Steady-State Kinetic Studies of the Negative Co-operativity and Flip-Flop Mechanism for *Escherichia coli* Alkaline Phosphatase

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(Received 3 February 1977)

1. A study of variations in experimental error of velocity measurement with substrate concentration for alkaline phosphatase reveals that the standard error is not constant or strictly proportional to velocity, but obeys a more complex dependence. 2. By using an approach based on error estimates at each individual substrate concentration, we show that the double-reciprocal plots in general are curved, necessitating a high-degree rate equation. The curves are analysed according to a recent classification of possible curve shapes for the 3:3 function, which is shown to be the lowest-degree rate equation satisfying the experimental data. 4. Other workers have supposed the enzyme to follow Michaelis–Menten kinetics, and it is shown that this assumption is approximately true at low temperatures in the absence of phosphate. 5. A study of the effects of phosphate concentration, pH and temperature on the kinetics shows that there is a gradual alteration in curve shape with these experimental variables, resulting in an apparent reduction in degree under certain special conditions, and particularly at low temperature. 6. It is shown that the steady-state kinetics do not require a flip-flop or half-of-sites reactivity mechanism as claimed, and a mechanism is proposed, a rate equation calculated and an analysis attempted. 7. An analysis of the product-inhibition effects for a linked two-sited Uni Bi enzyme is given. Alterations of asymptotic double-reciprocal slopes and limiting $(1/v)$ intercepts with products is discussed, and it is shown how the theory of product inhibition can be extended to complex kinetic situations to extract information as to molecular mechanism. 8. Deviations from Michaelis–Menten kinetics are expressed in terms of the magnitude of the appropriate Sylvester resultants.

Alkaline phosphatase from *Escherichia coli* is a dimeric zinc-containing enzyme that has been highly purified and subjected to exhaustive kinetic analysis in recent years. One reason for the considerable interest aroused by this enzyme is that it is put forward as an example of a flip-flop mechanism, a novel kind of extreme negative co-operativity with effective half-of-sites reactivity (Lazdunski *et al.*, 1971), and one important piece of evidence for this is the supposed Michaelis–Menten kinetics, despite the two non-independent sites. It is admitted that, under some circumstances, the double-reciprocal plots are curves (Lazdunski *et al.*, 1969; Chlebowski & Colman, 1974; Simpson & Vallee, 1970), but it is claimed that the enzyme obeys the Michaelis–Menten law at alkaline pH over a very wide range of substrate concentration (Lazdunski, 1972). Attempts have been made to reconcile this with the third-degree rate equation for a two-sited enzyme by seeking conditions under which reduction in degree can occur (Gache, 1974).

Recently we have become suspicious of supposedly linear double-reciprocal plots and expressed the view

that perhaps many of them are actually non-linear if examined critically (Bardsley & Childs, 1975; Bardsley, 1976). This view has been reinforced by a survey of the recent literature, which reveals that at least 800 enzymes have been reported as showing deviations from Michaelis–Menten kinetics and over 100 of these gave highly complex wavy types of initial-velocity curves (Hill *et al.*, 1977). In the present paper, we examine the experimental error in velocity measurements with *E. coli* alkaline phosphatase and conclude that a 3:3 rate equation is the minimum degree necessary to account for the complexity of curve shape. We also examine the conditions under which apparent Michaelis–Menten kinetics can occur, calculate a rate equation for an enzyme following a Uni Bi mechanism but having two linked sites, and attempt an explanation of the experimental data. In this analysis, we use the results of a recent study of the 3:3 function (Childs & Bardsley, 1976; Bardsley, 1977*a*) and apply Sylvester's dialytic method of elimination to the problem of reduction in degree of rate equations (Bardsley, 1977*b*).

Materials and Methods

E. coli alkaline phosphatase was supplied by Boehringer Corp. (London), Lewes, Sussex, U.K., and was found to be homogeneous on disc electrophoresis. We thank Dr. M. J. C. Crabbe of this Department for performing this experiment. *p*-Nitrophenyl phosphate was purchased from BDH Chemicals, Poole, Dorset, U.K., and fresh solutions were used. A Cary 118C recording spectrophotometer was used to measure rates of change of A_{398} , and it was equipped with a five-cell rotating turret for sample and blank cells and a thermostatically controlled circulator. Solutions were made up to a final volume of 2 ml and checked for pH and temperature before assay. The buffer used was 0.01 M-Tris/HCl/0.4 M-NaCl, pH 8.45, unless specifically stated otherwise. The pH was compensated for temperature changes. The initial velocity as a function of enzyme concentration at fixed (0.1 mM) substrate concentration was approximately linear over a wide range (0.00001–0.01 unit/ml) at 21 and 28°C, indicating absence of kinetically significant aggregation phenomena, but assays were conducted at fixed enzyme concentration (0.0008 or 0.00035 unit/ml for phosphate studies). Substrate inhibition occurs at substrate concentrations above 5 mM, but this was concluded to be an artifact and not studied further. Also, rates could not be reproducibly measured at a substrate concentration much below 0.0025 mM, and so data points lying outside the range 0.0025–5 mM were regarded as unreliable and not used.

Care is needed when working at higher substrate concentrations, and to obtain reproducible results it is necessary to measure the initial rate as quickly as possible after mixing enzyme and substrate. Complicated time-dependent effects were also obtained with *p*-nitrophenyl phosphate from alternative sources (e.g. Sigma 104), but the overall pattern of kinetics was the same. Phosphate concentration was not measured in the substrate solutions, but as all samples were slowly hydrolysing, fresh solutions were used and renewed about every 2 h.

Special methods

For convenience, we separately discuss the special experimental and theoretical procedures used under three separate headings, namely statistical considerations, a plausible rate equation, and finally some comments on the 3:3 function.

(1) *Statistical considerations.* Several authors have discussed statistical procedures in enzyme kinetics (Wilkinson, 1961; Cleland, 1967; Pettersson & Pettersson, 1970; Plowman, 1972; Storer *et al.*, 1975; Wong, 1975; Cornish-Bowden, 1976). Usually it has been assumed that attempts are being made to fit data to a simple model such as the Michaelis–

Menten equation, and weighting factors have generally been discussed as if standard error were constant or proportional to velocity. It has, however, been recognized that standard error could show a more complicated dependence on velocity and that error should really be estimated at each substrate concentration used, but this has not usually been done. Higher-degree rate equations have usually been discussed in terms of simple models such as saturation functions (Cornish-Bowden & Koshland, 1970), where the numerator is effectively the derivative of the denominator with respect to the logarithm of substrate concentration and the number of parameters is thereby much decreased, and in general, statistical fitting of general high-degree equations is difficult. It has even been suggested that postulated high-degree rate equations should be viewed with scepticism (Plowman, 1972), on account of the statistical difficulties in judging between, say, a 2:2, 3:3 or 4:4, function merely on the basis of minimizing sums of squares of weighted variances.

Accordingly, we decided to assay the enzyme under strictly controlled conditions of pH, ionic strength and temperature to discover the relationship between variance and velocity, v . We define the variance of the mean, $\sigma_{\bar{v}_j}^2$ to be

$$\sigma_{\bar{v}_j}^2 = \frac{\sum_i^n (v_{ij} - \bar{v}_j)^2}{(n-1)^2},$$

standard error to be $\sigma_{\bar{v}_j}$, and the relative error to be $\sigma_{\bar{v}_j}/\bar{v}_j$. At 28°C we performed ten replicate determinations of initial rates at each substrate concentration, and, at 21°C and 37°C, five determinations. In all cases, the initial rates were linear and easily measurable, and variance was calculated at each substrate concentration. Neither the standard error nor the relative error of velocity was constant or strictly proportional to velocity, but rather obeyed a more complex distribution. We have noticed a similar trend with other enzymes and, because of this, the following routine was adopted.

Experimental data were fitted by a non-linear regression program (kindly supplied by Dr. R. W. Foster of Manchester Medical School) using three separate procedures. Firstly, a Monte-Carlo search was used whereby the function was evaluated a number of times as specified by the user. For each call, all the values of the variable parameters were randomly chosen according to uniform distributions centred on best previous values and of widths depending on the given parameter errors and constraints. This increased the likelihood of approaching the global sum-of-squares minimum. This was followed by a Nelder & Mead (1967) minimization method using a simplex routine, which was slow but reliable. Finally, a rapid convergence based on the Davidson (1968) variable–

matrix algorithm proceeded towards the minimum by making successive approximations to the covariance matrix. This method involved only the calculations of the function and its first derivatives.

All substrate values were assumed to be error-free, and the errors in v_i , the velocity at the i th substrate concentration, were computed as standard errors from the repeated assays. The programmer supplied the experimental data and error estimates for v_i , parameter estimates, constraints and errors, the number of Monte-Carlo calls to the function, the criteria for convergence and a relevant model subroutine. The computer supplied the best-fit parameter values with error estimates, the internal covariance matrix and correlation coefficients, as well as the fitted data values.

A weighted sum of squares Q_j^2 for model j was estimated:

$$Q_j^2 = \sum_{i=1}^n q_{ij}^2 / \sigma_{ij}^2 (n - p_j)$$

where q_{ij} is the residual ($v_i - v'_i$), v'_i the calculated velocity, n the number of data values, p_j the number of model parameters, and σ_{ij}^2 the variance of v_i . This provided an estimate of the standard deviation of the errors expressed in terms of the individual errors calculated at each data point, and gave a measure of how closely the model fitted the 'average' data point. It has a value of around unity for a good fit. In addition, a model was not accepted unless it converged to a solution, its residuals were approximately normally distributed about a mean of zero, it did not reveal trends away from data, and the residuals were within the limits set by the experimental error.

(2) *A plausible rate equation.* In seeking a plausible rate equation for alkaline phosphatase, we note that some authorities claim the enzyme has two initially equivalent sites (Lazdunski, 1972), but that binding of phosphate shows negative co-operativity, and transient studies may suggest half-of-sites reactivity. Doubts have, however, been raised about the transient studies and the presence or absence of bound phosphate in the native enzyme, and so the half-of-sites reactivity and flip-flop mechanism is controversial at present (Bloch & Schlessinger, 1973). The reaction mechanism is actually Ping Pong Bi Bi, with addition of substrate (*p*-nitrophenyl phosphate, S), release of first product (*p*-nitrophenol, P), addition of water and finally release of phosphate (Q). Denoting the free enzyme as EE, then addition of substrate can give a species

where parentheses denote intermediate complexes. Similarly, addition of Q (phosphate) can give EEQ

and QEEQ with different equilibrium constants as required for negative co-operativity, and finally the species $\left(\begin{array}{c} \text{SEEQ} \\ \text{PQEEQ} \end{array} \right)$ is required to complete the stoichiometry requiring six nodes in all and a mechanism as shown in Fig. 1. This mechanism is put forward as an absolutely minimal scheme to account for the alkaline phosphatase kinetics and will be regarded as satisfactory until some piece of experimental evidence requires enlargement of the scheme. Certainly the transient and binding studies are accommodated by appropriate restrictions on rate-constant values, and we shall now see how the steady-state work can be adequately explained. The rate equation for this mechanism has not been given before, but can be calculated by using the Volkenshtein & Goldshtein (1966) procedure to be a 3:3 function in S in the absence of P and Q. We have checked the rate equation by using a computer method (Indge & Childs, 1976) and it has the following form:

$$\frac{v}{[E_0]} = \frac{\alpha_1[S] + \alpha_2[S]^2 + \alpha_3[S]^3}{\beta_0 + \beta_1[S] + \beta_2[S]^2 + \beta_3[S]^3}$$

$$\alpha_1 = 2k_{+1}k_{+3}k_{+8}k_{+9}(k_{-2} + k_{+4})(k_{+5} + k_{-6} + k_{+7})$$

$$\alpha_2 = 2k_{+1}k_{+8}\{k_{+2}k_{+4}k_{+9}(k_{-6} + k_{+7}) + (k_{+5} + k_{+7})[k_{+2}k_{+4}k_{+9} + k_{+3}k_{+6}(k_{-2} + k_{+4})]\}$$

$$\alpha_3 = 2k_{+1}k_{+2}k_{+4}k_{+8}(k_{+5} + k_{+7})$$

$$\beta_0 = k_{+8}k_{+9}(k_{-1} + k_{+3})(k_{-2} + k_{+4})(k_{+5} + k_{-6} + k_{+7})$$

$$\beta_1 = k_{+8}\{(k_{-2} + k_{+4})[2k_{+1}(k_{+3} + k_{+9})(k_{+5} + k_{-6} + k_{+7}) + k_{-1}k_{+5}k_{+6}] + k_{+2}k_{+4}k_{+9}(k_{-6} + k_{+7})\}$$

$$\beta_2 = k_{+1}\{k_{-6}(k_{-2} + k_{+4})[k_{+3}k_{+7} + 2k_{+8}(k_{+3} + k_{+5})] + k_{+2}[k_{+4}k_{+9}(k_{+7} + 2k_{+8}) + k_{+8}[2k_{+4} \times (k_{-6} + k_{+7}) + k_{+9}(k_{+5} + k_{-6} + k_{+7})]]\}$$

$$\beta_3 = k_{+1}k_{+2}k_{+6}[k_{+5}k_{+8} + k_{+4}(k_{+7} + 2k_{+8})]$$

Sigmoid curves arise when $\psi_{11}^{20} > 0$ (Bardsley, 1976) and final maxima require $\phi_{32} < 0$, and the appropriate expressions for these are:

$$\begin{aligned} \psi_{11}^{20} &= \alpha_2\beta_0 - \alpha_1\beta_1 \\ &= 2k_{+1}k_{+8}^2k_{+9}(k_{-2} + k_{+4})(k_{+5} + k_{-6} + k_{+7})\{k_{+3} \times (k_{-2} + k_{+4})[(k_{-1}k_{+6}k_{+7} - k_{+1}k_{+3}k_{-6}) + k_{+3}(k_{+5} + k_{+7})(k_{+6} - k_{+1})] \\ &\quad + k_{+4}k_{+9}[(k_{-1}k_{+2}k_{+7} - k_{+1}k_{+3}k_{-6}) + k_{+3}(k_{+5} + k_{+7})(k_{+2} - k_{+1})] \\ &\quad + (k_{+5} + k_{-6} + k_{+7})[k_{+9}(k_{-1}k_{+2}k_{+4} - k_{+1}k_{-2}k_{+3}) - k_{+1}k_{+3}(k_{-2} + k_{+4})(k_{+3} + k_{+9})]\} \end{aligned}$$

$$\begin{aligned}
\phi_{32} &= \alpha_3 \beta_2 - \alpha_2 \beta_3 \\
&= 2k_{+1}^2 k_{+2} k_{+6} k_{+8} \{k_{+5} k_{+6} k_{+8} (k_{-2} + k_{+4}) \\
&\quad \times (k_{+5} + k_{+7})(k_{+4} - k_{+3}) \\
&\quad + k_{+2} k_{+4}^2 [2k_{+8}(k_{-6} + k_{+7})(k_{+5} - k_{+9}) \\
&\quad + k_{+7}(k_{-6} + 2k_{+7})(k_{+8} - k_{+9}) \\
&\quad + k_{+4} k_{+8} [k_{+5}(k_{+4} k_{+5} k_{+6} - k_{+2} k_{+7} k_{+9}) \\
&\quad + k_{+4} k_{+7}(k_{+5} k_{+6} - k_{+2} k_{+9}) \\
&\quad + k_{+2} k_{+7} k_{+9}(k_{+7} - k_{+4}) \\
&\quad + k_{-2} k_{+5} k_{+6}(k_{+5} + k_{+7}) \\
&\quad + k_{+2} k_{-6} k_{+7}(k_{+4} + k_{+9})]\}
\end{aligned}$$

Note that sigmoidicity cannot result solely from allosterism of phosphate binding, since none of the inequalities in ψ_{11}^{20} depend on k_{+5} , k_{+8} or k_{+9} . A sigmoid curve would imply that allosterism must also be occurring elsewhere in the scheme. A final maximum (i.e. substrate inhibition) can result solely from allosterism of substrate binding, specifically if k_{+6} is sufficiently less than k_{+2} . Hence no matter how positively co-operative is the binding of second substrate ($k_{+2} > k_{+1}$), a maximum can result if the subsequent binding of S to species EQ is less favoured than that of S to species $\begin{pmatrix} \text{EES} \\ \text{EEPQ} \end{pmatrix}$ (i.e. $k_{+6} < k_{+2}$).

The scheme put forward by Lazdunski (1972) is specifically supposed to relate 'two apparently antinomic properties of the enzyme: the Michaelian kinetics and the negative co-operatively'. Inspection reveals that the kinetic features of this

We now comment further on the rate equation resulting from the flip-flop scheme of Fig. 1(b). If the node QRRQ be allowed, then, since no escape pathway is allowed via node F R_i Q, there is no steady-state solution for product zero. Instead of getting a steady-state initial rate when substrate is added to the enzyme, the enzyme species are all converted irreversibly into the final species QRRQ and eventually the reaction stops, and the only enzyme species present is the unproductive and catalytically inactive species QRRQ. So, to obtain a steady-state equation, it is further necessary to set $k_{+5} = 0$ in Fig. 1(b). If node STTS be allowed, then the resulting rate equation is a 1:2 function, i.e. dead-end substrate inhibition is given.

Saturating substrate concentration results eventually in complete population of the unproductive dead-end substrate-inhibition complex STTS. To avoid this and obtain Michaelis kinetics, it is then necessary to set $k_{+6} = 0$. It is not possible to carry out steady-state studies with any accuracy at *p*-nitrophenyl phosphate concentrations much above 5 mM, and we could not demonstrate substrate inhibition with any confidence, as discussed above.

So, to obtain Michaelis-Menten kinetics for the flip-flop mechanism, it is necessary to work with a four-node scheme, i.e. Fig. 1(b) with the dashed pathways non-existent, and in this scheme STRQ and QRTS are not distinguished kinetically. Then the rate equation is of the Michaelis-Menten type, but the kinetic constants bear no relationship to the constants of Michaelis theory. The equation is

$$v = \frac{k_{+1} k_{+2} k_{+3} k_{+4} [S][E_0]}{k_{+1} k_{+2} (k_{-3} + k_{+4}) + k_{-1} k_{+3} k_{+4} + k_{+1} k_{+3} (k_{+2} + k_{+4}) [S]} \quad (\text{rate constants as in Fig. 1b})$$

scheme are easily obtained from our more comprehensive scheme by certain assumptions, as shown in Fig. 1(b) where the nomenclature used by Lazdunski (1972) is used. From this it is clear that the flip-flop scheme amounts in kinetic terms to abolishing completely the pathways between certain nodes of Fig. 1(a), namely, $\text{EE} \rightleftharpoons \text{EEQ}$, $\text{EEQ} \rightleftharpoons \text{QEEQ}$ and

and, to obtain a first-degree rate equation, limiting the connections shown as dashed arrows in Fig. 1(b), i.e.

and so the apparent $V_{\max}$ is $k_{+2} k_{+4} / (k_{+2} + k_{+4})$ and the apparent K_m is $[k_{+1} k_{+2} (k_{-3} + k_{+4}) + k_{-1} k_{+3} k_{+4}] / k_{+1} k_{+3} (k_{+2} + k_{+4})$, and neither of these have the significance usually associated with $V_{\max}$ and K_m . It may be that some of the pathways omitted in obtaining Fig. 1(b) from Fig. 1(a) are difficult to demonstrate independently and that the nodes deleted may be sparsely populated at alkaline pH, but we consider that such a mutilation of the more comprehensive scheme would only be justified if the behaviour were truly Michaelian, which, we will argue below, is not the case.

We are also concerned with the effect of temperature and phosphate on these curves and the conditions under which curve shapes of lesser complexity can result from this rate equation, but before we can discuss this, it is necessary to outline some general properties of the 3:3 function.

Fig. 1. Mechanisms proposed for *E. coli* alkaline phosphatase

(a) The scheme is actually a two-sited co-operatively linked Ping Pong mechanism with *p*-nitrophenyl phosphate (S), *p*-nitrophenol (P) and phosphate (Q), but this becomes Uni Bi, since water is the second substrate. There are six nodes representing distinct enzyme species, parentheses denoting intermediate complexes. The sites are assumed to be identical initially, hence the free enzyme is labelled EE, but all other species are non-equivalent, allowing co-operative effects to be manifest. At low substrate concentrations, the loop $EE \rightleftharpoons \begin{pmatrix} EES \\ EEPQ \end{pmatrix} \rightleftharpoons EEQ$ is

(3) *The 3:3 function.* Methods for analysing complex kinetic graphs have been developed (Bardsley & Childs, 1976) and successfully applied to the problem of defining possible shapes for the 3:3 function (Childs & Bardsley, 1976; Bardsley, 1977a). Also whereas the usual methods for simplifying rate equations involve estimations of the relative magnitudes of rate constants (Gache, 1974) or assume rapid equilibrium (Cha, 1968), the general theory of Sylvester resultants has been developed to provide a comprehensive algebraic method for dealing with the problem which includes all possible contingencies leading to reduction in degree (Bardsley, 1977b). Since we claim that alkaline phosphatase obeys a 3:3 function, definitions are now given to explain such concepts as are needed in this particular instance. We require the identities:

$$\phi_{ij} = \begin{vmatrix} \alpha_i & \beta_j \\ \alpha_j & \beta_i \end{vmatrix}$$

$$\psi_{kl}^{ij} = \begin{vmatrix} \alpha_i & \alpha_k \\ \beta_l & \beta_j \end{vmatrix}$$

$$\varepsilon_{10} = \alpha_2 \psi_{11}^{20} - \alpha_1 \psi_{12}^{30}$$

$$\varepsilon_{11} = \alpha_3 \psi_{11}^{20} + \alpha_1^2 \beta_3$$

$$\varepsilon_{12} = \alpha_3 \psi_{12}^{30} + \alpha_1 \alpha_2 \beta_3$$

$$\varepsilon_{13} = \alpha_3 \phi_{31} - \alpha_2 \phi_{32}$$

mainly used, but higher concentrations of S drive reaction flux through the node $\begin{pmatrix} SEES \\ QPEEPQ \end{pmatrix}$, thus allowing for 'substrate activation'. S and Q compete for the nodes EE and EEQ, leading to 'pseudo-competitive product inhibition' in as much as the non-linear double-reciprocal plots converge on the $(1/v)$ axis. In the presence of Q, the v -versus-[S] curves are strongly sigmoid, since at low S values the loop $EEQ \rightleftharpoons \begin{pmatrix} SEEQ \\ QPPEEQ \end{pmatrix} \rightleftharpoons QEEQ$ is mainly used, whereas saturation with S leads to abolition of the Q effect and use of all the nodal possibilities. (b) Flip-flop scheme proposed by Lazdunski (1972). This is obtained by abolishing pathways in (a). QRRQ is required for negative co-operativity of phosphate binding, but its inclusion without the connection $QRRQ \rightleftharpoons FR_iQ$ (i.e. k_{+8} , k_{-8} of a) does not allow a steady-state solution. Similarly, species STTS, which does exist, would give dead-end substrate inhibition without the connection $STTS \rightleftharpoons STRQ$ (i.e. k_{+4} , k_{-4} of a). Species STRQ and QRST are not distinguished kinetically, since their breakdown and subsequent reactions are identical. Pseudo-Michaelis-Menten kinetics result if the dashed pathways are abolished, but the kinetic constants do not have the significance of V_{max} and K_m associated with Michaelis-Menten theory. [The terms R, T and F are defined by Lazdunski (1972).]

for then the general 3:3 function can be written, using $y = v/[E_0]$, $x = [S]$, in the equivalent forms

$$\begin{aligned}
 y &= \frac{\alpha_1 x + \alpha_2 x^2 + \alpha_3 x^3}{\beta_0 + \beta_1 x + \beta_2 x^2 + \beta_3 x^3} \\
 &= \frac{\alpha_1^2 x(\alpha_1 x + \alpha_2 x + \alpha_3 x^3)}{(\alpha_1 \beta_0 - \psi_{11}^{20} x)(\alpha_1 x + \alpha_2 x^2 + \alpha_3 x^3) + (\varepsilon_{10} x^2 + \varepsilon_{11} x^3)} \\
 &= \frac{\alpha_2^2 x(\alpha_1 x + \alpha_2 x^2 + \alpha_3 x^3)}{(\phi_{32} + \alpha_3 \beta_3 x)(\alpha_1 x + \alpha_2 x^2 + \alpha_3 x^3) + (\varepsilon_{12} x + \varepsilon_{13})} \\
 &= \frac{\alpha_1 \alpha_3 x(\alpha_1 x + \alpha_2 x^2 + \alpha_3 x^3)}{(\alpha_3 \beta_0 + \alpha_1 \beta_3 x)(\alpha_1 x + \alpha_2 x^2 + \alpha_3 x^3) + (\varepsilon_{12} x^2 - \varepsilon_{11} x)} \quad (1)
 \end{aligned}$$

Now the Sylvester resultants are:

$$R_0 = (\varepsilon_{11} \varepsilon_{13} - \varepsilon_{12}^2) / \alpha_3$$

$$R_1 = -\varepsilon_{13}$$

and under the constraint $R_0 = 0$, $R_1 \neq 0$ then the 3:3 function becomes a 2:2 function with decreased curve-shape possibilities, that is:

$$y \begin{pmatrix} 3:3 \\ 2:2 \end{pmatrix} = \frac{\varepsilon_{13}(\alpha_2 \varepsilon_{13} - \alpha_3 \varepsilon_{12})x + \alpha_3 \varepsilon_{13}^2 x^2}{(\beta_1 \varepsilon_{13}^2 - \beta_2 \varepsilon_{12} \varepsilon_{13} + \beta_3 \varepsilon_{12}^2) + \varepsilon_{13}(\beta_2 \varepsilon_{13} - \beta_3 \varepsilon_{12})x + \beta_3 \varepsilon_{13}^2 x^2}$$

When $R_0 = R_1 = 0$ then full reduction occurs and the osculating hyperbolas coalesce, leading to

$$\begin{aligned}
 y \begin{pmatrix} 3:3 \\ 1:1 \end{pmatrix} &= \alpha_1 \alpha_3 x / (\alpha_3 \beta_0 + \alpha_1 \beta_3 x) \\
 &= \alpha_1^2 x / (\alpha_1 \beta_0 - \psi_{11}^{20} x) \\
 &= \alpha_2^2 x / (\phi_{32} + \alpha_3 \beta_3 x)
 \end{aligned}$$

From the relations

$$\alpha_3 \varepsilon_{10} - \alpha_2 \varepsilon_{11} + \alpha_1 \varepsilon_{12} = 0$$

$$\alpha_3 \varepsilon_{11} - \alpha_2 \varepsilon_{12} + \alpha_1 \varepsilon_{13} = 0$$

and the alternative way of writing the 3:3 function in eqn. (1), it will be clear that as any two of ε_{10} , ε_{11} , ε_{12} , ε_{13} become small then the actual curve shape given by the 3:3 function becomes closer and closer to a Michaelis-Menten curve. Hence the magnitude of R_0 and R_1 are measures of the deviation from the

Michaelis-Menten equation and are of interest in the present case, since we are concerned with the deviation of alkaline phosphatase kinetics from a 1:1 function.

For the proposed mechanism we discover that:

$$\begin{aligned}
 \varepsilon_{11} &= 4k_{+1}^2 k_{+2} k_{+6} k_{+8}^2 k_{+9} (k_{-2} + k_{+4})(k_{+5} + k_{-6} + k_{+7}) \{ [k_{-2}(k_{-1} k_{+6} k_{+7} - k_{+1} k_{+3} k_{-6}) \\
 &\quad + k_{+7}(k_{-1} k_{+4} k_{+6} - k_{+1} k_{-2} k_{+3}) + k_{-2}(k_{-1} k_{+5} k_{+6} - k_{+1} k_{-6} k_{+9}) + (k_{-1} k_{+4} k_{+5} k_{+6} - k_{+1} k_{-2} k_{+7} k_{+9}) \\
 &\quad + k_{-2}(k_{+3} k_{+5} k_{+6} - k_{+1} k_{+7} k_{+9}) + k_{+4}(k_{+2} k_{+7} k_{+9} - k_{+1} k_{+3} k_{+5})] k_{+3} k_{+4} k_{+7} k_{+8} \\
 &\quad + 2k_{+4} k_{+8} k_{+9} [k_{+4} k_{+7}(k_{-1} k_{+2} k_{+7} - k_{+1} k_{+3} k_{-6}) + k_{+1} k_{+3} k_{+5}(k_{-2} k_{+3} - k_{+4} k_{-6})] \\
 &\quad + k_{+3} k_{+4} k_{+8}(k_{+3} k_{+5} k_{+6} - k_{+1} k_{+7} k_{+8}) [2k_{+4} k_{+7} + k_{+5}(k_{-2} k_{+4})] \\
 &\quad + k_{+3}^2 k_{+4} k_{+7} k_{+8} [k_{+7}(k_{-2} + k_{+4}) + k_{-2} k_{+5}](k_{+6} - k_{+1}) \\
 &\quad + k_{+4} k_{+8} k_{+9}(k_{-1} k_{+2} k_{+4} - k_{+1} k_{-2} k_{+3}) [(k_{+5} + k_{-6})(k_{+5} + k_{+7}) + 2k_{+5} k_{+7}] \\
 &\quad + k_{+3} k_{+4}^2 k_{+5} k_{+8} k_{+9}(k_{+2} - k_{+1})(k_{+5} + 2k_{+7} + k_{+1} k_{+3} k_{+5} k_{+8} k_{+9}(k_{+3} - k_{+4}) [k_{-2}(k_{+5} + k_{-6}) \\
 &\quad + k_{+4}(k_{+5} + k_{+7})] + k_{+1} k_{+3} k_{+4} k_{+7}(k_{+9} - k_{+8}) [k_{-2} k_{+3}(k_{+5} + k_{-6}) + k_{+3} k_{+4}(k_{+5} + k_{+7}) + k_{+3} k_{-6}^2] \\
 &\quad + 2k_{+1} k_{+3}^2 k_{+4} k_{+8}(k_{+9} - k_{+5})(k_{-2} k_{-6} + k_{+4}(k_{+5} + k_{+7}) + k_{+1} k_{+3}^2 [k_{-2} k_{+5} k_{+8}(k_{+7} k_{+9} - k_{+4} k_{+5}) \\
 &\quad + 2k_{+4} k_{+8}(k_{-2} k_{+7} k_{+9} - k_{+4} k_{+5} k_{-6}) + k_{+4} k_{+7}(k_{-2} k_{+7} k_{+9} - k_{+4} k_{-6} k_{+8}) \\
 &\quad + k_{+4} k_{+5} k_{+8}(k_{-6} k_{+9} - k_{-2} k_{+5}) + 2k_{+4} k_{+8}(k_{+4} k_{-6} k_{+9} - k_{-2} k_{+5} k_{+7})] \} \\
 \varepsilon_{12} &= 4k_{+1}^2 k_{+2} k_{+6} k_{+8}^2 k_{+9} (k_{-2} + k_{+4})(k_{+5} + k_{-6} + k_{+7}) \{ k_{+1} k_{+3} k_{+5}^2 k_{+6} k_{+8}(k_{-2} + k_{+4})(k_{+3} - k_{+4}) \\
 &\quad + k_{+1} k_{+2} k_{+3} k_{+4}^2 (k_{-6} + k_{+7}) [2k_{+8}(k_{+9} - k_{+5}) + k_{+7}(k_{+9} - k_{+8})] \\
 &\quad + k_{+1} k_{+3} k_{+7} k_{+8} [(k_{-2} k_{+3} k_{+5} k_{+6} - k_{+2} k_{+4} k_{-6} k_{+9}) + k_{+4}(k_{+3} k_{+5} k_{+6} - k_{+2} k_{+7} k_{+9})] \\
 &\quad + k_{+4} k_{+5} k_{+6} k_{+8}(k_{+5} + 2k_{+7}) [k_{+3} k_{+4}(k_{+2} - k_{+1}) + (k_{-1} k_{+2} k_{+4} - k_{+1} k_{-2} k_{+3})] \\
 &\quad + k_{+2} k_{+4}^2 k_{+7} k_{+8} [k_{+3} k_{+7}(k_{+6} - k_{+1}) + (k_{-1} k_{+6} k_{+7} - k_{+1} k_{+3} k_{-6})] \}
 \end{aligned}$$

There are four types of allostery possible in the absence of *P* (*p*-nitrophenol) and *Q* (phosphate), namely allostery in substrate binding, substrate release, *P* release and *Q* release. Analysis of ϵ_{11} and ϵ_{12} reveals that one particular set of conditions, namely $k_{+1} = k_{+2} = k_{+6}$, $k_{-1} = k_{-2} = k_{-6}$, $k_{+3} = k_{+4} = k_{+7}$ and $k_{+5} = k_{+8} = k_{+9}$ referring to the absence of each of the four types of allostery leads to the observed reduction in degree from 3:3 to a Michaelis–Menten hyperbola. However, as the reduction depends generally on the simultaneous vanishing of two expressions, each a non-linear function of 12 variables (rate constants) there are an infinite number of other conditions where $k_{+1} \neq k_{+2} \neq k_{+6}$, $k_{-1} \neq k_{-2} \neq k_{-6}$, $k_{+3} \neq k_{+4} \neq k_{+7}$ and $k_{+5} \neq k_{+8} \neq k_{+9}$ which satisfy the simultaneous equations $\epsilon_{11} = 0$ and $\epsilon_{12} = 0$. Hence, although disappearance of co-operativity of all four types leads to reduction to a hyperbola, the latter curve shape cannot be used to infer the absence of co-operativity either in part or totally, in the absence of additional evidence.

Results

The standard error was neither constant nor strictly proportional to velocity, but obeyed a more

complex distribution pattern. For this reason, individual error estimates were used in the curve-fitting procedure.

The two-sited mechanism under consideration is shown in Fig. 1 and has six nodes representing enzyme species.

In Fig. 2 are shown the *v*-versus-[*S*] data in semi-logarithmic space to which are fitted successively curves to degree 1:1, 2:2 and 3:3. Obviously, the 1:1 and 2:2 fits show trends, where the 3:3 fit goes through almost all experimental data. Increasing the degree to 4:4 produced no improvement, as shown by Table 1.

The same was true at other temperatures, and in Fig. 3 are shown the data for temperatures of 37, 28, 21 and 11°C. The curve flattens out as the temperature decreases and, whereas the shape is clearly

Table 1. Fitting of experimental data by *n*:*n* functions of degree 1:1, 2:2, 3:3 and 4:4

Model	(<i>n</i> − <i>p</i> _{<i>j</i>})	<i>Q</i> _{<i>j</i>} ²	<i>Q</i> _{<i>j</i>}
1:1	15	26.61	5.158
2:2	12	3.454	1.858
3:3	10	0.504	0.710
4:4	8	1.146	1.071

Fig. 2. Curve-fitting to experimental data

The results obtained at 28°C is shown in semilogarithmic space, each data point (○) representing the mean of ten separate determinations. The points were fitted as described in the text by using an *n*:*n* rational polynomial function of the following degrees: (i) 1:1; (ii) 2:2; (iii) 3:3. The fit with a 3:3 function is obviously superior to that with 2:2 or 1:1 functions, and no further improvement occurred with a 4:4 function.

Fig. 3. Alkaline phosphatase kinetics as a function of temperature

The curves shown are double-reciprocal plots for reactions at the following temperatures: ○, 37°C; △, 28°C; □, 21°C; ●, 11°C. Two scales for low (a) and high (b) concentration points are used. Solid lines are the best-fit 3:3 functions obtained by the statistical procedure described in the text.

high degree at 37°C and 28°C, this is not so clear at 11°C, where the double-reciprocal plot is almost linear.

Fig. 4 demonstrates that change in either pH or phosphate concentration emphasizes the deviation from Michaelis-Menten kinetics.

The extent to which the curves deviate from Michaelis-Menten kinetics is shown in Fig. 5, where the actual size of the resultants is plotted as a function

of temperature or phosphate concentration. The decrease in R_0 and R_1 as the temperature or phosphate concentration diminish is clear evidence that the kinetics become closer to being hyperbolic.

Table 2 gives the best-fit parameter values.

Discussion

It is clear that the experimental results presented require a rate equation of minimum degree 3:3 and, since this is the degree of the mechanism shown in Fig. 1, we shall proceed with analysis in these terms. First, we shall attempt an analysis of the experimental results and then subsequently an interpretation will be given in molecular terms.

Analysis of the experimental findings

As the temperature, pH or phosphate concentration change, then the alteration in curve shape is seen to result from stepwise changes in the ε functions and hence resultants R_0 and R_1 . It is possible to calculate the ε functions in terms of rate constants as shown in the text, but, in general, it has not proved possible to assign changes in shape predominantly in terms of one or two rate constants that offer a readily intuitive explanation. This is especially true when the Sylvester resultants are analysed. Already, however, some features can be identified as consistent with the rate equation and, as an example, we consider the effect of phosphate.

If we denote phosphate by Q, then it can be shown that the rate equation has the following functional dependence on [Q]:

$$\alpha_1 = a_0 + a_1[Q] + a_2[Q]^2$$

$$\alpha_2 = b_0 + b_1[Q]$$

$$\alpha_3 = c_0$$

$$\beta_0 = d_0 + d_1[Q] + d_2[Q]^2 + d_3[Q]^3$$

$$\beta_1 = e_0 + e_1[Q] + e_2[Q]^2$$

$$\beta_2 = f_0 + f_1[Q]$$

$$\beta_3 = g_0$$

where $a_i, b_i, \dots$ are collections of rate constants. Hence $\alpha_3/\beta_3 = c_0/g_0$ and is independent of [Q]. This means that, in the rate equation, Q is predicted as being a 'pseudo-competitive inhibitor' with respect to the limiting velocity, or the double-reciprocal plots are predicted to converge at the $1/v$ intercept for all phosphate concentrations, irrespective of the change in curve shape. This is essentially what is seen in Fig. 4. Also, $\alpha_1/\beta_0 = (a_0 + a_1[Q] + a_2[Q]^2)/(d_0 + d_1[Q] + d_2[Q]^2 + d_3[Q]^3)$ which is a 2:3 function in Q. This is also consistent with the experimental results. Consider also the inequality $\psi_{11}^{20} \geq 0$ dictating

Fig. 4. Effect of changes in pH and phosphate concentration on alkaline phosphatase kinetics

Solid lines are the best-fit 3:3 function fitted to the data as described in the text. In (a), (b) and (c) inserts are included so that high and low points may be shown. (a) At 21°C: ○, 0mm-phosphate; △, 5mm-phosphate; □, 20mm-phosphate; ●, 50mm-phosphate. (b) ○, At 11°C; ●, at 21°C. (c) At pH 10.2, in 0.1M-ethanolamine buffer, at 27°C. (d) At 11°C: ○, 0mm-phosphate; △, 5mm-phosphate; □, 10mm-phosphate; ●, 50mm-phosphate; ▲, 100mm-phosphate.

sigmoidicity, where we discover the function $\psi_{11}^{20}(Q)$ to be

$$\begin{aligned} \psi_{11}^{20} = & (b_0 d_0 - a_0 e_0) + (b_0 d_1 + b_1 d_0 - a_1 e_0 - a_0 e_1)[Q] \\ & + (b_0 d_2 + b_1 d_1 - a_0 e_2 - a_1 e_1 - a_2 e_0)[Q]^2 \\ & + (b_0 d_3 + b_1 d_2 - a_1 e_2 - a_2 e_1)[Q]^3 \\ & + (b_1 d_3 - a_2 e_2)[Q]^4 \end{aligned}$$

Hence we find that not only can a curve change from non-sigmoid to sigmoid as the concentration of Q increases, as found experimentally and as shown in Fig. 4 at both 11°C and 21°C, but this can actually happen up to four times at roots of $\psi_{11}^{20}(Q)$. This quite amazing fact would not have been predicted by chemical intuition alone. In other words, a curve can switch from sigmoid to non-sigmoid and vice versa several times as a result of the mathematical type of functional dependence of α_i , β_i in the original rate equation with product concentration. In this

case, phosphate is concerned and changes in curve shape have no direct interpretation in terms of co-operativity.

For completeness, we record the dependence of curve shape on *p*-nitrophenol alone, i.e. α_i , β_i as functions of a'_i , b'_i , ... and [P]. From the relationships

$$\begin{aligned} \alpha_1 &= a'_0 + a'_1[P] + a'_2[P]^2 \\ \alpha_2 &= b'_0 + b'_1[P] + b'_2[P]^2 \\ \alpha_3 &= c'_0 + c'_1[P] \\ \beta_0 &= d'_0 + d'_1[P] + d'_2[P]^2 + d'_3[P]^3 \\ \beta_1 &= e'_0 + e'_1[P] + e'_2[P]^2 + e'_3[P]^3 \\ \beta_2 &= f'_0 + f'_1[P] + f'_2[P]^2 + f'_3[P]^3 \\ \beta_3 &= g'_0 + g'_1[P] + g'_2[P]^2 \end{aligned}$$

we conclude that α_3/β_3 is a 1:2 function in P, which could be called a 'pseudo-uncompetitive' inhibition pattern, and α_1/β_0 is 2:3 in P.

Fig. 5. The resultants R_0 and R_1

As R_0 and R_1 decrease, then the curves become closer to obeying Michaelis-Menten kinetics. For convenience, the logarithm of 1 plus the modulus of R_0 or R_1 is plotted, owing to the enormous magnitude of the alterations and sign changes. R_0 and R_1 were calculated from the coefficients of the best-fit 3:3 function. $\circ$, R_0 ; $\triangle$, R_1 as defined in the text. (a) As a function of temperature; (b) as a function of phosphate concentration.

Hence the effects of product inhibitors on actual intercepts (β_n/α_n) and asymptotic slopes (β_0/α_1) of complex double-reciprocal plots are capable of analysis in both mathematical and intuitive terms for this particular mechanism. This is because α_1/β_0 represents the effect of very low substrate concentrations when only the $E_n + S = E_nS$ steps are under consideration whereas α_n/β_n represents the effects of very high substrate concentrations on steps such as $E_nS_{n-1} + S = E_nS_n$ and suggests that the rules for the effects of products on simple mechanisms (Cleland, 1970) might be capable of extension to more complex mechanisms.

Interpretation of the experimental findings in molecular terms

We have shown that all the steady-state data accumulated at 11, 21, 28 and 37°C at pH 8.45 and also at other pH values and phosphate concentrations can be satisfactorily analysed in mathematical terms by using the postulated rate equation, and so we turn now to the possible intuitive explanation of changes in curve-shape profile.

The addition of small amounts of phosphate immediately causes complex curves to reappear even at 11°C, indicating that intersubunit co-operative effects are easily manifest at the lower temperatures. In other words, the almost linear double-reciprocal plot at 11°C does not indicate that co-operativity has been 'frozen out' owing to decreased subunit flexibility and loss of enzyme 'fluidity' as would be commonly assumed, but rather indicates how the possibility of reduction in degree of a rate equation at specific values of rate constants, which has previously been discussed only from a theoretical point of view (Bardsley & Childs, 1975; Bardsley, 1977a,b,c) is now seen actually to occur. At 11°C

Table 2. Best-fit values and error estimates for the seven parameters of the 3:3 rate equation at various temperatures. The values were obtained by a non-linear regression program described in the text; α_i and β_i refer to the equation $v_i = [E_0] \sum \alpha_i x^i / \sum \beta_i x^i$. Q_i and Q_j^2 are estimates of the standard deviation and variance of the errors respectively. $(n-p_j)$ is the number of degrees of freedom involved in calculating Q_j^2 .

Parameter	...	α_1	α_2	α_3	β_0	β_1	β_2	β_3	Q_i	Q_j^2	$(n-p_j)$
Temp.											
11°C		0.5697 ±0.0057	0.8186 ±0.0575	0.6368 ±0.0353	6.372 ±0.1737	351.3 ±12.82	432.8 ±34.3	362.1 ±19.38	0.4293	0.1843	9
21°C		1.789 ±0.0238	1.918 ±0.1283	3.132 ±0.1171	5.780 ±0.1928	313.5 ±6.483	341.7 ±21.67	584.1 ±21.25	0.8782	0.9371	11
28°C		0.1288 ±0.0641	51.98 ±3.664	34.08 ±6.515	0.4256 ±0.1458	56.18 ±12.04	9453 ±269.6	1873 ±615.5	0.7098	0.5309	10
37°C		3.135 ±0.9974	67.22 ±40.67	121.9 ±19.65	3.554 ±0.9625	203.3 ±42.65	6873 ±3029	4017 ±402.5	0.9908	0.9817	12

the co-operative effects are still present, but the rate equation has become approximately 1:1 owing to temperature effects on the rate constants leading to specific values of α_i, β_i for which R_0 and R_1 are small. If $R_0 = R_1 = 0$, then the rate equation at 11°C would then be the theoretical parent hyperbola (Waight & Bardsley, 1977) described by

$$v = \frac{2k_{+1}k_{+3}k_{+4}k_{+8}(k_{+5}+k_{+7})[S][E_0]}{k_{+4}k_{+8}(k_{+5}+k_{+7})(k_{-1}+k_{+3})+k_{+1}k_{+3}(2k_{+4}k_{+8}+k_{+5}k_{+8}+k_{+4}k_{+7})[S]}$$

The pseudo-kinetic constants obtained by linear regression at 11°C would then be defined by this equation. As soon as the pH or phosphate concentrations change (Fig. 4), then α_i, β_i alter, the resultants increase, and so complex shapes result. We note that alkaline phosphatase is thought of as showing negative co-operativity of phosphate binding (Lazdunski, 1972), which would be positive co-operativity of phosphate binding when viewed in the direction of hydrolysis of *p*-nitrophenyl phosphate. Also we draw attention to the fact that, under most conditions, the double-reciprocal plots are concave down as $(1/[S]) \rightarrow 0$ ('apparent negative co-operativity'), concave-up as $(1/[S]) \rightarrow \infty$ ('apparent positive co-operativity'), and in the presence of phosphate, plots of v versus $[S]$ are sigmoid and of $(1/v)$ versus $(1/[S])$ concave-up ('apparent positive co-operativity'). We have shown that, for ligand binding, the condition that a $v/[S]$ curve be sigmoid is always stronger than the condition for positive co-operativity, but the concavity of $(1/v)$ -versus- $(1/[S])$ plots as $(1/[S]) \rightarrow 0$ is an exact indicator of final co-operativity in the binding of the last ligand (W. G. Bardsley & R. D. Waight, unpublished work). However, the concavity of $(1/v)$ -versus- $(1/[S])$ plots elsewhere, or particularly as $(1/[S]) \rightarrow \infty$, gives no reliable indication of co-operativity even with saturation functions, and in general with steady-state models there is no unambiguous relationship between curve shape and co-operativity at all, and hence no possibility of diagnosing molecular mechanism simply from concavity of double-reciprocal plots.

Nevertheless, the mechanism shown in Fig. 1 is consistent with the curve shapes found experimentally. Assuming that $k_{+8} \neq k_{+9}$ and $k_{-8} \neq k_{-9}$ is to propose a co-operative effect of binding of the first phosphate on binding the second, and in particular $k_{+9}/k_{-9} < 2k_{+8}/k_{-8}$ would give the negative co-operativity of binding required. Release of less than 2 mol of *p*-nitrophenyl-phosphate in transient studies could result from the predominant use of the loops

and the 'apparent substrate activation' at high concentrations of substrate could result from saturating concentrations of substrate driving the reaction flux into the nodes

Since, as we have argued, release of Q from QEEQ is more rapid than release of Q from EEQ, then eventual use of the loop

will give an overall faster rate at saturating S concentrations than the loop

which operates at low S concentrations. It is unfortunate that the term 'apparent substrate activation', which refers to v -versus- $[S]$ curves with an extended final section of positive gradient and double-reciprocal plots which tend to be concave-down at the $(1/v)$ intercept, has also become known as 'apparent negative co-operativity'. The confusion resulting from the equating of both 'apparent negative co-operativity' and 'apparent substrate activation' with a concave-down reciprocal plot is an unfortunate consequence of carrying the Michaelis-Menten equation over into non-linear cases where it is not applicable. In the Michaelis-Menten case, the double-reciprocal slope is constant at K_m/V , and it has been assumed that a double-reciprocal plot can bend down either because K_m is decreasing (hence negative co-operativity) or V is increasing (hence substrate activation) with substrate concentration. Such interpretation is worthless in steady-state analysis, where the complex curve shape is simply the manifestation of the high-degree rate equation and has no interpretation in terms of kinetic constants.

We can see in Fig. 1 how Q competes with S for nodes EE and also EEQ, and hence as $S \rightarrow \infty$ the initial velocity becomes independent of $[Q]$ and the double-reciprocal plots converge at β_3/α_3 and show a sort of competitive product inhibition. Adding Q will mean that the species EE is driven into the nodes EEQ and QEEQ, and hence when S is added the steady-state loop used would be

at low S concentrations, but as the S concentration increases, competition between S and Q will lead to the normal sequence

with resulting increase in rate. Hence, with phosphate present, the v -versus-[S] curves are sigmoid.

Conclusion

There seems little doubt that alkaline phosphatase from *E. coli* has two active sites that are co-operatively linked, as shown by several experimental approaches. When the rate equation is calculated for such a scheme then the degree is 3:3 and non-linear double-reciprocal plots are to be expected. However, in the belief that the double-reciprocal plots are linear, the flip-flop mechanism has been proposed whereby only one site is active at a time in an either/or fashion, and such a scheme can be derived from the comprehensive linked two-sited Uni Bi mechanism only by imposing drastic restrictions on rate-constant values as discussed in the text and illustrated in Fig. 1.

In the present study we have shown that *E. coli* alkaline phosphatase gives curved double-reciprocal plots at both 28°C and 37°C, but as the temperature decreases the curvature becomes less pronounced. However, the pseudo-Michaelian character of the curves is readily exposed by addition of phosphate or pH changes, and we are led to conclude that a 3:3 function is the minimum degree required to explain all the steady-state findings. The rate equation for a two-sited co-operatively linked Uni Bi mechanism accommodates all the experimental findings and in particular the decrease in curve-shape complexity with decreasing temperature. A new theoretical approach to this problem by using Sylvester's dialytic method of elimination is given. Such a change in curve shape would normally be interpreted as the disappearance of co-operativity, but we have shown how it is possible for this type of phenomenon to occur without loss of co-operativity. Indeed the disappearance of co-operativity is only one of a multitude of possible conditions under which a complex mechanism can appear to give Michaelis kinetics, and this has not been explicitly proved before. In particular, the effect of phosphate in perturbing the curve shape and the asymptotic behaviour of phosphate as a pseudo-competitive type of product inhibition is shown to be consistent with

the proposed mechanism, and suggests a novel, general approach to the use of product-inhibition studies in determining molecular mechanism for complex kinetic schemes giving non-linear double-reciprocal plots.

In concluding we note that whatever other evidence is adduced for *E. coli* alkaline phosphatase as a half-site enzyme with a flip-flop mechanism, this is not supported by, and is indeed quite contrary to, the results of the present steady-state study.

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