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AMINE COMPETITION FOR OXIDATION BY RAT LIVER MITOCHONDRIAL MONOAMINE OXIDASE

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Abstract—Mixed substrate experiments have been carried out with rat liver mitochondrial monoamine oxidase. The results of these studies are interpreted in terms of the known specificities and sensitivities to inhibition of the two kinetically distinguishable species present in this preparation. The results of this study are discussed in terms of the function of the enzyme *in vivo*.

Recently we have proposed a model for amine oxidation in rat liver mitochondria [1], based on the selective action of the irreversible inhibitor clorgyline [*N*-methyl-*N*-propargyl-3-(2,4-dichlorophenoxy-propylamine) HCl] and the reversible inhibitors benzyl cyanide and 4-cyanophenol. This model is similar to that proposed by Johnston [2], who postulated two enzyme species each with a distinct substrate specificity, these he designated the "A-enzyme", which was most sensitive to clorgyline and the "B-enzyme" which was less sensitive to clorgyline. However, Johnston's model would imply that elevation of the concentration of certain amines would be without effect on the metabolism, by monoamine oxidase, of some others. This study examines the properties of the rat liver system in terms of mixed substrate experiments, expanding a previous study with human brain monoamine oxidase [3], and demonstrates that mutual competition between amine substrates does indeed occur, although different amines will be affected to different extents. It is also pointed out that the interpretation of mixed substrate experiments may indeed be misleading.

METHODS

Mitochondrial outer membranes were prepared from rat liver mitochondria in the manner previously described [1, 4]. This preparation has been shown to contain 90 ± 5 per cent of the total mitochondrial monoamine oxidase activity originally present (independent of assay substrate), and to be free from enzyme activities that could interfere with the assay methods [1, 4]. Monoamine oxidase activity was assayed using either an oxygen electrode [5] or by using a coupled assay for aldehyde production, that followed the reduction of NAD^+ in the presence of beef liver aldehyde dehydrogenase [6]. All assays were carried out in 0·12 M potassium phosphate buffer, pH 7·2 and at 30°.

All materials were from BDH Ltd., Poole, Dorset, U.K., and were of the highest purity available, except for benzylamine free base which was obtained from Ralph N. Emanuel Ltd., Wembley, Middlesex, U.K., and was converted to its hydrochloride prior to use [4].

Values of the mixed substrate ratio, R, were determined from the formula [7],

$$R = \frac{\text{observed rate of oxidation of substrate mixture}}{\text{sum of individual rates of oxidation of each substrate in isolation}}$$

and the substrate concentrations were held at their Michaelis (K_m) concentrations which had previously been determined under these assay conditions [1].

We have previously shown [1] that the oxidation of adrenaline, noradrenaline, and their corresponding *m*-O-methyl derivatives and serotonin are carried out by "species A" monoamine oxidase, that benzylamine oxidation is carried out by "species B" monoamine oxidase and that tyramine and dopamine are substrates for both enzyme species. Also we have postulated that substrates of "species A" can bind the "species B" enzyme and inhibit oxidation of "species B" substrates in a competitive fashion, with K_i values similar to their K_m values for the "species A" enzyme. However it would appear that substrates of the "species B" enzyme can also bind to the "species A" enzyme, and thus inhibit oxidation of "species A" substrates, but in this case in a mixed fashion with K_i values similar to their K_m value for the "species B" enzyme. Thus we can consider a number of cases for the oxidation of substrate mixtures and define them in terms of steady state rate equations.

(*a*) *A mixture of two substrates of the same enzyme species*, e.g. 2-phenylethylamine and benzylamine, both

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"species B" substrates or serotonin and adrenaline, both "species A" substrates:

$$V^0_{mt} = \frac{\bar{V}_x[X]}{K^x_m(1 + [Y]/K^y_m) + [X]} + \frac{\bar{V}_y[Y]}{K^y_m(1 + [X]/K^x_m) + [Y]} \quad (1)$$

where V^o_{mt}, is the observed initial rate of oxidation of the substrate mixture; $\bar{V}_x$, $\bar{V}_y$ are maximum velocities; K^x_m, K^y_m are Michaelis constants; [X], [Y] are the two substrate components. These equations show that substrates for the same enzyme species, act as mutually competitive inhibitors.

(*b*) *A mixture of a substrate for the "species A" enzyme (X) and one and for the "species B" enzyme (Y)*, e.g. adrenaline (X) and benzylamine (Y):

$$V^0_{mt} = \frac{\bar{V}_x[X]}{K^x_m(1 + [Y]/K^y_i) + [X](1 + [Y]/K'^y_i)} + \frac{\bar{V}_y[Y]}{K^y_m(1 + [X]/K^x_i) + [Y]} \quad (2)$$

Here K^y_i, K'^y_i and K^x_i are the inhibitor constants, and the equation shows that adrenaline will competitively inhibit benzylamine oxidation by the "species B" enzyme; however, benzylamine will inhibit adrenaline oxidation by the "species A" enzyme in a mixed fashion.

(*c*) *A mixture of a substrate for both enzyme "species A and B" (X) and a substrate for "species B" only (Y)*, e.g. tyramine (X) and benzylamine (Y):

$$V^0_{mt} = \frac{\bar{V}_x[X]}{K^x_m(1 + [Y]/K^y_i) + [X](1 + [Y]/K'^y_i)} + \frac{\bar{V}'_x[X]}{K'^x_m(1 + [Y]/K'^y_i) + [X]} + \frac{V_y[Y]}{K^y_m(1 + [X]/K^x_i) + [Y]} \quad (3)$$

where $\bar{V}_x$ and K^x_m are those Michaelis parameters for that oxidation of tyramine (X) which is due to the "species A" enzyme, and $\bar{V}'_x$ and K'^x_m are those Michaelis parameters for that oxidation of tyramine (X) which is due to the "species B" enzyme.

Thus tyramine will competitively inhibit the oxidation of benzylamine, whereas benzylamine will apparently inhibit the oxidation of tyramine in a mixed fashion if the value of K^x_m is similar to K'^x_m (which would appear to be the case in rat liver [1]) or in a non-linear fashion if K^x_m is very different from K'^x_m.

(*d*) *A mixture of a substrate for both enzyme species (X) and a substrate for the "species A" enzyme only (Y)*, e.g. tyramine (X) and adrenaline (Y):

$$V^0_{mt} = \frac{\bar{V}_x[X]}{K^x_m(1 + [Y]/K^y_i) + [X]} + \frac{\bar{V}'_x[X]}{K'^x_m(1 + [Y]/K'^y_i) + [X]} + \frac{\bar{V}_y[Y]}{K^y_m(1 + [X]/K^x_i) + [Y]} \quad (4)$$

where $\bar{V}_x$, $\bar{V}'_x$ and K^x_m, K'^x_m are as defined in section (c). Here we can see that tyramine is a competitive inhibitor of benzylamine oxidation and benzylamine will appear to competitively inhibit tyramine oxidation if the value of K^x_m is close to that of K'^x_m, as would appear to be the case in rat liver [1].

(*e*) *A mixture of two substrates for both enzyme species*, e.g. tyramine and dopamine. These will appear to inhibit each other in a mutually competitive fashion in rat liver mitochondria, and we have discussed this in full elsewhere [3].

RESULTS AND DISCUSSION

The results of the mixed substrate experiments, carried out as described in Methods are shown in Table 1. The values of R observed are very close to that expected for a single enzyme catalysing the oxidation

Table 1. Observed and calculated values for the mixed substrate ratio R

Substrate pair	R-observed	Equation obeyed	R-calculated ($K^x_m = K^x_i$)	R-calculated ($2K^x_m = K^x_i$) or ($2K^x_m = K'^x_m = K^x_i$)
Adrenaline (X)–benzylamine (Y)	0·63	2	0·59	0·66
Noradrenaline (X)–benzylamine (Y)	0·66	2	0·59	0·66
m-*O*-Methylnoradrenaline (X)–benzylamine (Y)	0·66	2	0·59	0·67
m-*O*-Methyladrenaline (X)–benzylamine (Y)	0·66	2	0·60	0·67
Dopamine (X)–benzylamine (Y)	0·69	3	0·63	0·71
Tyramine (X)–benzylamine (Y)	0·69	3	0·63	0·69

of both substrate pairs, i.e. 0·67. However there is considerable evidence that more than one enzyme is responsible for the oxidation of these substrates (see e.g. Ref. 1), and thus we will reconsider the theory of mixed substrate experiments in terms of the equations given in the Methods section.

Equations 1 *and* 4. This situation has been dealt with previously [3], where mutually competitive substrates are present. Indeed when the K_m value shown by a particular substrate has a value close or equal to its K_i value shown towards the enzyme species that binds it but does not catalyse its oxidation, then the value of R tends to 0·67, that expected for a single enzyme catalysing the conversion of both substrates. This means that under the conditions of equation 4, then K_m^x, $K_m'^x$ and K_i^x must have similar values and K_m^y, K_i^y and $K_i'^y$ must also have similar values. Such a situation would appear to be the case [1], and would explain why the values of R observed for the dopamine–benzylamine and tyramine–benzylamine experiments (Table 1) are close to 0·67. Indeed, manipulation of the kinetic constants in equation 4 such that $2K_m^x = K_m'^x = K_i^x$, which would be predicted from our previous studies [1], yields a calculated value of R that agrees with the experimental one.

Equation 2. If we consider the case when $[Y] = K_m^y = K_i^y = K_i'^y$ and $[X] = K_m^x = K_i^x$, then the value of the mixed substrate ratio, R, is given by,

$$R = \frac{V_{mt}^0}{(V_x^0 + V_y^0)} = \frac{1}{2} + \frac{V_y^0}{6(V_x^0 + V_y^0)} \qquad (5)$$

where V_{mt}^o is the observed initial velocity of the substrate mixture, and V_x^o, V_y^o are the initial rates of oxidation of X by "species A" and Y by the "species B" enzyme respectively when assayed in isolation.

Under such conditions $R > 0{\cdot}5$; however, when $V_y^o \gg V_x^o$, then R tends to a value of 0·67, the value obtained for one enzyme catalysing the oxidation of both substrates [7]. In practice, the values of K_m^y, K_i^y, $K_i'^y$ and K_m^x, K_i^x need only be similar to achieve a value of R close to 0·67, and indeed we have shown that for benzylamine $K_m^y = 0{\cdot}245$ mM, $K_i^y = 0{\cdot}17$ mM and $K_i'^y = 0{\cdot}3$ mM. If we substitute these values in equation 2 and let the ratio $V_y:V_x = 10:7$ then we can derive values of R, which are in good agreement with those obtained experimentally even allowing for small variations in the $K_i^x:K_m^x$ ratio.

Equation 3. Under conditions such that $[Y] = K_m^y = K_i^y = K_i'^y = K_i''y$ and $[X] = K_m^x = K_m'^x = K_i^x$, the value of the mixed substrate ratio (R) is given by,

$$R = \frac{V_{mt}^0}{V_y^0 + V_x^0 + V_{x'}^0} = \frac{1}{2} + \frac{(V_y^0 + V_{x'}^0)}{6(V_y^0 + V_x^0 + V_{x'}^0)} \qquad (6)$$

where V_y^o, $V_{x'}^o$ are the initial velocities of oxidation of Y and X in isolation by the "species B" and V_x^o is that for the oxidation of X in isolation by the "species A" enzyme and V_{mt}^o is the initial rate of oxidation of the substrate mixture.

It is clear that under these conditions that R can have a value less than 0·67 if $V_x^o \gg (V_{x'}^o + V_y^o)$, i.e. R tends to 0·5. However, R tends to a value of 0·67, that expected for a single enzyme acting on both substrates [7] if:

$$(V_x^o + V_{x'}^o) \gg V_y^o,$$

$$\text{or } V_x^o \ll V_y^o \gg V_{x'}^o,$$

$$\text{or } V_x^o \ll V_{x'}^o \gg V_y^o$$

From our previously obtained data [1], it is likely that the kinetic constants delineated at the beginning of this section are likely to be similar, and that $(V_y^o + V_{x'}^o) > V_x^o$ for both benzylamine–dopamine and benzylamine–tyramine pairs [1], and indeed values for R similar to 0·67 are obtained (Table 1). Theoretical values of R are shown in Table 1, assuming that for benzylamine (Y) and dopamine (X), that $\bar{V}_y:\bar{V}_{x'}:\bar{V}_x = 100:67{\cdot}2:44{\cdot}8$ and for benzylamine (Y) and tyramine (X) this ratio is equal to 100:130:70 (see Ref. 1). Such an approach yields values of R that agree well with those obtained experimentally (Table 1), and indeed the observed value of R falls within the range calculated assuming $K_m^x = K_m'^x = K_i^x$ and that when $2K_m^x = K_m'^x = K_i^x$, reflecting the probable variation predicted by the use of clorgyline on the enzyme from this source [1].

We may conclude that an approach based upon our model for amine oxidation in rat liver mitochondria [1], accounts for these observations from mixed substrate experiments. This also would appear to support the validity of the irreversible inhibitor clorgyline as a useful tool in predicting the substrate specificity of monoamine oxidase. For indeed until now noradrenaline, adrenaline and their *m*-O-methyl derivatives have been assigned as substrates of the "species A" enzyme based solely on their sensitivity of their oxidation to clorgyline inhibition.

Similarly this kinetic treatment shows how interpretation of mixed substrate experiments in the manner described by Dixon and Webb [7], can be misleading under certain defined conditions.

It is clear that a change in concentration of a substrate for one enzyme species, whether it be caused by release of amine from storage granules, or nerve uptake, or increased synthesis, will not only affect the rate of oxidation of other substrates for the same enzyme species but also the rates of oxidation of substrates for the other enzyme species. However the mode of inhibition as explained in the Methods section is not a simple competitive event in all cases, but substrates for the "species B" enzyme inhibit oxidation of substrates for the "species A" enzyme in a mixed fashion.

In Fig. 1, the rates of oxidation of single components of substrate mixtures are examined using data for kinetic constants from Ref. 1, and equations 1–4. Where each curve is directly comparable as the fixed substrate in each case is at its K_m concentration, and increase in concentration of the variable substrate is shown as a

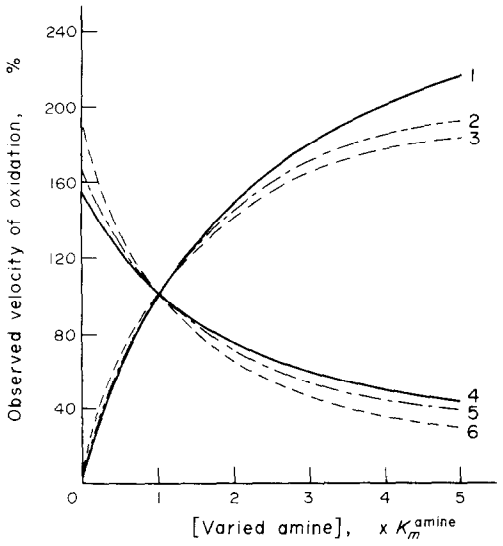

Fig. 1. Amine competition for oxidation. The observed velocity of oxidation of a single amine component of a two component mixture (as a percentage of that observed when both components are at their respective K_m concentrations) is plotted against increase in concentration of one such component (whose concentration is shown as a multiple of its K_m concentration). The non-varied component being present at a concentration equal to its K_m.

Curves 1–3 demonstrate the velocity of oxidation of the amine whose concentration is being varied in the mixture. Curves 4–6 demonstrate the velocity of oxidation of that amine whose concentration is constant (at a value equal to its K_m), with increase in concentration of the varied amine component.

Curve	Equation obeyed	Velocity of oxidation followed	Varied amine component	Fixed amine component
1	1	X	X	Y
1	1	Y	Y	X
1	2	X	X	Y
1	3	Y	Y	X
1	4	Y	Y	X
1	4	X	X	Y
2	3	X	X	Y
3	2	X	X	Y
4	1	X	Y	X
4	1	Y	X	Y
4	2	Y	X	Y
4	3	Y	X	Y
4	4	Y	X	Y
4	4	X	Y	X
5	3	X	Y	X
6	2	X	Y	X

multiple of its K_m concentration, curves 1 and 4 show the standard effect of mutually competitive substrates. This result is strikingly different from curves 3 and 6 (equation 2), demonstrating the effect of a substrate for "species B" (Y) on oxidation of a "species A" substrate (X). In this case it can be seen that if [X] = K_m^x an increase from K_m^y to 5 K_m^y results in a fall of 164 per cent in the velocity of X oxidation compared with 106 per cent for the competitive case. Similarly, if [Y] = K_m^y an increase in [X] from K_m^x to 5 K_m^x causes a rise in velocity of X oxidation of only 184 per cent compared with 214 per cent for the competitive case (equations 1, 4). Curves 2 and 5 are those determined using equation 3, considering the effect of a mixture of a substrate for "species B" and a substrate for both species and represent an intermediate position to the two states discussed above.

The absolute value of these differences depend on the relative concentrations of the amines present (i.e. amine concentration/K_m^{amine}), K_i values towards the enzyme species they bind but does not catalyse their oxidation, the maximal velocities of oxidation, and the ratio of activities of the two enzyme species in the case of amines oxidised by both enzyme species.

It is apparent that drugs effecting a change in the concentration of one particular amine will cause a change in the levels of other amines by inhibition of monoamine oxidase. The significance of this effect will depend on the kinetic parameters of the amine whose level is initially perturbed, and also the enzyme species it is oxidised by. Another important factor to be considered if the free steady state amine levels are perturbed, is that the corresponding metabolite pattern of various amines may well be altered. Indeed, in brain various workers [8, 9] have shown that biogenic aldehydes possessing a β-OH group are predominantly reduced, whereas those not possessing this moiety are predominantly oxidised. It may be of interest that all those possessing such a β-hydroxyl group are substrates for the "species A" enzyme only.

The minor amines β-phenylethylamine and tryptamine which are "species B" substrates may well effect perturbations in the levels of noradrenaline, dopamine and serotonin and although they only occur in low amounts in brain [10–14], they have low K_m values for monoamine oxidase [15, 16], and sharp local elevations in their levels may have similar effects to the action of the short-lasting reversible inhibitor harmine (see e.g. Ref. 17).

Thus monoamine oxidase may have a central importance for the interbalance of various amine levels through competition for oxidation. This may indeed also be regulated by product inhibition, as the "species B" enzyme has been shown to be peculiarly sensitive to aldehyde product inhibition [4]. However, as yet no aldehyde product inhibition studies have been carried out on the "species A" enzyme, and neither has the effect of the aldehyde products of the "species A" enzyme on the "species B" enzyme (and vice-versa) been investigated.

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