}H]pargyline that was reduced by treatment with 3×10^{-7} M clorgyline represented the corresponding 90% or more of the total MAO-A binding sites.

Samples of the control mitochondrial fractions, prepared as for the binding assays above, but without pretreatment with clorgyline, were used for some additional concurrent studies. These involved determinations of the in vitro sensitivity of benzylamine metabolism in these hearts towards inhibition by varying concentrations of clorgyline (10^{-9} to 10^{-3} M) and these experiments were performed as described in the previous section for rat liver mitochondrial fractions, except that the final benzylamine concentration in the assay was 0·5 mM.

RESULTS

Inhibition of rat liver mitochondrial MAO activity by clorgyline after pretreatment with deprenyl

Fig. 1 shows the inhibition of tyramine deamination by clorgyline in rat liver mitochondria which had been pretreated with 6×10^{-8} and 8×10^{-8} M deprenyl, and also in samples without deprenyl in the pretreatment procedure. In each case, a biphasic plot was obtained with a plateau region around 10^{-7} to 10^{-6} M clorgyline. These results indicated a ratio of about 30:70 for the contribution of MAO-A and MAO-B, respectively, towards tyramine metabolism in the absence of deprenyl.

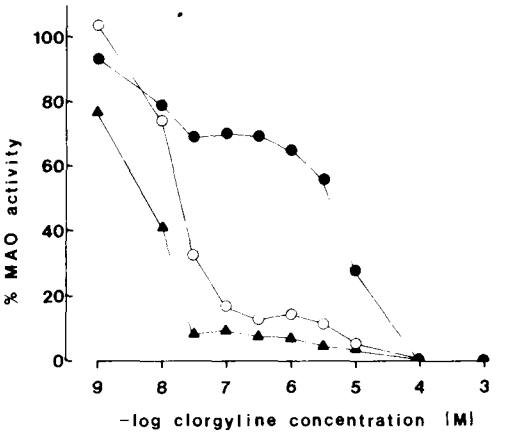

FIG. 1. In vitro inhibition by clorgyline of tyramine deamination in rat liver mitochondrial preparations pretreated with or without deprenyl. Deprenyl concentrations were zero (●——●), 6×10^{-8} M (○——○) and 8×10^{-8} M (▲——▲). Samples were assayed with 1 mM tyramine and activities are expressed as percentages of corresponding samples containing no clorgyline. Each point is the mean of sextuplicate determinations (standard errors of the ratio, omitted for clarity were $< \pm 6\%$).

After deprenyl pretreatment, this ratio changed to approximately 85:15 (6×10^{-8} M deprenyl) and 90:10 (8×10^{-8} M deprenyl), thus attesting to the progressive, selective removal of MAO-B activity as the deprenyl concentrations were increased by small increments. The specific enzyme activity (nmol h^{-1} [mg protein]$^{-1}$) towards tyramine in the control samples changed from 805 (without deprenyl) to 327 (6×10^{-8} M deprenyl) and 241 (8×10^{-8} M deprenyl).

When benzylamine was used as substrate to measure remaining MAO activity in these control samples, and also in some additional samples pretreated with slightly higher concentrations of deprenyl, the following specific activities (nmol h^{-1}

[mg protein]$^{-1}$) were measured: 341 (without deprenyl), 64 (6×10^{-8} M deprenyl), 28 (8×10^{-8} M deprenyl), 24 (9×10^{-8} M deprenyl) and 18 ($1{\cdot}5 \times 10^{-7}$ M deprenyl). Plots of the inhibition produced by clorgyline upon these samples are shown in Fig. 2A. In the absence of deprenyl pretreatment, the inhibition of benzylamine deamination required high concentrations and was monophasic, which indicated that the metabolism of benzylamine appeared to be brought about by MAO-B alone in this sample.

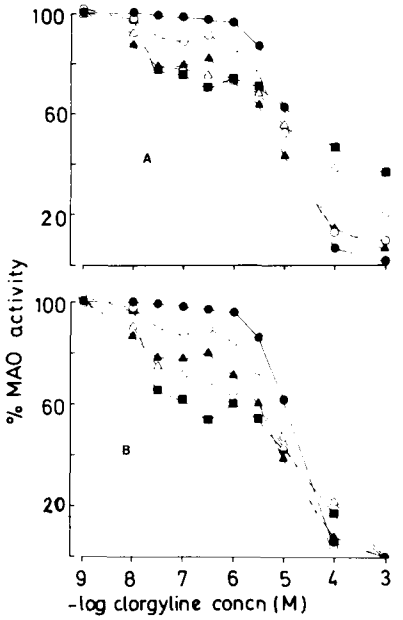

FIG. 2. A. In vitro inhibition by clorgyline of benzylamine deamination in rat liver mitochondrial preparations pretreated with or without deprenyl. Deprenyl concentrations were zero (●——●), 6×10^{-8} M (○- - -○), 8×10^{-8} M (▲——▲), 9×10^{-8} M (△- - -△) and $1{\cdot}5 \times 10^{-7}$ M (■——■). Samples were assayed with 1 mM benzylamine and activities are expressed as percentages of corresponding samples containing no clorgyline. Each point is the mean of sextuplicate determinations (standard errors of the ratio, omitted for clarity were $< \pm 4\%$).

B. Data of Fig. 2A plotted on normalized basis to exclude activity remaining in each sample after treatment with 10^{-3} M clorgyline.

In contrast, in samples pretreated with deprenyl, a distinct biphasic nature was revealed, indicating a contribution of MAO-A towards benzylamine deamination in these samples. From these plots, it appeared that a limiting proportion of about 20–25% of the total activities in these samples could be attributed to the MAO-A component, and this altered little following pretreatment with deprenyl concentrations above 8×10^{-8} M. However, at the same time, it is clear that an increasing proportion of benzylamine deamination in these samples remained resistant to inhibition by clorgyline at 10^{-3} M, a concentration which should be sufficient to inhibit both MAO-A and MAO-B activity completely. It therefore appears likely that these experiments are also revealing the activity of the residual, clorgyline-resistant amine oxidase which has been described in other rat tissues (Coquil et al 1973; Lyles & Callingham 1975; Lewinsohn et al 1978). Consequently, the inhibition of MAO activity was considered to represent the benzylamine deamination that could be inhibited by concentrations of clorgyline up to 10^{-3} M, and the inhibition curves were replotted on this normalized basis to exclude the contribution from the resistant enzyme (Fig. 2B). Here, it can be seen that the proportion of MAO-A increases from being undetectable (samples without deprenyl) to about 40% ($1{\cdot}5 \times 10^{-7}$ M deprenyl) of the total MAO activity towards benzylamine.

Inhibition of benzylamine deamination by clorgyline in rat heart mitochondrial fractions

Individual mitochondrial samples from the hearts of six rats were prepared as described in Materials and Methods, and the sensitivity of the benzylamine deamination in these samples towards inhibition by increasing concentrations of clorgyline was studied. Fig. 3 shows the overall data plotted as a mean inhibition curve from the six individual experiments.

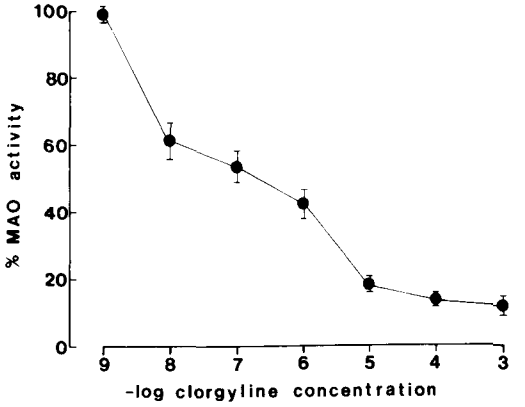

FIG. 3. In vitro inhibition by clorgyline of benzylamine deamination in rat heart mitochondrial preparations. Activities were assayed with 0·5 mM benzylamine and are expressed as percentages of corresponding samples containing no clorgyline. Each point is the mean ± standard error of the ratio from six rats, each assayed in triplicate.

Table 1. Specific activity and content of MAO in rat heart mitochondria. Activity in each heart mitochondrial preparation was assayed with 5-hydroxytryptamine (5-HT) and benzylamine (BZ) at 0·5 mM concentration. MAO-A and MAO-B content was estimated by the use of [^{3}H]pargyline as described in the text. Values are given as mean ±s.e.m. of six animals.

Body weight (g)	Heart weight (mg)	MAO specific activity [nmol (mg protein)$^{-1}$ h^{-1}]		MAO content [pmol (mg protein)$^{-1}$]	
		5-HT	BZ	MAO-A	MAO-B
310 ± 10	850 ± 32	540 ± 35	19·7 ± 1·0	8·87 ± 0·82	0·51 ± 0·22

In agreement with earlier findings for benzylamine metabolism by the rat heart (Lyles & Callingham 1975), a biphasic plot was obtained, and in these animals the plot indicated a contribution of approximately 50% and 40% for MAO-A and MAO-B, respectively, towards the total benzylamine deamination. In addition, the clorgyline-resistant amine oxidase represented about 10% of the total activity measured here.

[^{3}H]Pargyline binding to rat heart mitochondrial fractions

The concentrations of MAO-A and MAO-B active centres in the heart mitochondrial fractions from these six rats were estimated by means of the experiments described in Materials and Methods, which used [^{3}H]pargyline as a binding ligand. These results are shown in Table 1 along with the specific enzyme activities in these samples towards 5-HT and benzylamine, measured with the radiochemical assay. From the binding data, the contribution of MAO-A and MAO-B towards the total specific binding (9·38 pmol [mg protein]$^{-1}$) was estimated to be 95% and 5%, respectively.

DISCUSSION

It is clear from the present results that benzylamine is a substrate for MAO-A in the rat liver. This has been shown by selectively reducing the contribution of MAO-B towards the total benzylamine metabolism by the use of deprenyl, such that the MAO-A component now represents a significant proportion of the remaining activity towards this substrate. As a quantitative example of this point, it should be noted that the highest pretreatment concentration of deprenyl ($1·5 \times 10^{-7}$ M) caused a loss of 95% of the specific enzyme activity towards benzylamine, and from the inhibition by clorgyline of that remaining activity (5% of the original total), approximately 25% is represented by MAO-A, 40% by MAO-B, and 35% by the clorgyline-resistant activity (Fig. 2A). Thus, it is apparent that under normal circumstances, the proportional contributions of MAO-A and the clorgyline-resistant amine oxidase towards total benzylamine deamination in the rat liver are so tiny that they are masked by the preponderance of MAO-B, and to date, the corresponding inhibition curves have always been interpreted as single-sigmoid plots showing MAO-B alone to be active against benzylamine (e.g. Hall et al 1969). These results therefore suggest that the relative amounts of MAO-A and MAO-B (and the clorgyline-resistant amine oxidase) in a tissue may play a fundamental role in determining whether or not benzylamine metabolism by MAO-A can be seen.

Further evidence for this conclusion was sought in experiments with mitochondria from the rat heart, a tissue which was the first in which it was shown that benzylamine could be deaminated by MAO-A (Lyles & Callingham 1974, 1975). In the present study, the inhibition curves obtained by the use of clorgyline indicated that the relative proportions of MAO-A, MAO-B and clorgyline-resistant activity were approximately 50, 40 and 10%, respectively. By comparison, the use of [^{3}H]pargyline provided an estimate that MAO-A and MAO-B represented approximately 95 and 5%, respectively, of the total MAO binding sites in hearts from these animals. (It has been assumed in this work that [^{3}H]pargyline is unable to bind to the clorgyline-resistant enzyme). Thus, there appears to be a rather large discrepancy in the proportions of MAO-A and MAO-B detected by the two methods. However, a discussion of possible reasons for this discrepancy requires a clarification of the information available from inhibition curves obtained with clorgyline. These plots only allow an estimation of the proportions of the total metabolism of a substrate under the given assay conditions which can be attributed to MAO-A and/or MAO-B (and the clorgyline-resistant enzyme where appropriate). Restricting this discussion to MAO activity, the total metabolism (v_{total}) of a substrate (at concentration S) which is acted upon by both MAO-A and MAO-B, may be described by the sum of two Michaelis–Menten equations representing the reaction velocities (v_A and v_B) of each enzyme,

assuming no complications due to high substrate inhibition or product inhibition (see Peers et al 1980).

Thus,

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

and also,

$$\frac{v_A}{v_B} = \frac{V_{max_A}}{V_{max_B}} \cdot \frac{K_{m_B} + S}{K_{m_A} + S}$$

We have previously found evidence that K_{m_A} for benzylamine in the rat heart is very similar to K_{m_B}, being around 500 μM under our assay conditions (Lyles & Callingham 1975), and thus as an approximation we can consider that

$$\frac{K_{m_B} + S}{K_{m_A} + S} \simeq 1$$

Thus,

$$\frac{v_A}{v_B} \simeq \frac{V_{max_A}}{V_{max_B}}$$

Now, V_{max} for an enzymatic reaction is given by the term $k_p \cdot E_t$, where k_p represents the turnover rate constant for product formation, and E_t represents the total number of enzyme molecules (or active centres).

Hence,

$$\frac{v_A}{v_B} \simeq \frac{k_{p_A} \cdot E_{t_A}}{k_{p_B} \cdot E_{t_B}}$$

Our analysis of [^{3}H]pargyline binding indicates that E_{t_A}/E_{t_B} is 95/5 i.e. 19. In contrast, from the biphasic clorgyline plots v_A/v_B is 50/40 i.e. 1·25. Substitution therefore allows the approximation that k_{p_A}/k_{p_B} is 0·066, indicating that the turnover rate constant of MAO-B for benzylamine is approximately 15 times greater than that of MAO-A.

Clearly, this analysis has been made primarily on the basis of approximations of the parameters considered. Nevertheless, the data do point to the conclusion that in the example of benzylamine, both the turnover rate constant and the relative amounts of MAO-A and MAO-B appear to determine the eventual contributions of these enzyme forms towards the total benzylamine deamination. In contrast, studies with other substrates such as β-phenethylamine and isoamylamine suggest that large differences in K_{m_A} and K_{m_B} might also play a role in these determinations (Dial & Clarke 1979; Suzuki et al 1979; Peers et al 1980).

It is pertinent to include some additional comments about the clorgyline-resistant activity which metabolizes benzylamine in the rat heart and liver. In a previous study with the rat heart, this activity appeared to have a predominantly cytosolic subcellular distribution, although some activity was detectable in mitochondrial and microsomal fractions (Lyles & Callingham 1975). However, in the present experiments this activity has been recovered in well-washed mitochondrial membrane fractions from both heart and liver, and raises the possibility of a true membrane-bound location of part or all of this enzyme activity. In this context, Fowler & Callingham (1977) showed that the clorgyline-resistant enzyme which is responsible for virtually all of the benzylamine metabolism in the chick heart is recovered almost totally in membrane fractions, predominantly those containing the mitochondria. Clearly, the question as to the true subcellular site of this enzyme remains an important one to be answered.

In summary, our results suggest that benzylamine should be considered as a *relatively* specific substrate for MAO-B, mainly by virtue of a greater ability of this enzyme form than of MAO-A, to convert the bound substrate to product. However, in tissues such as the rat heart, the large excess of MAO-A enzyme content may more than compensate for the unfavourable k_p, and thus result in the observation that a significant proportion of substrate metabolism occurs by means of the A-form. This influence becomes particularly evident with age in the rat heart, in which the increasing specific activity of MAO in this organ appears to be due to selective increases in the activity and amount of MAO-A (Lyles & Callingham 1979; Fowler & Callingham 1979; Edwards et al 1979). A large excess of MAO-A over MAO-B content can also be produced experimentally in the rat liver, by almost total inhibition of its MAO-B by pretreatment with suitable concentrations of deprenyl. We have not attempted in the present study to determine the actual values of the turnover rate constants (k_{p_A} and k_{p_B}) for benzylamine, mainly because our assays of enzyme specific activity were not performed under V_{max} conditions. However, it would seem to be an obvious approach for future investigations to determine if these parameters are indeed constant from tissue to tissue, in order to provide information about whether or

not these enzyme forms from different tissues and animal sources show identical properties.

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