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# MIXED SUBSTRATE EXPERIMENTS WITH HUMAN BRAIN MONOAMINE OXIDASE

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**Abstract**—Michaelis constants for human brain monoamine oxidase have been determined with tyramine, benzylamine and dopamine as the substrates. In each case double reciprocal plots were linear over a 20-fold range of substrate concentrations. The method of mixed substrates failed to indicate heterogeneity in the enzyme preparation. The theory of the method of mixed substrates has been extended to cover systems in which two enzymes are each active toward two different substrates. It is shown that, regardless of the difference in $K_m$ values of each of the individual enzymes for the two substrates, if the $K_m$ values of the two enzymes are similar for each individual substrate the situation is indistinguishable from the case in which only a single enzyme is present. The observation that the mixed substrate experiments do not indicate the presence of more than one species of monoamine oxidase cannot therefore be regarded as providing firm evidence for homogeneity.

A NUMBER of preparations of the enzyme monoamine oxidase (monoamine:$O_2$ oxidoreductase (deaminating) E.C. 1.4.3.4) have been shown to contain several forms of the enzyme. These may be distinguished by the differential effects of thermal denaturation[1–3] and some irreversible inhibitors[4–7] on the activities towards different substrates as well as by the separation of different bands of activity on polyacrylamide gel electrophoresis.[2,3,7–10] Despite these differential effects the method of mixed substrates,[11] which usually provides a sensitive indicator for the presence of more than one enzyme, has failed to provide any evidence of heterogeneity in preparations which appeared to contain multiple forms as judged by other criteria.[1,2]

## MATERIALS AND METHODS

Human brain monoamine oxidase was prepared by the method previously reported,[7,10] and the enzyme was assayed at 30° in 0·05 M glycine–KOH buffer (pH 8·2), by following the reduction of $NAD^+$ spectrophotometrically at 340 nm as the aldehyde produced was oxidized in the presence of beef liver aldehyde dehydrogenase.[2] Human brains were obtained within 12 hr of post-mortem, from subjects with no recorded history of mental illness or of treatment with anti-depressant drugs.

$NAD^+$ was obtained from Biochemica, Boehringer, Mannheim, Germany. All other chemicals were from B.D.H. Ltd, Poole, Dorset, and were of the highest quality available. Benzylamine was converted into its hydrochloride, and was recrystallized before use.

## RESULTS

Double reciprocal plots were used to determine the $K_m$ and $V_{max}$ values for the three substrates tyramine, benzylamine and dopamine. These were found to be linear

TABLE 1. MICHAELIS CONSTANTS FOR HUMAN BRAIN MONOAMINE OXIDASE

| Substrate | $K_m$ ($\mu$M) | $V_{max}$ (nmoles/mg/min) | Substrate concn range (fold) | Correlation coefficient |
|---|---|---|---|---|
| Tyramine | 79 | 13·6 | 20 | 0·997 |
| Dopamine | 111 | 9·8 | 20 | 0·992 |
| Benzylamine | 91 | 17·1 | 20 | 0·998 |

Correlation coefficients were assessed on Lineweaver–Burke plots involving determinations of initial velocity at fifteen different substrate concentrations.

over a 20-fold range of substrate concentration for each substrate (correlation coefficients >0·99 in each case). The $K_m$ and $V_{max}$ values obtained are shown in Table 1.

The linearity of the reciprocal plots would suggest that if, as has been indicated by other studies,[7,10] this preparation contained more than one form of monoamine oxidase, these forms must have similar $K_m$ values for the three substrates and differ in their maximum velocities. To test this hypothesis the method of mixed substrates was carried out as described by Dixon and Webb.[11] The results of this study with mixtures of pairs of the three substrates is shown in Table 2. As in the case with previous studies[1,2] using this method the results gave no indication of the presence of more than one enzyme.

## DISCUSSION

The three substrates used in this study were chosen because the activities of human brain monoamine oxidase towards each of them had been shown to respond differently to a number of inhibitory procedures[7] and, in addition one of the electrophoretically separable forms of human brain monoamine oxidase appears to have a high specificity towards dopamine as a substrate.[8,10] The failure of the method of mixed substrates to indicate heterogeneity together with the linearity of the reciprocal plots over a wide range could indicate that the modifications leading to multiplicity had little effect on the $K_m$ or $V_{max}$ values of the parent enzyme. An alternative possibility arises, however, from a consideration of the theory behind the method of mixed substrates. This theory has so far only been developed in order to distinguish between a single enzyme acting on two substrates and two enzymes with different specificities. The case in which two enzymes are present each of which is active

TABLE 2. MIXED SUBSTRATE EXPERIMENTS

| Substrate pairs | Ratio × 100% |
|---|---|
| Benzylamine + tyramine | 70 |
| Benzylamine + dopamine | 65·5 |
| Tyramine + dopamine | 66·5 |

The ratio described is that of the observed mixed rate over the sum of the observed individual rates. At equal relative concentrations, equal to the $K_m$ concentrations, then the ratio should tend to 66·7% if one enzyme is responsible for the deamination of both substrate pairs.[11]

towards two substrates can, however, under certain circumstances lead to a result which is indistinguishable from the presence of a single enzyme.

If we consider the two enzyme species, E and F, catalysing the conversion of two substrates S and T:

$$E + S \rightleftharpoons ES \rightarrow E + P$$

$$E + T \rightleftharpoons ET \rightarrow E + Q$$

$$F + T \rightleftharpoons FT \rightarrow F + Q$$

where enzyme E can catalyse the conversion of substrates S and T into products P and Q respectively, whereas enzyme F can only catalyse the conversion of T into Q. In a mixed substrate experiment S and T may be considered to be competitive inhibitors with respect to each other for enzyme E, whereas if S does not bind to enzyme F it will obviously have no effect on the reaction catalysed by this enzyme. If the Michaelis constants for both enzymes for the substrate T are similar ($K_m^{ET} = K_m^{FT}$) then, when both substrates are present at their Michaelis constant concentrations, i.e. $K_m^{ET} = [T] = K_m^{FT}$ and $[S] = K_m^{ES}$, we can derive the expression:

$$R = \frac{v_{obs}}{(v_{ES}^0 + v_T^0)} = \frac{2}{3} + \frac{v_{FT}^0}{3(v_{ES}^0 + v_T^0)} \tag{1}$$

where $R$ is the ratio of the observed velocity of the total reaction in the presence of S and T($v_{obs}$) to the sum of the rates that would be observed if each substrate was assayed separately. Thus $v_{ES}^0$ represents the velocity of conversion of S by the enzyme E in the absence of T and $v_T^0$ represents the sum of the observed velocities for the conversion of T by enzymes E($v_{ET}^0$) and F($v_{FT}^0$) in the absence of S.

In such a situation $R$ is greater than 2/3 (0·67), whereas if a single enzyme catalyses the breakdown of both substrates,[11] $R$ would be equal to 2/3. Thus the method of mixed substrates would provide evidence for the presence of more than one enzyme in the preparation (Fig. 1), although its sensitivity would depend on the relative values of their maximum velocities $\bar{V}_{FT}$ and $\bar{V}_{ET}$. Under conditions where $\bar{V}_{FT}$ contributes only a minor proportion of the total rate of conversion of T and enzyme F is unable to bind S the mixed substrate method would give results which would tend to 66·7 per cent as the value of $\bar{V}_{FT}/\bar{V}_{ET}$ tends to zero. If, however, enzyme F were able to bind S as a competitive inhibitor with respect to T with a $K_i(K_i^{FS})$ value equal or close to $K_m^{ES}$ then it follows that when substrates are assayed at relative concentrations:

$$v_{obs} = \frac{v_{ES}^0(1 + \beta)}{1 + \delta + \beta} + \frac{v_{ET}^0(1 + \delta)}{1 + \delta + \beta} + \frac{v_{FT}^0(1 + \delta)}{1 + \delta + \beta} \tag{2}$$

where $\beta$ is the relative concentration $[S]/K_m^{ES} = [S]/K_i^{FS}$, and $\delta = [T]/K_m^{ET} = [T]/K_m^{FT}$. Such an equation reduces to:

$$R = \frac{v_{obs}}{(v_{ES}^0 + v_T^0)} = \frac{2}{3} \tag{3}$$

when substrates are held at their Michaelis concentrations ($\beta = \delta = 1$). Thus this situation would be indistinguishable from that in which only a single enzyme species

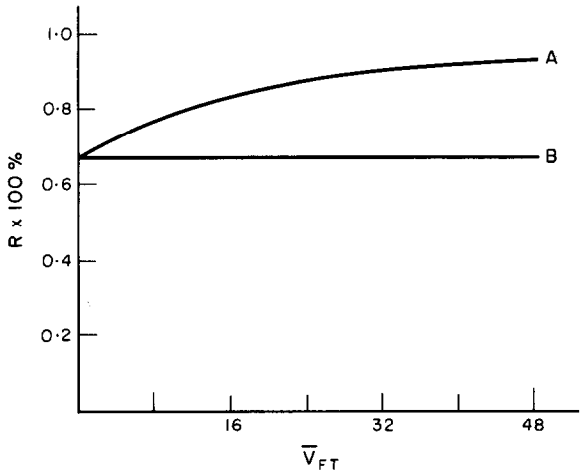


FIG. 1. Dependence of the ratio $R$ on the change in maximum velocity of the conversion of T by enzyme F. Line A represents the situation in which substrate S does not bind to enzyme F, and line B represents the situation in which S does bind to F. These data were obtained using the computer simulation program described by Illingworth.[13] Values for the Michaelis constants were $K_m^{ES} = K_i^{FS} = 111\ \mu$M. and $K_m^{ET} = K_m^{FT} = 79\ \mu$M. The maximum velocity for S conversion was set at $8{\cdot}0 \times 10^4\ \mu$moles/min ($V_{ES}$) and that for the conversion of T catalysed by E was $8{\cdot}0 \times 10^4\ \mu$moles/min ($V_{ET}$).

were involved. Similarly, if F were to bind S and convert it to P under conditions such that $K_m^{FS}$ was equal to (or, in practice, close to) $K_m^{ES}$, the value of the expression would be given by:

$$R = \frac{v_{obs}}{(v_S^0 + v_T^0)} = \frac{2}{3} \tag{4}$$

where $v_S^0 = v_{ES}^0 + v_{FS}^0$.

If such a situation were to exist, then reciprocal plots for the total conversion of T against [T], obtained at a series of fixed concentrations of S would indicate S to be a competitive inhibitor,[2] since:

$$\frac{1}{v_T^0} = \frac{K_m^{ET}(1 + [S]/K_m^{ES})}{[T](V_{ET} + V_{FT})} + \frac{1}{(V_{ET} + V_{FT})} \tag{5}$$

where $V_{ET}$ and $V_{FT}$ are maximum velocities. Dixon plots[12] of such inhibition data would be linear as would also be the case if only a single enzyme were involved. If, however, enzyme F did not bind S, the reciprocal plots obtained would be downwardly curving and the Dixon plots[12] would be non-linear, since:

$$\frac{1}{v_T^0} = \frac{[T](2K_m^{ES}K_m^{ET} + [S] + [T]K_m^{ES}) + (K_m^{ET})^2 K_m^{ES} + [S]K_m^{ET}}{K_m^{ES}(V_{ET} + V_{FT})([T] + K_m^{ET}) + [S]V_{FT}} \tag{6}$$

Thus the presence of two enzymes that catalyse the conversion of the two substrates would be indistinguishable from a single enzyme if the $K_m$ values of both enzymes were identical for each substrate pair regardless of differences in maximum velocities. In practice, of course, the $K_m$ values would need to be close rather than identical for such a situation to arise. Thus a situation in which a fraction of the enzyme preparation had impaired catalytic activity towards one substrate without its ability

to bind the substrate being greatly affected would be indistinguishable from a totally unimpaired preparation if the $K_m$ values of the enzymes for that substrate were close to the dissociation constant for the enzyme substrate complex ($K_s$).

If we consider the mixed substrate experiments with respect to, for example, tyramine and dopamine oxidation, then assuming two enzymes act on each substrate, the sum of the observed velocities of oxidation (in isolation) is given by

$$V^0_{itot} = \frac{\alpha \bar{V}_{ES}}{1+\alpha} + \frac{\alpha \bar{V}_{FS}}{1+\alpha} + \frac{\beta \bar{V}_{ET}}{1+\beta} + \frac{\beta \bar{V}_{FT}}{1+\beta} \tag{7}$$

and with substrate mixtures, for mutual competitive inhibition,

$$V^0_{mtot} = \frac{\alpha \bar{V}_{ES}}{1+\alpha+\beta} + \frac{\alpha \bar{V}_{FS}}{1+\alpha+\beta} + \frac{\beta \bar{V}_{ET}}{1+\alpha+\beta} + \frac{\beta \bar{V}_{FT}}{1+\alpha+\beta} \tag{8}$$

and the mixed substrate ratio, $R = V^0_{itot}/V^0_{mtot}$.

Where E and F are the two enzyme species, *S*-dopamine, *T*-tyramine, and $\alpha$ is the relative concentration for dopamine, and $\beta$ is the relative concentration for tyramine as defined previously. Some of the relationships between Michaelis parameters and the value of $R$, are examined in Table 3, when substrate concentrations are such that $\alpha = \beta = 1$. Such theoretical calculations must of course assume a single $K_m$ value regardless of any set of differences, between $K_m$ values. It is however difficult to detect nonlinearity of double-reciprocal plots and the situation in which two enzymes were present with $K_m$ values differing by factor of 3 could

TABLE 3. SOME VALUES OF THE MIXED SUBSTRATE RATIO, $R$ WITH CHANGES IN MICHAELIS PARAMETERS

| $K_m$ condition \ $V$ condition | Mixed substrate ratio, $R$(%) | | | |
|---|---|---|---|---|
| | $\bar{V}_{ES} = \bar{V}_{ET}$<br>$\bar{V}_{FS} = \bar{V}_{FT}$<br>$\bar{V}_{ES}/\bar{V}_{FS} = 1$ | $\bar{V}_{ES} = \bar{V}_{FS}$<br>$\bar{V}_{ET} = \bar{V}_{FT}$<br>$\bar{V}_{ES}/\bar{V}_{ET} = 4{\cdot}9/6{\cdot}8$ | $10\bar{V}_{ES} = \bar{V}_{FS}$<br>$10\bar{V}_{ET} = \bar{V}_{FT}$<br>$\bar{V}_S/\bar{V}_T = 9{\cdot}8/13{\cdot}6$ | $\bar{V}_{ES} = 10\bar{V}_{FS}$<br>$\bar{V}_{ET} = 10\bar{V}_{FT}$<br>$\bar{V}_S/\bar{V}_T = 9{\cdot}8/13{\cdot}6$ |
| $K_m^{ES} = K_m^{FS}$<br>$K_m^{ET} = K_m^{FT}$ | 66·7 | 66·7 | 66·7 | 66·7 |
| $K_m^{ES} = 2K_m^{FS}$<br>$K_m^{ET} = K_m^{FT}$ | 68·8 | 68·0 | 73·0 | 67·3 |
| $K_m^{ES} = K_m^{FS}$<br>$K_m^{ET} = 2K_m^{FT}$ | 68·8 | 67·3 | 69·6 | 66·9 |
| $K_m^{ES} = 2K_m^{FS}$<br>$K_m^{ET} = 2K_m^{FT}$ | 72·2 | 71·7 | 72·0 | 67·0 |
| $K_m^{ES} = 3K_m^{FS}$<br>$K_m^{ET} = K_m^{FT}$ | 70·7 | 71·6 | 77·2 | 67·5 |
| $K_m^{ES} = K_m^{FS}$<br>$K_m^{ET} = 3K_m^{FT}$ | 70·7 | 70·5 | 72·2 | 67·1 |
| $K_m^{ES} = 2K_m^{FS}$<br>$K_m^{ET} = 3K_m^{FT}$ | 70·9 | 76·3 | 75·2 | 68·6 |
| $K_m^{ES} = 3K_m^{FS}$<br>$K_m^{ET} = 2K_m^{FT}$ | 70·9 | 76·0 | 76·8 | 67·5 |
| $K_m^{ES} = 3K_m^{FS}$<br>$K_m^{ET} = 3K_m^{FT}$ | 76·1 | 74·8 | 77·6 | 67·3 |

give rise to plots which appeared to be linear within experimental error[11] giving an apparent $K_m$ value between the two actual values and biased by the relative values of their maximum velocities.

In Table 3 it is assumed that $\alpha = [S]/K_m^{ES}$ where $[S] = K_m^{ES}$, and $\beta = [T]/K_m^{ET}$ where $T = K_m^{ES}$. It can be seen that under certain conditions the value of $R$ is relatively insensitive to small fluctuations in $K_m$ values, and in many cases is within the expected experimental error of that for a single enzyme catalysing both reactions ($R = 66{\cdot}7$ per cent). Variations will also depend on a relative value of the ratio $\bar{V}_S/\bar{V}_T$, the ratio of the total rates of oxidation for dopamine and tyramine, and also for the ratios, $\bar{V}_{ES}/\bar{V}_{FS}$ and $\bar{V}_{ET}/\bar{V}_{FT}$.

If it is assumed that dopamine can competitively inhibit tyramine T, then under the conditions examined in equation (5) a linear Dixon plot would result unless, one enzyme (F) did not bind dopamine when a non-linear Dixon plot would be obtained [equation (6)]. In addition if F binds dopamine but $K_m^{FS} \neq K_m^{ES}$, then a non-linear result may be obtained (Fig. 2). The degree of non-linearity depends on the concentration of the fixed substrate (tyramine), the degree of dissimilarity between $K_m^{FS}$ and $K_m^{ES}$, and the relative maximum velocities. Also, the ratio $K_m^{FT}/K_m^{ET}$ is important, for the theoretical curves (Fig. 2) show either a "damping" ($K_m^{FT} > K_m^{ET}$ Fig. 2b) or "accentuation" ($K_m^{ET} > K_m^{FT}$ Fig. 2c) of non-linearity depending on this ratio. However it is obvious that deviations in $K_m$ values of 2 or 3 fold yield Dixon plots that would no doubt appear linear assuming a nominal experimental error. This exemplifies the need to use wide ranges of both inhibitor and substrate concentrations when studying inhibition kinetics by Dixon analysis.[12]

Thus the results of the mixed substrate experiments with monoamine oxidase are not necessarily incompatible with the results of Johnston[4] which indicated that two

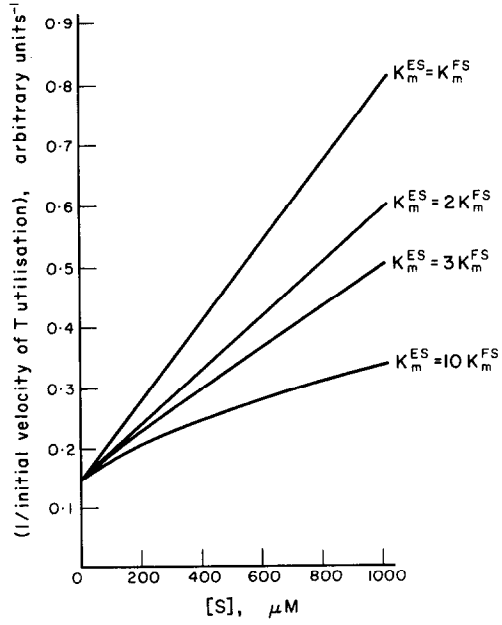


FIG. 2(a).

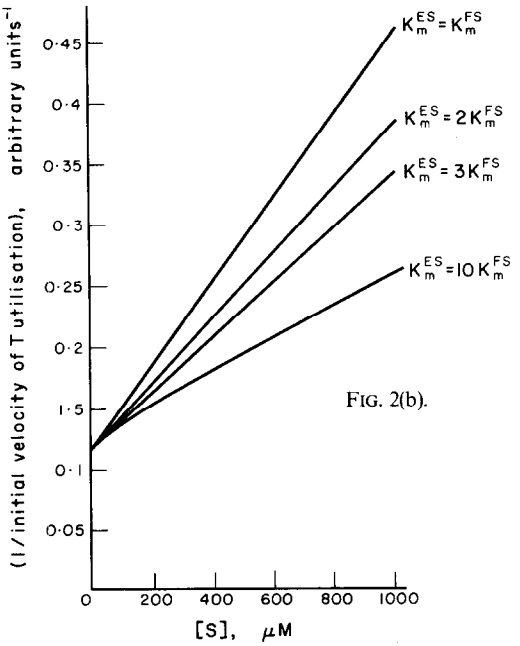


FIG. 2(b).

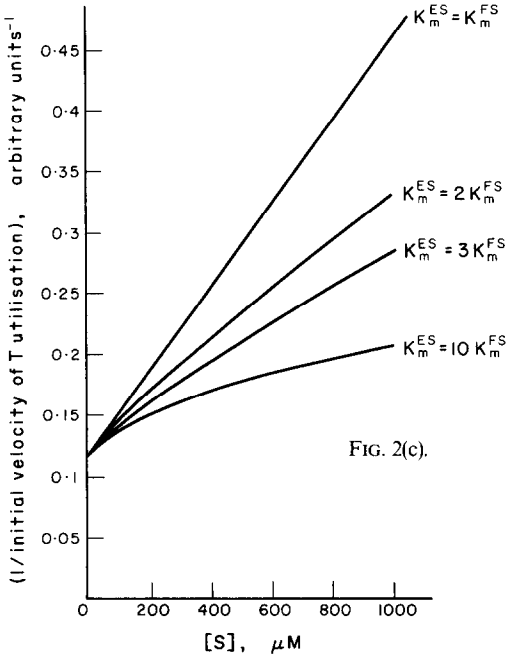


FIG. 2(c).

FIG. 2. Theoretical Dixon plots showing the inhibition of tyramine deamination by dopamine. These results are obtained by substitution in the equation

$$V_T^0 = \frac{\bar{V}_{ET}[T]}{K_m^{ET}(1 + [S]/K_m^{ES}) + [T]} + \frac{V_{FT}[T]}{K_m^{FT}(1 + [S]/K_m^{FS}) + [T]}$$

where we assume that $\bar{V}_{ET} = \bar{V}_{FT}$ and that $[T] = K_m^{ET} = 79\,\mu M$. The curves obtained using a number of ratios of $K_m^{ES}/K_m^{FS}$ are examined, and $K_m^{ES}$ is fixed as $111\,\mu M$. (a) $K_m^{ET} = K_m^{FT}$; (b) $2K_m^{ET} = K_m^{FT}$; (c) $K_m^{ET} = 2K_m^{FT}$.

enzyme species existed in such preparations of the enzyme. The presence of two kinetically distinct enzymes with similar $K_m$ (or $K_i$) values for the individual amines would give no indication of heterogeneity when examined by the mixed substrate method. Such a situation may account for the apparently conflicting evidence concerning the heterogeneity of monoamine evidence. Since the binding of membrane material has been demonstrated to affect the properties of the enzyme[2,7] it may be that such binding affects the maximum catalytic activity without significantly affecting the $K_m$ values of the species generated.

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