The Interpretation of Non-Hyperbolic Rate Curves for Two-Substrate Enzymes

A POSSIBLE MECHANISM FOR PHOSPHOFRUCTOKINASE

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1. A theoretical appraisal of the alternative pathway mechanism for a two-substrate enzyme shows that this mechanism is capable of giving rise to apparent substrate inhibition or substrate activation (Dalziel, 1958). It has now been shown that these phenomena may occur simultaneously in the following ways. With certain relationships between the kinetic parameters and the constant concentration of one substrate, A, the plot of initial rate, v , against the concentration of the other substrate, B, may show substrate 'activation' at low concentrations of B and substrate 'inhibition' at high concentrations of B. In other circumstances the plot of v against [B], with [A] constant, may be sigmoid (substrate activation), whereas the plot of v against [A], with [B] constant, may pass through a maximum (substrate inhibition). 2. Kinetic data for phosphofructokinase are of the latter type and it is suggested that the mechanism of this enzyme may involve a kinetically preferred pathway. It is emphasized that the phenomena of substrate inhibition and activation need not necessarily involve more than one binding site for each substrate on the enzyme molecule, nor more than one monomer per molecule.

The current interest in regulatory enzymes, many of which exhibit curves of initial velocity against substrate concentration that are either sigmoid or pass through maxima, stimulated a search for models of enzyme mechanism that would explain such kinetic data.

Where sigmoid curves have been found they have often been interpreted in terms of co-operative sub-unit interactions of the type described by Adair (1925) for haemoglobin. Though this is probably correct in many cases, Atkinson & Walton (1965) have pointed out that '... sigmoid rate curves... do not necessarily require sub-unit aggregation or more than one catalytic site per molecule'. Further, Dalziel (1957) has shown that an alternative pathway mechanism for a two-substrate enzyme may in certain cases give rise to substrate activation.

Similarly, where rate curves have been found to pass through a maximum it has often been concluded that a second binding site for substrate is necessary to explain the substrate inhibition at high concentrations (e.g. Alberty, 1956). Such a conclusion is only one of several possible ones, as has been shown previously (Dalziel, 1957, 1958; Theorell & McKinley-McKee, 1961; Raval & Wolfe, 1963; Massey & Veeger, 1963).

The purpose of the present paper is to investigate the general mechanism for a two-substrate enzyme

and to determine under what special conditions it may give rise to substrate activation (a sigmoid rate curve) or substrate inhibition (a rate curve with a maximum) without postulating more than one binding site for each substrate on the enzyme molecule. The general mechanism considered (see eqn. 1) is one where each substrate can combine with the free enzyme to give a binary complex, the binary complexes each then combining with the other substrate to give the same ternary complex, which breaks down to products. Alternative pathways to the ternary complex are thus available. Dalziel (1958) has pointed out that when treated by the steady-state method '... substrate "activation" and "inhibition" are inherent in this mechanism'.

$$k_{+1} \ k_{-2} \ k_{+3} \ k_{-4} = k_{-1} \ k_{+2} \ k_{-3} \ k_{+4}$$

The initial rate in the absence of products and in the presence of a constant concentration, b , of the second substrate, B , is given by:

$$v = \frac{v'}{e} = \frac{ia^2 + ja}{k + la^2 + ma} \quad (2)$$

where a is the concentration of A , e is the total enzyme concentration and i, j, k, l and m are functions of b and the rate constants for the various steps shown in eqn. (1) (Ingraham & Makower, 1954; Dixon & Webb, 1964).

Differentiation of the rate eqn. (2) gives:

$$\frac{dv}{da} = \frac{a^2(im - jl) + 2kia + kj}{(k + la^2 + ma)^2} \quad (3)$$

There will be maxima or minima in the plot of v against a when $dv/da = 0$, i.e. when $a = \pm \infty$ or when the numerator of eqn. (3) is zero. In the latter case the roots are given by:

$$a = - \frac{ki \pm \sqrt{k^2i^2 - kj(im - jl)}}{(im - jl)}$$

These roots will be negative and therefore of no practical interest, except when $im < jl$, when there will be one positive root and one negative root.

The second differential of the initial rate is given by:

$$\frac{1}{2} \frac{d^2v}{da^2} = - \frac{l(im - jl)a^3 - 3kila^2 - 3kjla + k(ki - mj)}{(k + la^2 + ma)^3} \quad (4)$$

This shows that points of inflexion will be present in the plot of v against a when $a = \pm \infty$ or when the numerator of eqn. (4) is zero, i.e. when:

$$a^3 + \frac{3ki}{(im - jl)} \cdot a^2 + \frac{3kj}{(im - jl)} \cdot a + \frac{k}{l} \cdot \frac{(mj - ki)}{(im - jl)} = 0 \quad (5)$$

The roots of eqn. (5) will, once more, be negative and of no practical interest, except when either of the following conditions is met:

Condition (i): $ki > mj$ and $im > jl$

Condition (ii): $im < jl$

In the second condition, from the earlier consideration, there will also be a maximum at a positive value of a . If, at the same time, $ki < mj$ there will be a single point of inflexion at a positive value of a (ii a), whereas if $ki > mj$ there will be two positive points of inflexion (ii b). If $ki = mj$ there will be one point of inflexion at the origin and one at a

Fig. 1. Shapes of the initial rate curves for the alternative pathway mechanism (eqns. 1 and 2 in the text) with various relationships among the constants i, j, k, l and m of eqn. (2). Botts (1958) has also described the general shape of the curves that may arise from eqn. (2), in connexion with various single-substrate enzyme models.

positive value of a . The expected rate curves will therefore have the appearance shown in Fig. 1.

Fig. 1 (ii b) indicates that this mechanism could give rise to a rate curve showing substrate activation at low substrate concentrations and substrate inhibition at high substrate concentrations. Each of the constants i, j, k, l and m is itself a very complex function of the rate constants and of b (see Dixon & Webb, 1964), so that conditions (i) and (ii) are extremely complex in terms of the rate constants and b . It has not been possible to reduce these conditions to a form in which obvious relationships among the rate constants can be seen. It appears that for either condition k_{+5} should be large, that part of condition (i) is $k_{+1} > k_{+4}$ and that part of condition (ii) is $k_{+2} > k_{+3}$. However, a number of hypothetical cases were inspected to see whether any plausible combinations of the rate constants would meet either of the above conditions for points of inflexion. As would be expected, no case where breakdown of EXY to products is rate-limiting (i.e. 'rapid-equilibrium' or Michaelis-Menten-Haldane kinetics) will meet either condition; nor will any steady-state situation, where both routes to EXY (see eqn. 1) are equally favoured, or where one route is obligatory.

On the other hand, the following situation meets condition (i). Let the values of the rate constants in eqn. (1) be as follows:

$$k_{+1} = k_{+3} = 10^5; k_{+2} = k_{+4} = 10^4; k_{+5} = 10^6 \\ k_{-1} = k_{-3} = 10^{-1}; k_{-2} = k_{-4} = 10^{-2}$$

(the rate constants and concentrations have not been given any dimensions, but any consistent set of dimensions would be acceptable). The reaction now has a kinetically preferred pathway:

Table 1. Numerical values of the quantities involved in conditions (i) and (ii) (see the text) for various values of b , in the case of the hypothetical enzyme described in the text

b	ki	mj	im	jl
10^{-2}	1.00×10^{29}	1.00×10^{28}	1.00×10^{31}	1.00×10^{30}
10^{-3}	1.00×10^{26}	1.01×10^{25}	1.00×10^{29}	1.01×10^{28}
10^{-4}	1.02×10^{23}	1.13×10^{22}	1.02×10^{27}	1.11×10^{26}
10^{-5}	1.21×10^{20}	2.52×10^{19}	1.20×10^{25}	2.10×10^{24}
10^{-6}	4.00×10^{17}	3.60×10^{17}	3.00×10^{23}	1.20×10^{23}
10^{-7}	1.21×10^{16}	2.33×10^{16}	2.10×10^{22}	1.11×10^{22}

but the other pathway is not forbidden:

The dissociation constants K_1 , K_2 , K_3 and K_4 for the scheme (see eqn. 1) are all equal to 10^{-6} , so that there is no thermodynamic preference for any one step.

If b is set at a constant value, the values of i , j , k , l and m may be calculated from the equations set out by Dixon & Webb (1964). These values lead to the relationships shown in Table 1, and these satisfy condition (i) in all cases, except when $b = 10^{-7}$. This demonstrates that for values of $b \geq 10^{-6}$ there will be a point of inflexion in the graph of v against a , which is shown for $b = 10^{-3}$ in Fig. 2(a). The points have been calculated from eqn. (2), by substituting the calculated values of i , j , k , l and m . There is a point of inflexion at $a = 4.6 \times 10^{-4}$.

If the points shown in Fig. 2(a) had been obtained experimentally and had been assumed to follow the equation:

$$v = V_{\max.} \frac{a^n}{k + a^n}$$

the maximum value obtained for n by plotting $\log[(V_{\max.} - v)/v]$ against $-\log a$ would have been 1.53. This equation is analogous to the Hill equation for the oxygenation of haemoglobin (Wyman, 1948, 1963), in which n is taken as a measure of the co-operative effects present in the haemoglobin molecule.

A further property of the enzyme system considered above is that, if now a were to be held constant while b was increased, the plot of v against b would be of different form. (This is equivalent to the situation where the values of k_{+1} and k_{+2} , k_{-1} and k_{-2} , k_{+3} and k_{+4} , k_{-3} and k_{-4} have all been interchanged, and b is held constant and v plotted against a as before.) The new relationships are shown in Table 2.

In every case, except for $a = 10^{-7}$, the relationships satisfy condition (ii) and we should expect both a point of inflexion and a maximum in the

Fig. 2. Initial rate plots for a hypothetical two-substrate enzyme where the rate constants for various steps (see the text) are such as to give a kinetically preferred pathway:

(a) Constant concentration of substrate $B = 10^{-3}$; concentration of substrate A increasing (i.e. $s = a$.)

(b) Constant concentration of $A = 10^{-3}$; concentration of B increasing (i.e. $s = b$.)

Table 2. Variation in the values of the quantities involved in conditions (i) and (ii) (see the text) with different values of a , for the hypothetical enzyme described in the text

a	$k'i'$	$m'j'$	$i'm'$	$j'l'$
10^{-2}	1.00×10^{28}	1.00×10^{29}	1.00×10^{30}	1.00×10^{31}
10^{-3}	1.00×10^{25}	1.00×10^{26}	1.00×10^{28}	1.00×10^{29}
10^{-4}	1.02×10^{22}	1.03×10^{23}	1.02×10^{26}	1.01×10^{27}
10^{-5}	1.21×10^{19}	1.33×10^{20}	1.20×10^{24}	1.11×10^{25}
10^{-6}	4.00×10^{16}	4.24×10^{17}	2.02×10^{22}	2.10×10^{23}
10^{-7}	1.20×10^{15}	2.40×10^{15}	2.00×10^{21}	1.20×10^{21}

plot of v against b . This is shown in Fig. 2(b) for $a = 10^{-3}$.

If the initial rate eqn. (2) is recast in the form:

$$\frac{1}{v} = \frac{la^2 + ma + k}{ia^2 + ja} = \frac{l + m/a + k/a^2}{i + j/a} \quad (6)$$

and if $1/v = y$ and $1/a = x$, then:

$$y = \frac{l + mx + kx^2}{i + jx} \quad (7)$$

$$\frac{dy}{dx} = \frac{kjx^2 + 2kix + (im - jl)}{(i + jx)^2} \quad (8)$$

$$\frac{d^2y}{dx^2} = \frac{2[ki^2 - j(im - jl)]}{(i + jx)^3} \quad (9)$$

These equations show that when $1/v$ is plotted against $1/a$ the curve intercepts the $1/v$ axis at l/i with a slope of $(im - jl)/i^2$. As $1/a$ approaches ∞ , the slope approaches k/j . Further, from eqn. (8) there will be a minimum when:

$$\frac{1}{a} = \frac{ki + \sqrt{k^2i^2 - kj(im - jl)}}{kj}$$

If $im < jl$ this will be at a positive value of $1/a$, and the condition is the same as for a maximum in the plot of v against a , i.e. condition (ii). When condition (i) applies the reciprocal plot will have its concave side uppermost, i.e. the slope increases as $1/a$ increases, but there will be no minimum, maximum or point of inflexion. For a reciprocal plot to have its convex side uppermost, the condition is that $im > jl$ and $ki < mj$, which is also necessary for a linear reciprocal plot. In the latter case eqn. (9) shows that, in addition, $ki^2 = j(im - jl)$, which may be rearranged to give $j^2l = i(mj - ki)$. In this special case the slope will be k/j .

A similar treatment shows that for the plot of a/v against a the intercept on the a/v axis will be k/j , and there will be a minimum when $ki > mj$,

which is part of the condition for a sigmoid plot of v against a . As before, the plot will be linear when $ki^2 = j(im - jl)$ and under these circumstances the slope will be l/i .

The most interesting of the customary methods of plotting kinetic data is the one where v is plotted against v/a . In this case the curve intercepts the v axis at i/l with a slope of $(jl - im)/il$, and the v/a axis at j/k with a slope of $kj/(ki - mj)$. When $im < jl$ there will be a maximum (as in the plot of v against a), but if $ki > mj$ the curve will double back on itself. The data for Fig. 2 are replotted in Fig. 3 in this manner, and case (a), which gave a sigmoid curve in Fig. 2, is bow-shaped in Fig. 3. Once again, if $ki^2 = j(im - jl)$ the plot will be linear, with slope $-ki/jl$.

A qualitative understanding of the properties of the 'preferred order' system shown in Figs. 2 and 3 can be arrived at as follows. In case (a) of Fig. 2, that substrate, B, is held at a constant concentration which binds less readily to the free enzyme. As the concentration of the other substrate, A, is increased from very low values, at first most of it will react with EB, which will exist at a relatively high concentration, and the route followed will be almost entirely:

At higher concentrations of A, however, the other, kinetically preferred, route can begin to take over, thus accounting for the upward lift in the rate curve. At very high concentrations of A the enzyme becomes saturated with A and the rate will level off. This is shown in Table 3.

In the second case the situation is reversed and the substrate, A, that is held at a constant concentration binds more readily to the free enzyme. But once the concentration of B becomes high enough to overcome the kinetic factor favouring the formation of EA more and more of the slower-reacting EB will be formed, thus reducing the overall velocity observed.

It is tempting to suppose that any steady-state system where there is a kinetically preferred pathway would give rise to the kind of sigmoid plot shown in Fig. 2(a) when the substrate, B, that is held at a constant concentration is the second one that is added on in the preferred pathway. Also, it could be supposed that holding the concentration of A constant in such a system would always give the type of curve shown in Fig. 2(b). Such suppositions can hardly be justified in view of the complexity of the conditions (i) and (ii) that govern the appearance of such curves. It is, however, reasonable to suppose that if one starts by considering the scheme of eqn. (1), with the concentration of B held constant, and chooses rate

Fig. 3. Data from Fig. 2 replotted as v against v/s .

Table 3. *Distribution of enzyme among the different intermediates, and the relative flow through the two pathways shown in eqn. 1, for the hypothetical enzyme whose rate curve is shown in Fig. 2(a)*

The relative concentrations of the intermediates were calculated by solving the simultaneous steady-state equations for the hypothetical enzyme. Relative rates of flow through the two pathways were calculated by comparing the net rates of formation of EA and EB from E. Line 3 shows the situation at the point of inflexion of Fig. 2(a); here EA:EB=rate via EB:rate via EA=4.6.

<i>a</i>	Percentage of total enzyme present				Percentage of total rate proceeding	
	As E	As EA	As EB	As EXY	Via EA	Via EB
10 ⁻⁵	1.09	0.01	98.91	0.000	9.9	90.1
10 ⁻⁴	9.09	0.91	90.00	0.002	50.0	50.0
4.6 × 10 ⁻⁴	27.60	12.80	59.80	0.011	82.2	17.8
10 ⁻³	33.34	33.31	33.31	0.020	91.6	8.4
10 ⁻²	9.01	90.00	0.90	0.089	99.0	1.0
10 ⁻¹	0.99	98.90	0.01	0.100	99.9	0.1

constants that make the uppermost pathway obligatory, and then gradually changes the rate constants so as to allow the lower pathway to become possible, a point will be reached when a sigmoid plot of *v* against *a* will appear. At some later point in this process of shifting the emphasis, as the lower pathway begins to approach parity with the upper one, the condition will disappear. Later, when the lower path has assumed the greater kinetic importance a maximum will appear in the plot, finally disappearing as the lower pathway approaches the condition of being obligatory. If the whole process were now to be repeated with the concentration of A held constant, a maximum would be expected to appear first, then to disappear and be followed by a sigmoid curve that would finally disappear. The appearance of a maximum with the concentration of A held constant would probably not coincide with the appearance of a sigmoid curve when the concentration of B was held constant, because the conditions for a sigmoid curve are more stringent than those for a maximum. Presumably there is a whole range of ways of conducting the gradual transference of kinetic preference from one path to the other.

A previous investigation of limiting cases for the general rate equation (King, 1956) showed that 'unusual' kinetic consequences arise when most of the enzyme is present in the form of an intermediate that does not lie on the pathway by which most of the product is being formed. The term 'unusual' was used there to describe cases where the order of the rate equation fails to indicate the composition of the activated complex. It appears very likely that 'unusual' situations exist at or near the points of inflexion or maxima in the kinetic situations described above (see Table 3).

In work on aspartate transcarbamoylase, the elegant experiments of Gerhart & Pardee (1964), who used maleate as a competitor for aspartate,

suggest very strongly that here sub-unit interactions are responsible for the sigmoid rate curves. Further, the sigmoid curve obtained for this enzyme is a fairly symmetrical one, whereas in the 'preferred pathway' cases so far examined the sigmoid curves have all been unsymmetrical with the final plateau reached only at concentrations of A much higher than those present in the region of the point of inflexion.

The kinetic data for phosphofructokinase (Atkinson & Walton, 1965; Underwood & Newsholme, 1965) are very similar to the curves shown in Fig. 2, and it may well be that the mechanism of this enzyme action involves a kinetically preferred pathway. It is true that the effects of modifiers such as AMP would require an 'effector' site on the enzyme molecule somewhere other than the active site, as suggested by Atkinson & Walton (1965). It may, however, be unnecessary to postulate other binding sites for the substrates, which would simplify the picture of the phosphofructokinase molecule considerably.

Other enzymes that show similar kinetic properties and whose mechanisms could perhaps be explained on the basis described above are NAD-isocitrate dehydrogenase of yeast (Hathaway & Atkinson, 1963) and heart malate dehydrogenase (Wolfe & Neilands, 1956; Davies & Kun, 1957). Many other enzymes that show the phenomenon of substrate inhibition when one substrate concentration is varied and the other held constant may also be of this type, even though they do not show sigmoid curves when the substrates are interchanged.

Kinetic preference may arise from effects other than conformational changes in the enzyme molecule when it combines with substrates. Further, in the case considered (Figs. 2a and 2b) the kinetic preference was unaccompanied by thermodynamic preference for any of the steps in the mechanism.

Such a situation might arise in Fig. 2(a) if the substrate A was a small molecule of positive charge binding to a rigid, negatively charged, active site, whereas B was a large negatively charged molecule binding at an uncharged part of the rigid active site. Other situations can be imagined in which conformational changes attendant on the binding of one substrate would affect the binding of the other in the manner described by the 'induced fit' hypothesis (e.g. Koshland, 1963). The latter situation would bear a close resemblance to the interaction postulated for haemoglobin and aspartate transcarbamoylase, except for the relatively minor difference that the interaction would be 'monosteric' rather than allosteric.

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REFERENCES

- Adair, G. S. (1925). *J. biol. Chem.* **63**, 529.
Alberty, R. A. (1956). *Advanc. Enzymol.* **17**, 1.
Atkinson, D. & Walton, G. M. (1965). *J. biol. Chem.* **240**, 757.
Botts, D. J. (1958). *Trans. Faraday Soc.* **54**, 593.
Dalziel, K. (1957). *Acta chem. scand.* **11**, 1706.
Dalziel, K. (1958). *Trans. Faraday Soc.* **54**, 1247.
Davies, D. D. & Kun, E. (1957). *Biochem. J.* **66**, 307.
Dixon, M. & Webb, E. C. (1964). *Enzymes*, 2nd ed., pp. 95-98. London: Longmans, Green and Co.
Gerhart, J. C. & Pardee, A. B. (1964). *Fed. Proc.* **23**, 727.
Hathaway, J. A. & Atkinson, D. E. (1963). *J. biol. Chem.* **238**, 2875.
Ingraham, L. L. & Makower, B. (1954). *J. phys. Chem.* **58**, 266.
King, E. L. (1956). *J. phys. Chem.* **60**, 1375.
Koshland, D. E., jun. (1963). *Cold Spr. Harb. Symp. quant. Biol.* **28**, 473.
Massey, V. & Veeger, C. (1963). *Annu. Rev. Biochem.* **32**, 620.
Raval, D. N. & Wolfe, R. G. (1963). *Biochemistry*, **2**, 220.
Theorell, H. & McKinley-McKee, J. S. (1961). *Acta chem. scand.* **15**, 1834.
Underwood, A. H. & Newsholme, E. A. (1965). *Biochem. J.* **95**, 868.
Wolfe, R. G. & Neilands, J. B. (1956). *J. biol. Chem.* **221**, 61.
Wyman, J. (1948). *Advanc. Protein Chem.* **4**, 407.
Wyman, J. (1963). *Cold Spr. Harb. Symp. quant. Biol.* **28**, 483.