BBA 66660

KINETIC STUDIES WITH PHOSPHOTRANSACETYLASE

IV. INHIBITION BY PRODUCTS

S. A. KYRTOPOULOS AND D. P. N. SATCHELL
Department of Chemistry, King's College, Strand, London WC2R 2LS (Great Britain)

(Received February 14th, 1972)

SUMMARY

1. We have studied product inhibition in the phosphotransacetylase (acetyl-CoA:orthophosphate acetyltransferase, EC 2.3.1.8) catalysed reactions:

Acetyl phosphate + CoA ⇌ acetyl-CoA + phosphate

Acetyl-CoA + arsenate → acetyl arsenate + CoA

2. The effect of CoA is to inhibit acetyl-CoA competitively and the other (non-nucleotide) substrate non-competitively in accordance with an equation (9) based on our previous model for these two-substrate, two-product reactions.

3. Acetyl phosphate and phosphate both produce similar (and complex) inhibition patterns in their respective reactions. A very similar pattern is also observed when acetyl phosphate is used to inhibit the arsenate reaction. These results support our contention that the reactions above all proceed by essentially the same mechanism, but reveal that this mechanism must be more complicated than hitherto believed.

4. The complex inhibition pattern involves, as the relevant inhibitor concentration is increased, two regions of competitive inhibition of the structurally similar substrate, separated by a region displaying partial recovery of activity. This pattern had not been observed previously in any enzymatic reaction under conditions of reversible inhibition. In the present system it requires for its explanation adsorption of the non-nucleotide species by the enzyme at (at least) three sites (A, B and C) two of which (A and C) can both lead to product formation via interaction of the adsorbed non-nucleotide substrate with a single, locally adsorbed nucleotide molecule; adsorption of non-nucleotide at the other site (B) does not lead to products directly, but to the steric acceleration of product formation via site C when product replaces substrate on B. Depending on whether the acetyl group is being transferred to, or from, the nucleotide, this acceleration is provided by a local decrease, or increase, in steric crowding around the nucleotide. The best agreement between experimental and theoretical inhibition curves is obtained when it is assumed that there exist two or more sites like B which must be simultaneously occupied to provide maximum acceleration.

5. The mechanism requires that, at high substrate concentrations in the absence of significant inhibition, at least four (probably six) molecules of substrate (including one molecule of nucleotide) are taken up by the enzyme's active centre. Approximate K_m values are given for the various substrates at the different types of site. The rate of the surface reaction via site C is calculated to be approximately that via site A when the B sites are largely occupied by substrate; the factor rises to roughly twenty when product replaces substrate on these sites.

INTRODUCTION

We have recently studied the kinetics of the forward and reverse processes of the phosphotransacetylase (acetyl-CoA:orthophosphate acetyltransferase, EC 2.3.1.8) catalysed equilibrium (1), and of the related process (2). These enzymatic reactions were all shown

$$\text{Acyl phosphate} + \text{CoA} \overset{\text{enzyme}}{\rightleftharpoons} \text{acyl-CoA} + \text{phosphate} \qquad (1)$$

$$\text{Acetyl-CoA} + \text{arsenate} \overset{\text{enzyme}}{\longrightarrow} \text{acetyl arsenate} + \text{CoA} \qquad (2)$$

to be random bimolecular in type, in which the two substrates enjoy independent sites and are probably taken up under pre-equilibrium (Michaelis–Menten) conditions[1–3]. Our studies included the effects on the Michaelis parameters of changes in pH and in the structure of the acyl group, and we reached certain conclusions concerning the details of the reaction on the enzyme surface. One finding was that CoA is more strongly bound to the enzyme than is acyl-CoA, the same K_m values being obtained from both Reactions 1 and 2. Also, acetyl phosphate is more strongly bound than is phosphate. In keeping with these results, Reaction 2 and the reverse process of equilibrium (1) display easily detectable inhibition by their respective products. We have now made a detailed examination of product inhibition in these systems. Previous studies of this aspect of phosphotransacetylase activity are scant and contradictary[4,5].

MATERIALS AND METHODS

(i) Chemicals

Enzyme and all other chemicals were obtained from the sources previously listed[1,3].

(ii) Kinetic methods

All experiments were at 25 °C and pH 8.32. The different reactions were monitored as previously described[1–3] and their initial velocities calculated as in Part II[2]. For a random bimolecular mechanism, the substrates having independent sites (Eqns 3–7), the initial velocity is given[1,6], in the absence of significant amounts of deliberately added inhibitor, by Eqn 8, where k is the rate of transformation of the enzyme–substrate

$$E + \text{A} \rightleftharpoons E\text{A} \qquad K_\text{A} \text{ fast} \qquad (3)$$

$$E + \mathrm{B} \rightleftharpoons E\mathrm{B} \qquad K_{\mathrm{B}} \text{ fast} \tag{4}$$

$$E\mathrm{A} + \mathrm{B} \rightleftharpoons \begin{matrix} & \mathrm{A} \\ E & \\ & \mathrm{B} \end{matrix} \qquad K_{\mathrm{B}} \text{ fast} \tag{5}$$

$$E\mathrm{B} + \mathrm{A} \rightleftharpoons \begin{matrix} & \mathrm{A} \\ E & \\ & \mathrm{B} \end{matrix} \qquad K_{\mathrm{A}} \text{ fast} \tag{6}$$

$$\begin{matrix} \mathrm{A} & & \mathrm{X} \\ & E & \\ \mathrm{B} & & \mathrm{Y} \end{matrix} \qquad k \text{ slow} \tag{7}$$

ternary complex, A–E–B, to the corresponding product complex, X–E–Y, $K_{\mathrm{A}} = [E]\,[\mathrm{A}]/[E\mathrm{A}] = [E\mathrm{B}]\,[\mathrm{A}]/[E\mathrm{AB}]$, $K_{\mathrm{B}} = [E]\,[\mathrm{B}]/[E\mathrm{B}] = [E\mathrm{A}]\,[\mathrm{B}]/[E\mathrm{AB}]$, and $[E_0]$ is the total enzyme concentration.

$$v_0 = k\,[E_0]\,[\mathrm{A}]\,[\mathrm{B}]/\{([\mathrm{A}] + K_{\mathrm{A}})\,([\mathrm{B}] + K_{\mathrm{B}})\} \tag{8}$$

In the presence of a given added concentration of a product (say X) which resembles A and therefore inhibits the adsorption of A competitively, but does not affect the binding of B, it can readily be shown that the initial velocity equation becomes (9). Hence, so far as B is concerned, the inhibition by X appears as a non-competitive effect. Similar considerations apply for the

$$v_0 = k[E_0]\,[\mathrm{A}]\,[\mathrm{B}]/\left\{[\mathrm{A}] + K_{\mathrm{A}}\left(1 + \frac{[\mathrm{X}]}{K_{\mathrm{X}}}\right)\right\}\,([\mathrm{B}] + K_{\mathrm{B}}) \tag{9}$$

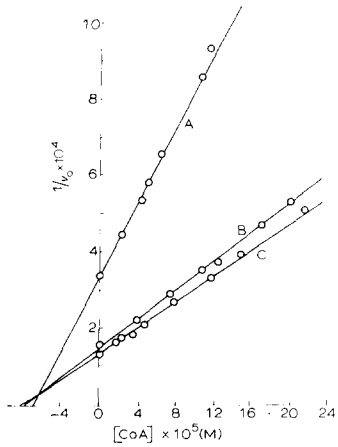
other product Y if it resembles B, but does not affect the adsorption of A. In such circumstances the equation representing inhibition by both products is (10). (The analysis of product inhibition patterns given by Morris *et al.*[7], based

$$v_0 = k[E_0]\,[\mathrm{A}]\,[\mathrm{B}]/\left\{[\mathrm{A}] + K_{\mathrm{A}}\left(1 + \frac{[\mathrm{X}]}{K_{\mathrm{X}}}\right)\right\}\left\{[\mathrm{B}] + K_{\mathrm{B}}\left(1 + \frac{[\mathrm{Y}]}{K_{\mathrm{Y}}}\right)\right\} \tag{10}$$

on ref. 8, does not appear to allow for the possibility of independent sites. Our equations are, however, largely covered by Clark's analysis[9]). Our earlier studies therefore led us to expect compliance of the product inhibition with Eqn 10—at least to a first approximation. Diagnostic experiments were arranged along lines suggested by Dixon[6] for single substrate systems. The various patterns of inhibition actually observed are illustrated in the figures. Their implications are discussed in the next section.

RESULTS AND DISCUSSION

In examining the effects of the nucleotides it is more convenient to use CoA as the inhibitor than to use acyl-CoA, owing to the stronger adsorption of the former by the enzyme. Addition of CoA to the reaction mixture at the start of Reaction 2, or of the reverse reaction of equilibrium (1), leads to inhibition. Typical results for the phosphate system are in Figs 1 and 2. It is evident[6] that CoA acts as a competitive in-



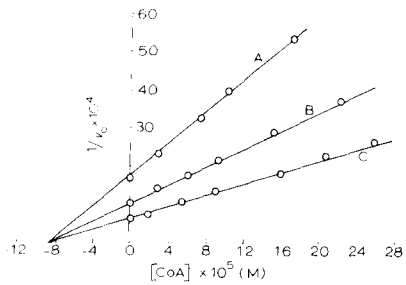


Fig. 1. Competitive inhibition between CoA and acetyl-CoA in Reaction 1. A, B and C correspond to [acetyl-CoA] at 35, 85 and 125 μM, respectively; [phosphate] = $6.1 \cdot 10^{-2}$ M; [Na^+] = $13.2 \cdot 10^{-2}$ M; [NH_4^+] = $14.4 \cdot 10^{-3}$ M; [enzyme] = $5.0 \cdot 10^{-8}$ M; pH 8.32; ionic strength = 0.3. The plots give $K_{ACA} = 1.1 \cdot 10^{-3}$ M and $K_{CA} = 0.75 \cdot 10^{-4}$ M.

Fig. 2. Non-competitive inhibition between CoA and phosphate in Reaction 1. A, B and C correspond to [phosphate] at 20, 40 and 60 mM, respectively; [acetyl-CoA] = $7.4 \cdot 10^{-5}$ M; [Na^+] = 0.12 M; [enzyme] = $1.7 \cdot 10^{-8}$ M; pH 8.32; ionic strength = 0.26 ± 0.02. The plots give $K_{Pi} = 0.3$ and $K_{CA} = 1.0 \cdot 10^{-4}$ M.

hibitor towards acetyl-CoA and as a non-competitive inhibitor towards phosphate. Michaelis constants calculated from Figs 1 and 2, using Dixon's general method[6] and Eqn 9 with A, B and X representing acetyl-CoA, phosphate and CoA respectively, are in good agreement with those previously determined at pH 8.32 in the absence of inhibition[1-3]. These results therefore support all our previous conclusions.

However, experiments on inhibition of the foregoing processes by acetyl phosphate, and also of the forward step of equilibrium (1) by phosphate, reveal unexpected complexities. Typical inhibition curves are in Figs 3, 4 and 5. They show that as the inhibitor concentration rises a region of inhibition is followed by one of partial recovery of activity, this being followed in turn by a further final region of inhibition Our results appear to be the first example of this type of inhibition pattern to be found involving reversible inhibitors. That we find very similar patterns for the three separate, although related, reactions argues strongly for the reality of the phenomena.

It does not seem possible to explain these phenomena in terms of adsorption of the inhibitor at a single site for which it competes with the relevant, structurally similar substrate. Our previous ideas about the mechanism have therefore to be radically revised. Nor does it seem realistic to invoke some combination of such competitive inhibition with favourable conformational changes induced by adsorption at a second site, the reason being that both phosphate and acetyl phosphate would have to be considered capable of inducing this change, so that it should also affect the kinetic behaviour on increasing the substrate concentration in the absence of inhibitor, yet there is no evidence for such an accelerating effect in any of these three reactions[1-3]. Conformational changes there probably are during the course of the reactions, but it is unlikely that they underlie the present phenomena.

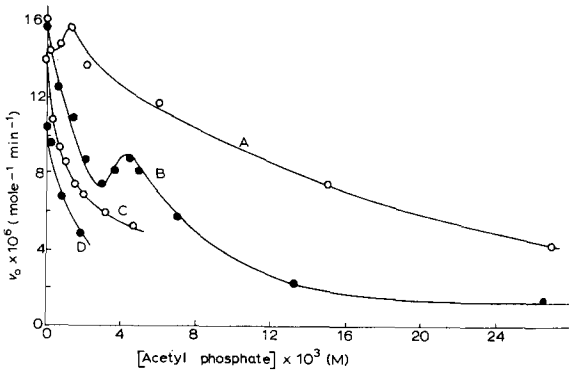


Fig. 3. Effect of added acetyl phosphate on the acylation of phosphate by acetyl-CoA. A, B, C and D correspond to [phosphate] at 900, 400, 150 and 76 mM, respectively; [acetyl-CoA] = $4.4 \cdot 10^{-5}$ M; $[NH_4^+] = 7.2 \cdot 10^{-3}$ M; $[K^+] = 1.2$ M; [enzyme] = $2.5 \cdot 10^{-8}$ M; pH 8.32.

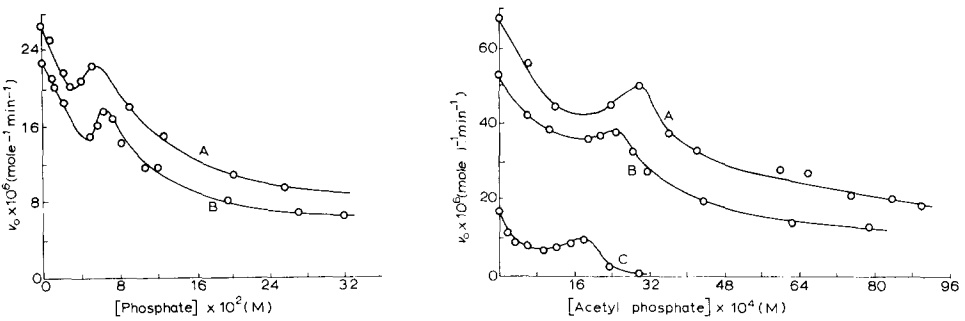


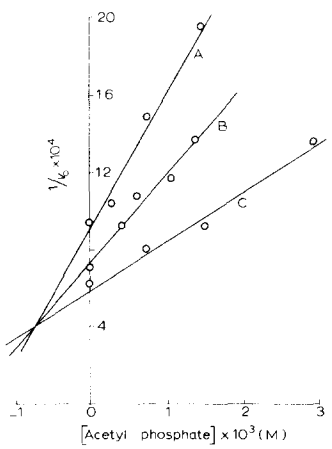
Fig. 4. Effect of added phosphate on the acylation of CoA by acetyl phosphate. A and B correspond to [acetyl phosphate] at 4.13 and 1.96 mM, respectively; [CoA] = $6 \cdot 10^{-5}$; $[NH_4^+] = 3.6 \cdot 10^{-2}$ M; $[Na^+] = 76.5 \cdot 10^{-2}$ M; pH = 8.32; ionic strength = 1.3; [enzyme] = $12.5 \cdot 10^{-8}$ M.

Fig. 5. Effect of added acetyl phosphate on the acylation of arsenate by acetyl-CoA. A, B and C correspond to [arsenate] at 20.6, 10.3 and 2.1 mM, respectively; [acetyl-CoA] = $6.9 \cdot 10^{-5}$ M; [enzyme] = $2.5 \cdot 10^{-8}$ M; $[NH_4^+] = 7.2 \cdot 10^{-3}$ M; $[K^+] = 2.1 \cdot 10^{-2}$ M; pH 8.32; ionic strength = 0.16.

A possible, and chemically attractive, explanation which is also nicely compatible with the appearance of the effects in product inhibition but not elsewhere in the reaction kinetics, is suggested by our earlier finding[1] that the steric bulk of the acyl group has an important effect in determining the rate of the surface reaction. In its simplest form this explanation runs as follows. Adsorption of phosphate (or acetyl phosphate or arsenate) can occur at three sites A, B and C, all spatially adjacent to the site which carries the CoA species, with adsorption at site A stronger than at B or C. Considering first the forward step of equilibrium (1), the product-forming process is considered to involve the interactions between adsorbed CoA and acetyl phosphate located on either site A or C. The changing occupation of site B provides a steric acceleration[10]. Thus, as the concentration of inhibitor (phosphate) is increased, at fixed concentrations of all other species, the observed initial rate of acetyl-CoA formation will at first fall owing to competition between acetyl phosphate and phosphate for site A, the site of strongest adsorption of phosphate species generally. This provides the

first region of inhibition. However, if the replacement of acetyl phosphate by phosphate at site B permits the acyl group to be transferred to CoA from any acetyl phosphate on site C more easily (owing to a reduction in steric crowding around CoA), then a temporary recovery in the rate of acetyl-CoA formation is likely as more phosphate is added. When sufficient phosphate is present to significantly compete with acetyl phosphate for site C (the site of weakest general adsorption) then the rate will fall again and will not recover, thus providing the second region of inhibition. An exactly complementary argument applies for the reverse reaction. Here product formation involves the transfer of the acyl group from acetyl-CoA to phosphate. When acetyl phosphate rather than phosphate occupies site B this transfer will be accelerated by the increased steric crowding around the acetyl-CoA. Similar considerations should apply to the effect of acetyl phosphate on the arsenate reaction, (2), if this reaction does, as we believe[2], employ the same sites and mechanism as Reaction 1. That the inhibition patterns for (1) and (2) are indeed so similar is thus further evidence for the essential identity of these two reactions.

The foregoing discussion provides a qualitative rationalisation of the shapes of Figs 3, 4 and 5. If correct, it has two important implications. First, that the directly determined Michaelis curves for phosphate, arsenate and acetyl phosphate all represent the composite effect of adsorption at the two sites A and C. Secondly, that the two regions where v_0 is falling in Figs 3–5 represent competitive inhibition at these sites. Such inhibition is demonstrated in Figs 6 and 7, where the relevant parts of Fig.



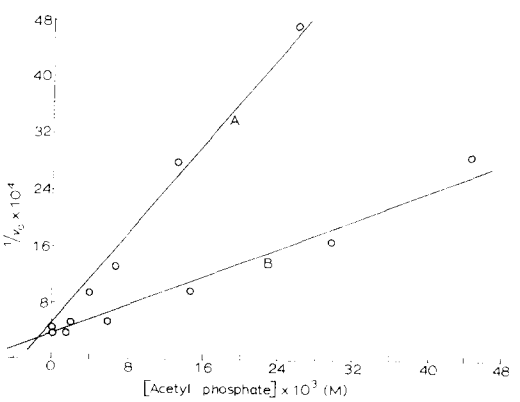


Fig. 6. Competitive inhibition by acetyl phosphate in the first region of inhibition. A, B and C correspond to [phosphate] at 76, 150 and 400 mM, respectively; general conditions as for Fig. 3.

Fig. 7. Competitive inhibition by acetyl phosphate in the second region of inhibition. A and B correspond to [phosphate] at 400 and 900 mM, respectively; general conditions as for Fig. 3.

3 are plotted according to Dixon's procedure[6]. Each of the sets of inhibition curves (Figs 3–5) leads to satisfactory reciprocal plots of this type. The (approximate) K_m values at the two sites can be obtained from these plots. The derived constants are collected in Table I where they are compared with the Michaelis constants obtained directly from Lineweaver–Burke (or similar) plots in the absence of inhibition[1–3]. It

can be seen that the K_m values for the two sites differ by a factor of between 4 and 7 (depending on the substrate) and that they bracket the value determined directly.

The question now arises: "Can adsorption at two independent sites lead to behaviour indistinguishable from that appropriate for a single site of intermediate K_m value?" The answer depends upon the relative K_m values of the two sites and upon their relative contributions (V values) to the overall velocity[6,11]. In principle, for two (or more) sites, except when their K_m values are the same, some curvature of a Lineweaver–Burk (or other) reciprocal plot is expected, especially at the extremes of the substrate concentration range. In practice this curvature is often too slight to detect, particularly over the easily accessible concentration range. If the ratio of the K_m values is approx. 5 (Table I) and that of the V values approx. 1–2 (as we shall show below is probable) then negligible curvature is to be expected in any reciprocal plot over the substrate concentration ranges we have used to determine K_m values. (Very high substrate concentrations relative to K_m are not normally feasible for phosphate, arsenate and acetyl phosphate). Our various results are thus not inconsistent. It can be shown that, for the specified relative K_m and V values at the two sites, the apparent K_m value should be the (approximate) average of the K_m values for the separate sites. Table I shows that our apparent (directly determined) K_m values tend to lie closer to that for site C than to that for site A, but the inevitable uncertainty* in the values for sites A and C makes this result of doubtful significance.

TABLE I

AVERAGE K_m VALUES FROM INHIBITION BY PHOSPHATE AND ACETYL PHOSPHATE

K_{AP}, K_{Pi} and K_{As} represent the Michaelis constants for acetyl phosphate, phosphate and arsenate, respectively. Units of K are mole·l^{-1}; temperature 25 °C; pH 8.32.

	K_{AP}	K_{Pi}	K_{As}	
Inhibition experiments	$(6.6 \pm 1) \cdot 10^{-4}$	0.05 ± 0.02	$(8.0 \pm 1.5) \cdot 10^{-3}$	Site A
	$(5.0 \pm 1.5) \cdot 10^{-3}$	0.20 ± 0.04	$(4.0 \pm 0.5) \cdot 10^{-2}$	Site C
Direct determination in absence of inhibition	$(3.0 \pm 0.2) \cdot 10^{-3}$	0.24 ± 0.07	$(1.4 \pm 0.1) \cdot 10^{-2}$	

While the multi-site mechanism described above thus provides a satisfactory outline explanation of the observed inhibition patterns, it remains to show that it is also satisfactory at a more quantitative level. However, to derive the full rate equation for the proposed mechanism, while perhaps feasible, would certainly lead to an expression too complex to permit of convincing test. An indication that the mechanism may be along the right lines can nevertheless be obtained by the following approximate procedure. The overall initial rate of product formation (v_0) at given, fixed concentrations of substrate and inhibitor, will be the sum of three separate rates: that involving site A (v_1), the unaccelerated rate involving site C (v_2), and the accelerated rate for site C (v_3). Remembering that there will be competition between the non-

* Only if the K_m values at the two sites are very different can the regions of inhibition be regarded as effectively independent, as assumed by Figs 6 and 7. The region of activation also inevitably has a disturbing effect on the inhibition regions, so that Figs 6 and 7 can only be approximate.

nucleotide substrate (S) and inhibitor (I) at each site, rates v_1 and v_2 will be controlled by expressions of the form (11) and (12), in which K^A and K^C represent the

$$v_1 = k_1[E_0]\,[S]/\left\{[S] + K_s^A\left(1 + \frac{[I]}{K_i^A}\right)\right\} \tag{11}$$

$$v_2 = k_2[E_0]\,[S]/\left\{[S] + K_s^C\left(1 + \frac{[I]}{K_i^C}\right)\right\} \tag{12}$$

Michaelis constants for sites A and C respectively, and k_1 and k_2 are constants proportional to the velocity of the surface reactions (7) at the two sites for any fixed nucleotide concentration. Rate v_3 can be regarded as proportional to the concentration of non-nucleotide substrate on site C, to the concentration of I on site B, and to an acceleration factor x, such that $k_3 = xk_2$. Thus v_3 is controlled by an equation like (13).

$$v_3 = \left(k_3[E_0]\,[S]/\left\{[S] + K_s^C\left(1 + \frac{[I]}{K_i^C}\right)\right\}\right)\left([I]/\left\{[I] + K_i^B\left(1 + \frac{[S]}{K_s^B}\right)\right\}\right) \tag{13}$$

Using Eqns 11–13 and appropriate, reasonable values for the ratios k_1/k_2, k_2/k_3, K^A/K^C, K^B/K^C, and $[S]/K^A$ it is possible to calculate v_0 ($= v_1 + v_2 + v_3$) as a function of $[I]$. A typical plot is given in Fig. 8; it bears some resemblance to the experimental curves (*e.g.* Fig. 5), but suffers from the particular disadvantage that the minimum and maximum are too greatly separated along the $[I]$ axis. While other features of the plot in Fig. 8 can be varied by suitable choice of the above ratios, this particular

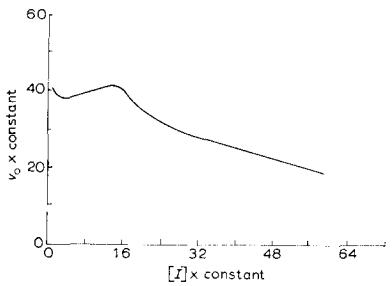


Fig. 8. Typical theoretical velocity–inhibitor profile based on Eqns 11–13.

defect cannot be overcome using our simple three site model. It may be that this model is essentially correct ,but that the detailed shape of the plot is defective owing to the approximations inherent in the use of Eqns 11–13. However, if the model is elaborated somewhat, more satisfactory theoretical plots can be obtained.

The essence of the problem is the rapid onset of the recovery of reactivity following the first phase of inhibition. If it is assumed that not one, but two, or preferably three, sites like B must be simultaneously occupied by the "inhibitor" before material steric acceleration is produced, then v_3 is given (approximately) by an equation like (14). A set of theoretical curves,

$$v_3 = \left(k_3[E_0]\,[S]/\left\{[S] + K_s^C\left(1 + \frac{[I]}{K_i^C}\right)\right\}\right)\left([I]/\left\{[I] + K_i^B\left(1 + \frac{[S]}{K_s^B}\right)\right\}\right)^3 \tag{14}$$

based on this equation for v_3, which give the expected inhibition patterns for certain

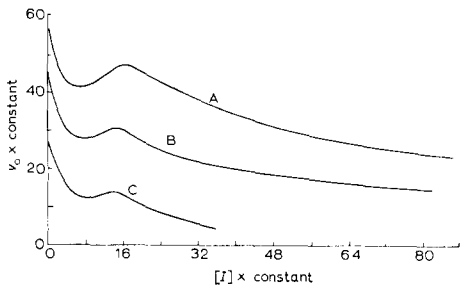


Fig. 9. Theoretical velocity–inhibitor profiles for a series of substrate concentrations under otherwise fixed conditions based on Eqns 11, 12 and 14. A, B and C correspond to ([S] × constant) at 6.0, 3.0 and 1.0 M, respectively.

fixed ratios k_1/k_2, k_2/k_3, K^A/K^C, and K^B/K^C at a series [S] values, are given in Fig. 9. Without being a perfect representation these curves simulate the experimental results (*e.g.* Fig. 5) rather well. An important feature of the experimental plots, especially noticeable when $[S] \gtrsim K_s^C$, is that v_0 falls very slowly at high [I] values. In our explanation this is due to the opposing effects of the very powerful acceleration obtained as the B sites fill up with "inhibitor" and the inevitable fall in v_0 as site C is occupied. If $K^B \simeq K^C$ and $k_3 \simeq 20k_2$ this phenomenon is well reproduced.

REFERENCES

1 F. Hibbert, S. A. Kyrtopoulos and D. P. N. Satchell, *Biochim. Biophys. Acta*, 242 (1971) 39.
2 S. A. Kyrtopoulos and D. P. N. Satchell, *Biochim. Biophys. Acta*, 268 (1972) 334.
3 S. A. Kyrtopoulos and D. P. N. Satchell, *Biochim. Biophys. Acta*, 276 (1972) 376.
4 E. R. Stadtman, *J. Biol. Chem.*, 196 (1952) 527.
5 H. U. Bergmeyer, G. Holtz, H. Klotzsch and G. Lang, *Biochem. Z.*, 338 (1963) 114.
6 M. Dixon and E. C. Webb, *Enzymes*, 2nd ed., Longmans, London, 1964.
7 D. Morris, A. Maneckjee and C. Hebb, *Biochem. J.*, 125 (1971) 857.
8 W. W. Cleland, *Biochim. Biophys. Acta*, 67 (1963) 104.
9 A. G. Clark, *Biochem. J.*, 117 (1970) 997.
10 C. K. Ingold, *Structure and Mechanism in Organic Chemistry*, Bell, London, 1953.
11 J. M. Reiner, *Behaviour of Enzyme Systems*, 2nd ed., Van Nostrand, New York, 1969.