The probability that complex enzyme kinetic curves can be caused by activators or inhibitors

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Numerous chemical compounds are known that alter the rate of conversion of substrates into products in enzyme-catalysed reactions by interacting with the enzyme rather than substrates. Where this takes place in such a way that the effect is reversible on removing the compound, say by dialysis, and where the compound is unchanged chemically by the enzyme system, we refer to such a compound as a modifier. So protons, inorganic salts, activators, inhibitors or even specific allosteric effectors would all be modifiers, and any chemically reasonable kinetic scheme that is proposed to account for such effects is referred to as a modifier mechanism. Three versions of a modifier mechanism of enzyme action are studied. The implicit representation is 2:2 in [S] (with $\alpha_0 = 0$) and 2:2 in [M] (with $\alpha_0 \neq 0$), and this is a short-hand scheme for the minimum chemical formulation, the explicit one, involving discrete ES and EP species, which is 2:2 in [S] (with $\alpha_0 = 0$) and 3:3 in [M] (with $\alpha_0 \neq 0$). If m extra steps are allowed between interconversion of ES and EP species, the degree of the rate equation remains 2:2 in [S] (with $\alpha_0 = 0$), but increases to degree $(m+3):(m+3)$ in modifier (with $\alpha_0 \neq 0$). It is proved that this increase in degree is genuine and that highly complex $v([M])$ (i.e. v -versus-[M]) curves can occur. Computation of the probabilities of the five possible double-reciprocal plots in $1/v$ versus $1/[S]$ show that all of these formulations of the modifier mechanism give similar probabilities, and these are characteristic for the mechanism and quite distinct from the intrinsic curve-shape probabilities. It is also established that the probabilities of alternative complex $v([M])$ plots are similar for the various formulations, and again the probabilities of the allowed complex curves for the mechanism are quite distinct from the intrinsic probabilities of the ten possible $v([M])$ curves for a 2:2 function (with $\alpha_0 \neq 0$). The computer studies reported lead to several conclusions about the probability of modifiers leading to inhibition or activation or causing changes in $v([S])$ curve shapes, and suggest that differentiation between model mechanisms may be facilitated by knowledge of the intrinsic curve-shape probabilities for the appropriate degree rational function and the characteristic way that this is altered by specific mechanisms. It is shown that, although in some instances new curve-shape complexities are possible when schemes are considered that allow for interconversion of ES and EP species, these are highly improbable and, for theoretical purposes, schemes formulated with node compression provide good approximations to the more complicated explicit schemes. By node compression we refer to the procedure whereby enzyme kinetic schemes are simplified by replacing sequences of steps such as $ES \rightleftharpoons X_1 \rightleftharpoons X_2 \rightleftharpoons \dots \rightleftharpoons EP \dots$ by a single step $\dots ES/EP \dots$ that does not formally recognize the existence of the intermediate species. We show that the modifier mechanism studied is one where this process alters the form of the rate equation.

On a number of occasions we have claimed that enzymes do not usually obey the Michaelis–Menten equation, but, generally, the experimental points can

be fitted by rational functions (Bardsley & Childs, 1975; Bardsley, 1976, 1977a; Hill *et al.*, 1977; Bardsley *et al.*, 1980). If this point of view is

accepted, then it is necessary to attempt to study model reaction mechanisms leading to non-linear double-reciprocal plots to see whether there are any specific features about particular plausible schemes that could be exploited to discriminate between alternative possibilities. The usual approach to elucidating enzyme mechanisms (Cleland, 1970) is largely based on the assumption that, by replotting slopes and intercepts of supposedly linear double-reciprocal plots, it is possible to determine the order of substrate addition and product desorption; however, the fact that no similar routine procedures exist for non-linear plots must discourage many people from recognizing the potential mechanistic information that may be discarded through failure to take note of experimental points that highlight deviations from Michaelis kinetics. In a previous paper (Solano-Muñoz *et al.*, 1981), we studied the one-site/two-state, substrate-modifier and random Bi Bi mechanisms in order to see whether these chemically reasonable schemes might have specific diagnostic features. The results of that approach were encouraging, and have prompted us to develop the computational approach adopted in that study and apply it to another special mechanism: the modifier mechanism. A number of interesting results have emerged from this project, and these are now detailed in the present paper.

Theoretical

Aspects of the modifier mechanism

Let us suppose that a model one-substrate/one-product enzyme exists with a binding site other than the active site that can be occupied by a ligand that is not the substrate. When this binding site is occupied, the kinetic properties of the enzyme may change in such a way as to give either inhibition or activation. Indeed, a ligand may activate at one concentration and inhibit at another by diverting reaction flux into alternative pathways, and so we propose to use the traditional and less-specific term modifier for this alternative ligand. This model mechanism was one of the first to be studied, and we refer at this point to the pioneer studies by Botts & Morales (1953) and Botts (1958). These workers solved numerous questions related to this particular mechanism, and also several other important theoretical studies (Segal *et al.*, 1952; Frieden, 1964; Whitehead, 1979) have been published. These have dealt with special conditions leading to reduction in degree or have used the rapid-equilibrium assumption, but we shall demonstrate that the availability of computation now allows us to solve a number of important problems associated with this mechanism that have lain unanswered during the last 20 years. At this stage we draw attention to the nodal scheme for this mechanism, as shown in Fig.

Fig. 1. Modifier mechanism

The various formulations of the modifier mechanism that are discussed in the text are illustrated, and some relevant information on the chemistry involved in these reaction mechanisms and the degree of the resulting pseudo-steady-state rate equations is given below. Mechanism 1(a). The traditional representation of the implicit mechanism is shown on the right along with an appropriate nodal scheme emphasizing the stoichiometry. No allowance is made for the $ES \rightarrow EP$ or $EMS \rightarrow EMP$ steps. The rate equation is 2:2 in $[S]$ and 2:2 in $[M]$ with $\alpha_0 \neq 0$. Mechanism 1(b). Nodal scheme for the explicit formulation in which the steps $ES \rightarrow EP$ and $EMS \rightarrow EMP$ are formally recognized. The rate equation is 2:2 in $[S]$ but now has become 3:3 in $[M]$ with $\alpha_0 \neq 0$. Mechanism 1(c). Nodal scheme for the double-explicit formulation in which the distinct steps $ES \rightarrow X \rightarrow EP$ and $EMS \rightarrow Y \rightarrow EMP$ are allowed. This has the effect of giving a rate equation of degree 2:2 in $[S]$ and 4:4 in $[M]$ with $\alpha_0 \neq 0$. Mechanism 1(d). Nodal scheme for a multi-explicit formulation. This scheme would be appropriate for several distinct enzyme-substrate or enzyme-product complexes formed in sequence as in $ES \rightarrow X_1 \rightarrow X_2 \rightarrow \dots \rightarrow X_m \rightarrow EP$ and $EMS \rightarrow Y_1 \rightarrow Y_2 \rightarrow \dots \rightarrow Y_m \rightarrow EMP$. Now the rate equation is still 2:2 in $[S]$ but $(m+3):(m+3)$ in $[M]$ with $\alpha_0 \neq 0$.

1. Fig. 1(a) illustrates the mechanism as usually formulated, and we refer to this as the implicit scheme, since it admits no formal recognition of the need to have a chemical reaction interconverting species ES and EP. Fig. 1(b) shows the nodal diagram appropriate when such a step is considered, which we refer to as an explicit mechanism (Waight & Bardsley, 1977). Of course, there may be two distinct chemical interconversion steps involved as in $ES \rightarrow X \rightarrow EP$ in the double-explicit scheme of Fig. 1(c) or even a whole succession of steps such as $ES \rightarrow X_1 \rightarrow X_2 \rightarrow \dots \rightarrow X_m \rightarrow EP$ as shown in Fig. 1(d). At this point we point out that the pseudo-steady-state rate equation, in the absence of product, will take the form of a surface, $v = v([S], [M])$, in Euclidean three-space defined by the real rational function:

$$v([S], [M]) = \frac{\left\{ \left(\sum_{i=0}^{m+3} \alpha_{1i} [M]^i \right) [S] + \left(\sum_{i=0}^{m+2} \alpha_{2i} [M]^i \right) [S]^2 \right\} [E_0]}{\left(\sum_{i=0}^{m+3} \beta_{0i} [M]^i \right) + \left(\sum_{i=0}^{m+3} \beta_{1i} [M]^i \right) [S] + \left(\sum_{i=0}^{m+2} \beta_{2i} [M]^i \right) [S]^2} \quad (1)$$

Note that $m = -1$ in the implicit scheme of Fig. 1(a).

Regarding $[M]$ as constant, say $[M] = [M_0]$, we have a 2:2 function:

$$v([S], [M_0]) = \frac{\{\alpha_1([M_0])[S] + \alpha_2([M_0])[S]^2\} [E_0]}{\beta_0([M_0]) + \beta_1([M_0])[S] + \beta_2([M_0])[S]^2} \quad (2)$$

whereas if $[S]$ is fixed at $[S_0]$ we have an alternative type of $n:n$ function with $n = m + 3$ and $\alpha_0 \neq 0$:

$$v([S_0], [M]) = \frac{\{\alpha_0([S_0]) + \alpha_1([S_0])[M] + \dots + \alpha_n([S_0])[M]^n\} [E_0]}{\beta_0([S_0]) + \beta_1([S_0])[M] + \dots + \beta_n([S_0])[M]^n} \quad (3)$$

The serious study of complex curves in enzyme kinetics began when Botts (1958) applied co-ordinate geometry to the problem of possible curves given by eqns. (2) and (3), presumably motivated by the hope that a knowledge of the conditions governing curve shape might assist in discriminating between model mechanisms. Let us now see if we can formulate some of the questions that remain unanswered concerning this mechanism.

Question 1. It has been shown that each of the four $v([S])$ or five $1/v$ -versus- $1/[S]$ curves predicted by eqn. (2) have 'intrinsic' probabilities (Solano-Muñoz *et al.*, 1981). How do these probabilities change when the coefficients α_{ij}, β_{ij} are calculated according to the mechanism of Fig. 1? Can this mechanism, for instance, give all of the possible

$v([S])$ and double-reciprocal plots that are available to a general 2:2 function?

Question 2. Eqn. (3) predicts ten distinct $v([M])$ plots. What are the intrinsic probabilities of these curves and can all of them occur for the modifier mechanism of Fig. 2?

Question 3. It is evident that the degree of the $v([S])$ curve predicted by eqn. (2) is unaffected when proceeding from the implicit scheme of Fig. 1(a) to the general one of Fig. 1(d). However, the degree of the $v([M])$ curves predicted by eqn. (3) increase as we include the extra steps for conversion of substrate into product. Is this increase in degree real, or do the Sylvester resultants (Bardsley, 1977b) vanish so that this feature is not kinetically significant?

Question 4. What new behaviour is predicted by the explicit schemes of Fig. 1, and are these extra

possibilities sufficiently probable to require us to abandon the simple scheme of Fig. 1(a) in attempting to understand the modifier mechanism?

Question 5. It is frequently observed that curve-shape features change as the concentration of

activators or inhibitors is altered. The graphical behaviour of the $v([S])$ and $v([M])$ curves are governed by the sign of curve-shape determinants (Bardsley, 1977c; Bardsley & Waight, 1978), and these are polynomials in the concentrations of fixed reagent, which can have positive zeros. What are the probabilities that these curve-shape determinants can realize the possible positive zeros resulting in changes in curve shapes that may be diagnostically useful?

In the present paper a polynomial, $f(x)$, of degree n , is said to have k positive zeros, $k \leq n$, if there exist k positive numbers $x_1, x_2, \dots, x_k$ that are roots of the equation $f(x) = 0$, i.e. $f(x_1) = f(x_2) = \dots = f(x_k) = 0$, where coincident roots are counted according to multiplicity.

Intrinsic probability of complex curves

In a previous paper we showed that it was possible to calculate the intrinsic probabilities associated with the four $v([S])$ and five $(1/v)$ -versus- $(1/[S])$ curves that are possible for eqn. (2), i.e. with modifier concentration fixed (Solano-Muñoz *et al.*, 1981). This analysis rests on the sign of the four curve-shape determinants:

$$\begin{aligned}\phi_{21} &= \alpha_2\beta_1 - \alpha_1\beta_2 \\ \psi_{11}^{20} &= \alpha_2\beta_0 - \alpha_1\beta_1 \\ -R_0^{2:2} &= \alpha_2\psi_{11}^{20} + \alpha_1^2\beta_2 \\ &= \alpha_2^2\beta_0 - \alpha_1\phi_{21}\end{aligned}$$

and the probability of these having particular signs is dependent on the probability density function for the α_i, β_i coefficients. In the present paper we adopt the classification used previously (Solano-Muñoz *et al.*, 1981), but it is now necessary to choose a classification scheme appropriate to the $v([M])$ curves with $[S]$ fixed. The required curve-shape determinants and geometric arguments are now presented.

Let the real rational function $y = f(x)/g(x)$ be defined by:

$$y = \frac{\alpha_0 + \alpha_1 x + \alpha_2 x^2}{\beta_0 + \beta_1 x + \beta_2 x^2}; \quad \alpha_i, \beta_i \geq 0, \forall i$$

where $(f, g) = 1$, i.e.

$$\begin{vmatrix} \alpha_2 & \alpha_1 & \alpha_0 & 0 \\ 0 & \alpha_2 & \alpha_1 & \alpha_0 \\ 0 & \beta_2 & \beta_1 & \beta_0 \\ \beta_2 & \beta_1 & \beta_0 & 0 \end{vmatrix} = \phi_{21}\phi_{10} - \phi_{20}^2 \neq 0$$

The derivatives are:

$$\begin{aligned}y' &= [\phi_{10} + 2\phi_{20}x + \phi_{21}x^2]/(\beta_0 + \beta_1 x + \beta_2 x^2)^2 \\ y'' &= 2[(\beta_0\phi_{20} - \beta_1\phi_{10}) - 3\beta_2\phi_{10}x - 3\beta_2\phi_{20}x^2 - \beta_2\phi_{21}x^3]/(\beta_0 + \beta_1 x + \beta_2 x^2)^3\end{aligned}$$

and so the appropriate curve-shape determinants are:

$$\begin{aligned}\theta_1 &= \phi_{10} \quad (\theta_1 > 0 \Rightarrow y'(0) > 0) \\ \theta_2 &= \phi_{20} \quad (\theta_2 > 0 \Rightarrow y(0) < y(\infty)) \\ \theta_3 &= \phi_{21} \quad (\theta_3 > 0 \Rightarrow y'(x) > 0 \text{ as } x \rightarrow \infty) \\ \theta_4 &= \beta_0\phi_{20} - \beta_1\phi_{10} \quad (\theta_4 > 0 \Rightarrow y''(0) > 0)\end{aligned}$$

However, these curve-shape determinants are not algebraically independent, since:

$$\begin{vmatrix} \beta_0 & \beta_1 & \beta_2 \\ \beta_0 & \beta_1 & \beta_2 \\ \alpha_0 & \alpha_1 & \alpha_2 \end{vmatrix} = \beta_0\phi_{21} - \beta_1\phi_{20} + \beta_2\phi_{10} = 0$$

and

$$\begin{vmatrix} \beta_0 & \beta_1 & \beta_2 \\ \alpha_0 & \alpha_1 & \alpha_2 \\ \alpha_0 & \alpha_1 & \alpha_2 \end{vmatrix} = \alpha_0\phi_{21} - \alpha_1\phi_{20} + \alpha_2\phi_{10} = 0$$

so evidently:

$$\begin{aligned}\phi_{21} \geq 0, \phi_{10} \geq 0 &\Rightarrow \phi_{20} \geq 0 \\ \phi_{21} \leq 0, \phi_{10} \leq 0 &\Rightarrow \phi_{20} \leq 0\end{aligned}$$

and y' can have only one positive zero and y'' can have at most two positive zeros.

Fig. 2 shows the ten shapes that are possible, depending on the sign of θ_i and the use of the limiting expressions:

$$\begin{aligned}y(0) &= \alpha_0/\beta_0 \\ y'(0) &= \phi_{10}/\beta_0^2 \\ y''(0) &= 2(\beta_0\phi_{20} - \beta_1\phi_{10})/\beta_0^3\end{aligned}$$

valid at $x = 0$ and the appropriate approximations:

$$\begin{aligned}y &\approx \alpha_2/\beta_2 \\ y' &\approx \phi_{21}/\beta_2^2 x^2 \\ y'' &\approx -2\phi_{21}/\beta_2^2 x^3\end{aligned}$$

for large values of x .

Note that four shapes not included in Fig. 2 might seem to be possible, namely: (a) two inflexions, no turning point and $y'(0) > 0$; (b) two inflexions, no turning point and $y'(0) < 0$; (c) two inflexions, one turning point and $y'(0), y''(0) > 0$; (d) two inflexions, one turning point and $y'(0), y''(0) < 0$. These shapes involve inconsistencies with Descartes's rule of signs or the induced inequalities presented above.

Suitable probability density functions for coefficients and rate constants

In the calculation of intrinsic probabilities, the values of α_i, β_i were calculated according to a

simulated continuous rectangular distribution on $[0, 1]$ as described elsewhere (Solano-Muñoz *et al.*, 1981). Values of k_i were calculated in accordance with a log-normal distribution, i.e. x_i values were distributed $N(\mu, \sigma)$ with $\mu = 2, \sigma = 4$, k_i values were calculated in accordance with $k_i = 10^{x_i}$ and all x_i values falling outside 2 standard deviations were rejected. Where appropriate, $[M]$ and $[S]$ were generated randomly as 10^{x_i} , where x_i was distributed $N(\mu, \sigma)$ with $\mu = -2, \sigma = 1$, values of x_i greater than 0 being rejected. Each time the computer generated a satisfactory set of k_i values, dependent k_i values were calculated in accordance with detailed balancing and were rejected if outside

Fig. 2. Ten $v([M])$ plots with $[S]$ fixed

Analysis of eqn. (3) in the text shows that the ten possible $v([M])$ curves at fixed $[S]$ can be classified depending on the sign of four curve-shape determinants. These are $\theta_1 = \phi_{10}$, $\theta_2 = \phi_{20}$, $\theta_3 = \phi_{21}$ and $\theta_4 = \beta_0\phi_{20} - \beta_1\phi_{10}$, where $\phi_{ij} = \alpha_i\beta_j - \alpha_j\beta_i$. Symbols used have these meanings: O, turning point; Δ , inflexion point; ---, asymptote. The geometric features are as follows: $\theta_1 > 0 \Rightarrow v'(0) > 0$, the curve slopes upwards at the origin; $\theta_2 > 0 \Rightarrow$ final asymptote $> v(0)$; $\theta_3 > 0 \Rightarrow v'([M]) > 0$ for large $[M]$; $\theta_4 > 0 \Rightarrow v''(0) > 0$; the curve is concave-up at the origin. Reversing the sign of these θ_i reverses the geometric feature, and the signs of θ_i required to determine the ten shapes are now listed.

Shape	θ_1	θ_2	θ_3	θ_4
1	+	+	—	+
2	+	+	+	+
3	+	+	—	—
4	+	+	+	—
5	+	—	—	—
6	—	—	—	—
7	—	—	+	—
8	—	+	+	+
9	—	—	—	+
10	—	—	+	+

$[10^{-6}, 10^{10}]$. The program used all possible cycles of microscopic reversibility in sequence, since we found that failure to do this results in a slight bias.

We emphasize that, in this procedure for exploring all possible cycles of microscopic reversibility with comparable probability, the ranges of values of $[S]$, $[M]$ and k_i were $0 < [S] < 1M$, $0 < [M] < 1M$ and $10^{-6} < k_i < 10^{10} s^{-1}$ or $M^{-1} \cdot s^{-1}$ as appropriate. All choices of k_i values satisfied the algebraic equations required for detailed balancing in the mechanisms.

Details are provided in the Appendix, where the rate equations are also discussed.

Results

Probability of alternative $v([S])$ plots with modifier concentration fixed

In Table 1 the results of the present study are listed, and a number of conclusions can be made. First of all, it is clear that the mechanisms have quite different curve-shape probabilities from the intrinsic ones. For instance, substrate inhibition or sigmoidicity both have an intrinsic probability of 50%, but the mechanistic probabilities are much lower, about 5–10%. Again, the intrinsic probability of a concave-up double-reciprocal plot is halved for the mechanism. Another important conclusion is that the curve-shape probabilities for the implicit, explicit and double-explicit mechanisms are comparable and all of the intrinsically possible shapes can occur.

Probability of alternative $v([M])$ plots with substrate concentration fixed

The results of interest are shown in Table 2 but, before we discuss the significance of these, we have to deal with the problem of analysing $v([M])$ data for the explicit and double-explicit versions of the modifier mechanism. These are rational functions of degree 3:3 and 4:4 respectively and cannot therefore be analysed according to the ten shapes of Fig. 2.

There is a complication arising when we attempt to compare the $v([M])$ plots for the explicit mechanism, since, whereas the implicit mechanism is 2:2 and can have only ten shapes, the explicit mechanism gives 3:3 curves and the double-explicit mechanism 4:4 curves. Now, intuition suggests that the probability of the $v([M])$ curves actually showing the additional complexities that are possible (Bardsley & Childs, 1975; Bardsley, 1976, 1977a) is small. To establish this we have computed the probability of $v'([M])$ having more than one positive zero or $v''([M])$ having more than the two positive zeros

This classification neglects the cases $\theta_i = 0$, since these lead to an inordinate increase in the special cases to be considered, none of which is fundamentally distinct from shapes 1–10.

Table 1. Probability of various complex curves with concentration of substrate varied and that of modifier fixed for the modifier mechanism

The modifier mechanism of Fig. 1 gives a 2:2 $v([S][M_0])$ curve of the form $v([S], [M_0]) = \{\alpha_1([M_0])[S] + \alpha_2([M_0])[S]^2\} / \{\beta_0([M_0]) + \beta_1([M_0])[S] + \beta_2([M_0])[S]^2\}$, where $[M_0]$ is the fixed modifier concentration and the curve shapes possible depend on the signs of the curve-shape determinants ϕ_{21} , ψ_{11}^{20} , $-R_0^{2:2}$ (Bardsley, 1977c; Solano-Muñoz *et al.*, 1981). The intrinsic probabilities are for α_i, β_i varied according to a rectangular distribution on $[0, 1]$, and k_i and $[M_0]$ obey the log-normal distribution described in the text. The range of k_i values was $10^{-6} < k_i < 10^{10}$ and that of $[M_0]$ was $0 < [M_0] < 1$, values outside this range being rejected. The numbers of satisfactory cycles of random numbers are given in parentheses.

Type of 2:2 curve (with $\alpha_0 = 0$) according to the sign of ϕ_{21} , ψ_{11}^{20} and $-R_0^{2:2}$	Intrinsic probability (% in 10000 cases)	Implicit mechanism (Fig. 1a) (% in 4278 cases)	Explicit mechanism (Fig. 1b) (% in 6237 cases)	Double-explicit mechanism (Fig. 1c) (% in 2399 cases)
+ - +	7.99	19.7	21.03	25.51
+ - -	12.81	58.92	59.32	58.27
- - +	29.34	5.14	8.56	9.79
+ + +	28.9	15.98	10.77	6.08
- + +	20.96	0.23	0.30	0.17
Substrate inhibition ($\phi_{21} < 0$)	50.3	5.37	8.86	9.96
Sigmoid curve ($\psi_{11}^{20} > 0$)	49.86	16.21	11.07	6.25
Concave-up double-reciprocal plot ($-R_0^{2:2} > 0$)	87.19	41.08	40.68	41.73

allowed for in 2:2 functions. As can be seen, the probability is indeed low, and this suggests a 'pseudo classification' of 3:3 and 4:4 $v([M])$ by using the following 'pseudo curve-shape determinants':

$$\theta_{1(3)} = \theta_1 \quad [\theta_1 > 0 \Rightarrow v'(0) > 0]$$

$$\theta_{2(3)} = \phi_{30} \quad [\theta_{2(3)} > 0 \Rightarrow v(0) < v(\infty)]$$

$$\theta_{3(3)} = \phi_{32} \quad [\theta_{3(3)} > 0 \Rightarrow v'([M]) > 0 \text{ as } [M] \rightarrow \infty]$$

$$\theta_{4(3)} = \theta_4 \quad [\theta_4 > 0 \Rightarrow v''(0) > 0]$$

for the 3:3 plots and:

$$\theta_{1(4)} = \theta_1 \quad [\theta_1 > 0 \Rightarrow v'(0) > 0]$$

$$\theta_{2(4)} = \phi_{40} \quad [\theta_{2(4)} > 0 \Rightarrow v(0) < v(\infty)]$$

$$\theta_{3(4)} = \phi_{43} \quad [\theta_{3(4)} > 0 \Rightarrow v'(\infty) > 0]$$

$$\theta_{4(4)} = \theta_4 \quad [\theta_4 > 0 \Rightarrow v''(0) > 0]$$

for the 4:4 plots.

Owing to the low probability, shown in Table 2, for highly complex 3:3 and 4:4 curves, the values marked with an asterisk in Table 2 can be regarded as good approximations to the possible curves. In other words, it is highly likely that this mechanism will give a $v([M])$ curve of overall shape indistinguishable from that of the ten shapes possible for a 2:2 function with $\alpha_0 \neq 0$. The signs of the $\theta_{i(j)}$ classify geometrically similar features for $j:j$ functions with $\alpha_0 \neq 0$.

An interesting point can be seen in the data of Table 2. Shape 7 does not occur for the modifier mechanism, but its mirror image, shape 1, does. We cannot say that the shape is impossible, only that it is extremely improbable. A more extended computer

search for this shape was performed with extended ranges of k_i values and with relaxed constraints on limits. These alternative searches do change the mechanistic curve-shape probabilities somewhat, but no example of shape 7 was unearthed, and it seems likely that there may be mathematical reasons why this curve shape is impossible for the modifier mechanism. Algebraic analysis was attempted but seemed intractable.

Bearing in mind the reservations about the pseudo-classification adopted for 3:3 and 4:4 curves where the values are given with an asterisk (*) in Table 2, we now draw attention to the principal findings. First, we note that the intrinsic probabilities are not the same as the mechanistic ones. Further, along any given line of the Table representing the probabilities of any particular curve shape, we see that the probabilities for the implicit, explicit and double-explicit mechanisms are very similar.

Next, we consider corresponding pairs of curves. Notice in Fig. 2 that the ten curves can be thought of as two sets of mirror images, i.e. the pairs (1,7), (2,6), (3,10), (4,9) and (5,8). Apart from the pair (5,8), these corresponding pairs do not have identical intrinsic probabilities. However, if we consider the pairs consisting of sums of corresponding mirror images, i.e. the pairs (6+9, 2+4), (5+6+7+9+10, 1+2+3+4+8) and (7+8+10, 1+3+5), then these do have identical probabilities. Turning now to the mechanistic probabilities we see that the simple pairs (1,7), (2,6), (3,10), (4,9) and (5,8) do not have identical probabilities, and this is also the case with the compound pairs except for the pair (5+6+7+9+10, 1+2+3+4+8).

Table 2. Probability of various complex $v([M])$ curves with $[S]$ fixed for the modifier mechanism

The modifier mechanism of Fig. 1 gives a $v([M])$ curve with $[S] = [S_0]$ of the form $v([M]) = \{\alpha_0([S_0]) + \alpha_1([S_0])[M] + \dots + \alpha_n([S_0])[M]^n\} / \{\beta_0([S_0]) + \beta_1([S_0])[M] + \dots + \beta_n([S_0])[M]^n\}$, and the ten possible curve shapes as discussed in the text depend on the signs of four curve-shape determinants $\theta_1, \theta_2, \theta_3$ and θ_4 . The values for mechanisms 1(b) and 1(c) are marked with an asterisk (*), since they are for a pseudo-analysis involving θ_{1j} . The ranges of values were $10^{-6} < k_i < 10^{10}$ and $0 < [S_0] < 1$, and the numbers of cycles of the program are given in parentheses. Note that the entry 'Impossible' indicates mathematical grounds for probability 0. The entry 0 merely indicates that no example was discovered in the computer search.

Curve shape (Fig. 1)	$\theta_1 \theta_2 \theta_3 \theta_4$	Intrinsic probability (% in 10000 cases)	Implicit mechanism (Fig. 1a) (% in 12846 cases)	Explicit mechanism (Fig. 1b) (% in 5818 cases)	Double-explicit mechanism (Fig. 1c) (% in 2344 cases)
1	+ + - +	8.85	0.37	0.72*	0.94*
2	+ + + +	12.82	1.54	1.15*	0.94*
3	+ + - -	7.63	15.22	22.98*	27.64*
4	+ + + -	3.64	32.72	25.16*	19.62*
5	+ - - -	16.43	6.82	12.15*	16.94*
6	- - - -	7.78	0.51	0.51*	1.07*
7	- - + -	1.85	0	0 *	0 *
8	- + + +	17.10	0.08	0.13*	0.08*
9	- - - +	8.78	42.50	36.19*	32.64*
10	- - + +	15.12	0.22	0.36*	0.13*
Uniform inhibition (6 and 9)		16.56	43.01	36.70*	33.71*
Uniform activation (2 and 4)		16.46	34.26	26.31*	20.56*
Overall inhibition (5, 6, 7, 9 and 10)		50.96	50.05	49.21*	50.78*
Overall activation (1, 2, 3, 4 and 8)		49.04	49.93	50.14*	49.22*
Inhibition followed by activation (7, 8 and 10)		34.07	0.3	0.49*	0.21*
Activation followed by inhibition (1, 3 and 5)		32.91	22.41	35.85*	45.52*
Number of turning points $v'([M]) = 0$				(% in 2932 cases)	(% in 2372 cases)
0		33.02	77.27	64.00	54.00
1		66.98	22.71	35.52	44.73
2		Impossible	Impossible	0.46	1.22
3		Impossible	Impossible	Impossible	0.04
Number of inflexion points, $v''([M]) = 0$				(% in 2945 cases)	
0		12.42	75.22	61.35	44.64
1		76.88	24.31	37.07	44.98
2		10.70	0.37	1.56	8.90
3		Impossible	Impossible	0	1.48

It is interesting to compare particular pairs. For instance, curve 4 (smooth activation) is less probable than curve 9 (smooth inhibition) for both intrinsic and mechanistic cases. Also, uniform activation (2+4) is less probable than uniform inhibition (6+9) for the mechanism. Chemical intuition might have led us to expect that it was equally likely for any chemical substance to activate or inhibit after binding to the enzyme, but this result shows that this is not so. It is more probable to produce inhibition than activation.

A final point concerns $v([M])$ curves with turning points. Note that, whereas the compound pair

(7+8+10, 1+3+5) have comparable probability for the intrinsic scheme, the situation is dramatically altered for the mechanism. Again, intuition might suggest that activation followed by inhibition was as likely as inhibition followed by activation. However, the results show that it is quite likely for a compound to produce first an activation and then an inhibition, but very unlikely for it to produce inhibition followed by activation. As might be expected, there is an equal likelihood that $v([0]) > v([\infty])$ or $v([0]) < v([\infty])$, but nevertheless it seems that is highly unlikely for any compound to cause a smooth inhibition (9) or smooth activation (4). It is very

likely that the $v([M])$ profile will show inflexions, and quite likely that the $v([M])$ profile will show turning points if the range of $[M]$ is sufficiently wide. For these reasons we suggest that the term modifier is preferred to the alternatives activator and inhibitor, since it is unlikely for a substance to be simply an activator or inhibitor over a sufficiently large range of $[S]$ and $[M]$.

Curve-shape determinants as functions of modifier concentration

It is clear from Fig. 1 that, when $[M] = 0$, the rate equation is 1:1, since only the top line of nodes is involved. Similarly, when $[M]$ is very large, only the bottom row of nodes is involved, and 1:1 behaviour results, i.e. $\psi_{11}^{20} < 0$, $\phi_{21} > 0$ and $-R_0^{2:2} = 0$. It seems reasonable to expect that intermediate values of $[M]$ could lead to zeros in these curve-shape determinants, e.g. a curve could be sigmoid and then, as $[M]$ increases, it could become non-sigmoid. The number of times this can occur is limited by the maximum number of positive zeros of the curve-shape determinants viewed as polynomials in $[M]$. Here we adopt the convention that for $P([M])$, defined by:

$$P([M]) = p_0 + p_1[M] + p_2[M]^2 + \dots + p_n[M]^n$$

a polynomial with $p_0 = p_1 = p_2 = \dots = p_r = 0$, $p_{r+1} \neq 0$, will be said to have a zero root of multiplicity $r + 1$, whereas a polynomial with $p_{n-s} = p_{n-s+1} = \dots = p_n = 0$, $p_{n-s-1} \neq 0$, will be said to have an infinite root of order s . This is also discussed in the Appendix. We can present an algebraic analysis of this problem for the implicit mechanism of Fig. 1(a). However, it is necessary to resort to computation to solve this problem for the explicit scheme, owing to the increased complexity.

The rate equation for mechanism 1(a) and the expressions for $\phi_{21}([M])$, $\psi_{11}^{20}([M])$ and $-R_0^{2:2}([M])$ are given in the Appendix. From these it is evident that ϕ_{21} , ψ_{11}^{20} or $R_0^{2:2}$ can have up to two positive zeros. In Table 3 are given the types of polynomials we are dealing with and probabilities that these zeros can fall in the chemically reasonable range $0 < [M] < 1$. It is clear that an experimentalist has a good chance of changing the curve shape by changing $[M]$, and this could be a useful way to confirm or refute this mechanism. Obviously the explicit mechanisms of Figs. 1(b) and 1(c) have zero and infinite roots according to the intuitive arguments outlined above. However, information about these has been obtained not by algebra but by computation. In the explicit schemes of mechanisms 1(b) and 1(c) it is possible for these curve-shape determinants to have more than the maximum number of two positive zeros allowed for the implicit scheme. So in Table 3 we also list the cases where more than two zeros occur even if these are for $[M] > 1$, since these

features prove the rate equations to be genuinely of degree greater than 2:2, i.e. the Sylvester resultants do not vanish identically (Bardsley, 1977b).

Discussion

At this point we pause to consider the answers than can now be given to the questions concerning the modifier mechanism that were posed at the beginning of the present paper.

Answer to question 1. The modifier mechanism can certainly give all the $v([S])$ and $1/v$ -versus- $1/[S]$ curves that are possible for a 2:2 function with $\alpha_0 = 0$. The probabilities of the various shapes are quite different from those computed for arbitrary coefficients α_i, β_i and are sufficiently characteristic to be useful in discriminating between possible rival mechanisms.

Answer to question 2. Only nine of the plots possible for an arbitrary 2:2 function with $\alpha_0 \neq 0$ were shown to be possible for the modifier mechanism. Shape 7 of Fig. 2 was not found during an extensive search involving mechanisms 1(a), 1(b) and 1(c) of Fig. 1 and, although we have not proved that this curve shape is impossible, we have established that it is highly improbable. Surprisingly, it transpires that uniform inhibition is more probable than uniform activation, and inhibition followed by activation is highly improbable, whereas activation followed by inhibition is quite likely to be encountered.

Answer to question 3. The increase in degree that occurs in v as a function of $[M]$ with $[S]$ fixed as we proceed from mechanism 1(a) to 1(b), 1(c) and 1(d) is real. The Sylvester resultants do not vanish identically, and numerator and denominator of the rate equation do not have common factors. Thus, in principle, highly complicated $v([M])$ curves are possible.

Answer to question 4. It is actually possible to explain wavy $v([M])$ curves by invoking multi-explicit mechanisms as in Fig. 1(d). However, since the formulation of mechanism 1(a) is a good approximation for deviations from hyperbolic kinetics that occur as a result of the effects of the binding of activator or inhibitor ligands at a single binding site other than the active site, the more complex models are probably unnecessary. A $v([M])$ profile more complicated than the curves of Fig. 2 would indicate the need to consider an alternative model.

Answer to question 5. The modifier mechanism predicts Michaelis-Menten kinetics when $[M]$ is zero or very large, but it is quite likely for the $v([S])$ curve shape to change as $[M]$ is varied between these limits. For instance, a curve showing substrate inhibition could change into one showing activation as $[M]$ is varied. Indeed, this could happen several times at positive zeros of $\phi_{21}([M]) = 0$. Similarly,

Table 3. *Probability of changes in types of curve-shape complexities as modifier concentration is varied*

The curve-shape determinants ϕ_{21} , ψ_{11}^{20} and $-R_0^{2:2}$ are polynomials in $[M]$ and can change sign at positive zeros. The type of polynomial and probability of positive roots are given. Values are given in parentheses for positive roots less than $[M] = 1$, i.e. those that could be measured experimentally.

Curve-shape determinant	Implicit mechanism (Fig. 1a)	Explicit mechanism (Fig. 1b)	Double-explicit mechanism (Fig. 1c)
$\phi_{21}([M])$; the meaning is as follows: $\phi_{21} > 0 \Rightarrow$ substrate activation $\phi_{21} < 0 \Rightarrow$ substrate inhibition			
Type of polynomial			
Probability of positive zeros:			
0 (for $[M] < 1$)	$a_0(\pm)a_1[M] + a_2[M]^2 + 0[M]^3$; $a_1 \geq 0$ (% in 3540 cases) 91.02 (92.65)	$a_0 + a_1[M] + \dots + a_4[M]^4 + 0[M]^5$ (% in 5587 (1867) cases) 83.53 (87.73)	$a_0 + a_1[M] + \dots + a_6[M]^6 + 0[M]^7$ (% in 2348 (1882) cases) 82.28 (89.74)
1 (for $[M] < 1$)	0 (5.93)	2.06 (9.69)	3.92 (9.62)
2 (for $[M] < 1$)	8.98 (1.41)	14.23 (2.35)	13.25 (0.64)
3 (for $[M] < 1$)	Impossible	0.16 (0)	0.30 (0)
4 (for $[M] < 1$)	Impossible	0.02 (0)	0.25 (0)
5 (for $[M] < 1$)	Impossible	Impossible	0 (0)
6 (for $[M] < 1$)	Impossible	Impossible	0 (0)
$\psi_{11}^{20}([M])$; the meaning is as follows: $\psi_{11}^{20} > 0 \Rightarrow$ sigmoid $v([S])$ curve $\psi_{11}^{20} \leq 0 \Rightarrow$ non-sigmoid $v([S])$ curve			
Type of polynomial			
Probability of positive zeros:			
0 (for $[M] < 1$)	$-a_0(\pm)a_1[M](\pm)a_2[M]^2$ $-a_3[M]^3 - a_4[M]^4$; $a_1 \geq 0$ (% in 4305 cases) 73.9 (76.95)	$a_0 + a_1[M] + \dots + a_5[M]^5 - a_6[M]^6$; $a_6 > 0$ (% in 5587 (1867) cases) 82.24 (83.4)	$a_0 + a_1[M] + \dots + a_7[M]^7 - a_8[M]^8$; $a_8 > 0$ (% in 2348 (1916) cases) 86.75 (88.10)
1 (for $[M] < 1$)	0 (15.26)	0.04 (9.64)	0 (5.74)
2 (for $[M] < 1$)	26.1 (7.78)	17.70 (6.75)	13.20 (6.16)
3 (for $[M] < 1$)	Impossible	0 (0)	0 (0)
4 (for $[M] < 1$)	Impossible	0.02 (0)	0.04 (0)
$-R_0^{2:2}([M])$; the meaning is as follows: $-R_0^{2:2} > 0 \Rightarrow$ concave-up double-reciprocal plot $-R_0^{2:2} < 0 \Rightarrow$ concave-down double-reciprocal plot			
Type of polynomial			
Probability of positive zeros:			
0 (for $[M] < 1$)	$[M]((\pm)a_0(\pm)a_1[M] \pm a_2[M]^2 + 0[M]^3 + 0[M]^4)$; $a_1 \geq 0$ (% of 4347 cases) 78.93 (91.58)	$[M](a_0 + a_1[M] + \dots a_5[M]^5 + 0[M]^6 + 0[M]^7)$ (% in 5587 (4710) cases) 57.88 (85.93)	$[M](a_0 + a_1[M] + \dots a_8[M]^8 + 0[M]^9 + 0[M]^{10})$ (% in 2298 (1882) cases) 46.08 (78.85)
1 (for $[M] < 1$)	19.90 (7.8)	30.61 (12.89)	31.85 (17.53)
2 (for $[M] < 1$)	1.17 (0.62)	10.91 (1.11)	18.62 (3.24)
3 (for $[M] < 1$)	Impossible	0.51 (0.07)	3.00 (0.37)
4 (for $[M] < 1$)	Impossible	0.08 (0)	0.44 (0)
5 (for $[M] < 1$)	Impossible	0 (0)	0 (0)

sigmoid curves can become non-sigmoid, and concave-up double-reciprocal plots can be concave-down at specific $[M]$ values. Indeed, for the explicit schemes $\psi_{11}^{20}([M])$ and $-R_0^{2:2}([M])$ can even have four positive zeros, although this is unlikely to be diagnostically useful. Hence it is quite unlikely to find a chemical that will simply bind to a modifier site giving a uniform $v([M])$ curve at all $[S]$ values and giving the same type of $v([S])$ curve at all $[M]$ values. The term modifier is perhaps to be preferred for this reason, since, according to the experimental conditions chosen, any given compound may lead to either activation, inhibition or a mixture of different effects at different concentrations.

Perhaps the most reassuring result to emerge from these studies of the probability of various curve shapes is that each mechanism dictates a distinctive pattern of probabilities. These can be determined by the methods we have now reported and constitute a 'fingerprinting' process for theoretical mechanisms that has considerable predictive and diagnostic power.

Let us give examples of how the finding we have described could be used for this purpose. Suppose an experimentalist obtained a $v([M])$ curve like that of curve 7 in Fig. 2 at several $[S]$ values. The modifier mechanism discussed in the present paper could then be eliminated from consideration as a possibility. Again, there are numerous occasions when compounds have been found to activate at low concentration and then inhibit at higher concentration. In fact, the work described in the present paper grew out of an attempt to prove that such behaviour is consistent with the modifier mechanism and that this mechanism is a possible one for pig plasma benzylamine oxidase (Kelly *et al.*, 1981). The essential idea is merely an extension to non-linear mechanisms of the idea of replotting slopes and intercepts to differentiate Ping Pong from Ordered mechanisms. If we have Tables like Tables 1 and 2 of the present paper available for a particular mechanism, we might reasonably claim to have the kinetic potential of the mechanism. If our experimental results resemble those that are probable for a particular mechanism, then this supports us in considering such a mechanism as a possible one. As usual, the technique is rather more powerful in eliminating mechanisms from consideration, namely those mechanisms for which experimental findings require improbable curves.

Conclusion

We have repeatedly argued that enzymes do not, in general, obey the Michaelis–Menten equation, so it behoves us to try to understand the kinetic behaviour possible for chemically reasonable model mechanisms. However, the rate equations for such

schemes are enough to make strong men weep. The rate equation for mechanism 1(c) of Fig. 1, for instance, contains 1672 terms, each being a product of seven or eight rate constants, and it is small wonder that previous authors have resorted to sweeping assumptions in order to make savage economies in the algebra necessary for analysis. For the first time, the computer studies reported in the present paper generate a ray of hope. We have faced up to the problem of handling the complex equations necessary to describe the enzyme kinetics of a model enzyme with a site for binding activators or inhibitors when separate steps for the interconversion of substrate and product are considered, and we have shown such mechanisms can indeed lead to highly complex curves. However, the probability of obtaining such complexities is reassuringly low, and, by and large, it seems that the behaviour predicted by mechanism 1(a) of Fig. 1 is that which is typical of the family of reaction mechanisms involving discrete steps for interconversion of ES and EP complexes, i.e. Fig. 1(d).

Evidently there are some topological features of the scheme that dominate the kinetic potentialities of the mechanisms. After all, the principal feature of the modifier mechanism is that there are two nodes involving substrate addition and two involving product desorption. Somehow detailed balancing in the cycles of the mechanism means that the extra directed 'trees' involved in the explicit formulations are dependent in such a way that the essential feature of the four-node scheme of mechanism 1(a) dominates all others. It remains for the future to see why this is so, and to explain why the extra degrees of freedom in explicit nodal schemes are not likely to be detected experimentally.

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APPENDIX

Computer programs

The programs used in the present study were developed in FORTRAN by using the Manchester University CYBER 72 interactive system. The rate equations were calculated by using a PASCAL program that lists coefficients α_{ij} and β_{ij} as functions of all the rate constants in the mechanism. Each cycle of a mechanism imposes a restriction on the rate constants because of detailed balancing and all the constants used satisfied these constraints. Pilot studies showed that, not surprisingly, the probabilities are somewhat dependent on the particular set of rate constants that are solved in terms of the independent ones. Although the effect is slight, we circumvented it by including a step in the programs so that all possible choices for solving for dependent rate constants were exploited in sequence. The rate equation for mechanism 1(a) is given subsequently, and that of mechanism 1(b) is obtained by replacing k_2 and k_4 by $k_2[M]$ and $k_4[M]$ in eqn. 1(a) of Bardsley *et al.* (1980), noting the corrections reported by Solano-Muñoz *et al.* (1981), since the case $[M] = 1$ is formally the same as the one-site/two-state mechanism. The rate equation for mechanism 1(c) is too large to print, since it contains 1672 terms, and in any case human life is too short for algebraic analysis of equations of this nature.

Choice of random values for α_i , β_i , k_i , $[M]$ and $[S]$

The RANF procedure was used for generating random numbers with six digits according to a rectangular distribution, but the NAG library subroutines were employed for numbers obeying a Gaussian distribution.

The rectangular distribution is discontinuous, but can be approximated by the probability density function:

$$f(x) = \begin{cases} 1 & \text{for } 0 \leq x \leq 1 \\ 0 & \text{otherwise} \end{cases}$$

whereas the transformation $y = 10^x$ can be approximated by:

$$g(y) = \begin{cases} (\log e)/y & \text{for } 1 \leq y \leq 10 \\ 0 & \text{otherwise} \end{cases}$$

These distributions have been used to generate α_i, β_i values according to $\alpha_i, \beta_i = x$ or y , but to obtain a log-normal distribution for k_i we generated x according to $N(\mu, \sigma)$, i.e.:

$$f(x) = \frac{1}{\sqrt{(2\pi)\sigma}} \cdot \exp - \left(\frac{x - \mu}{\sqrt{2\sigma}} \right)^2$$

with $\mu = 2, \sigma = 4$. Setting $y = 10^x$ and $k_i = y$ shows that the approximate probability density function for the k_i was:

$$g(y) = \left[\frac{(\log e)}{\sqrt{(2\pi)\sigma}} \cdot \exp - \left(\frac{(\log e) \cdot \ln y - \mu}{\sqrt{2\sigma}} \right)^2 \right] / y$$

and any choice of k_i less than 10^{-6} or greater than 10^{10} was rejected. Where necessary $[M_i]$ and $[S_i]$ were selected by a similar routine with $\mu = -2$ and $\sigma = 1$, and values greater than 1 were rejected. We found that after 50–100 satisfactory cycles the computed probabilities approached steady values, and in no case was it really necessary to run a program for more than 1000 completed cycles. Naturally programs for Fig. 1(c) involved a high proportion of rejections.

Algebraic analysis

We now give the rate equation for the mechanism of Fig. 1(a) and the curve-shape determinants as functions of modifier concentration. Following the style of eqn. 1 of the text, we have:

$$\beta_{00} = k_{-1}k_{-2}k_{-3} + k_{-1}k_{-2}k_q + k_{-1}k_{-2}k_{-4} + k_{-2}k_{-4}k_p + k_{-2}k_{-3}k_p + k_{-2}k_p k_q$$

$$\beta_{10} = k_{+1}k_{-2}k_{-3} + k_{+1}k_{-2}k_q + k_{+1}k_{-2}k_{-4} + k_{+3}k_{-1}k_{-4} + k_{+3}k_{-4}k_p$$

$$\beta_{20} = k_{+1}k_{+3}k_{-4}$$

$$\beta_{01} = k_{+2}k_{-1}k_{-3} + k_{+2}k_{-1}k_q + k_{+4}k_{-2}k_{-3} + k_{+4}k_{-2}k_q + k_{+2}k_{-1}k_{-4} + k_{+2}k_{-4}k_p + k_{+2}k_{-3}k_p + k_{+2}k_p k_q$$

$$\beta_{11} = k_{+2}k_{+3}k_{-1} + k_{+1}k_{+4}k_{-2} + k_{+1}k_{+4}k_{-3} + k_{+1}k_{+4}k_q + k_{+2}k_{+3}k_{-4} + k_{+2}k_{+3}k_p$$

$$\beta_{21} = k_{+1}k_{+3}k_{+4}$$

$$\beta_{02} = k_{+2}k_{+4}k_{-3} + k_{+2}k_{+4}k_q$$

$$\beta_{12} = k_{+2}k_{+3}k_{+4}$$

$$\alpha_{10} = k_{+1}k_p k_{-2}k_{-3} + k_{+1}k_p k_{-2}k_q + k_{+1}k_p k_{-2}k_{-4}$$

$$\alpha_{20} = k_{+1}k_{+3}k_{-4}k_p$$

$$\alpha_{11} = k_{+2}k_{+3}k_{-1}k_q + k_{+1}k_{+4}k_{-2}k_q + k_{+2}k_{+3}k_{-4}k_p + k_{+2}k_{+3}k_p k_q$$

$$\alpha_{21} = k_{+1}k_{+3}k_{+4}k_q$$

$$\alpha_{12} = k_{+2}k_{+3}k_{+4}k_q$$

$$\phi_{21}([M]) = k_{+1}k_{+3}\{k_{+3}k_{-4}^2(k_{-1} + k_p)k_p + [k_{+4}k_q(k_{+3}k_{-4}(k_{-1} + k_p) + k_{+1}k_{-2}(k_q - k_p)) + k_{-4}k_p(k_{+2}k_{+3}(k_p - k_q) + k_{+1}k_{+4}(k_{-3} + k_q))][M] + k_{+1}k_{+4}^2k_q(k_{-3} + k_q)[M]^2\}$$

$$\begin{aligned} \psi_{11}^{20}([M]) = & -k_{+1}^2k_{-2}^2k_p(k_{-4} + k_{-3} + k_q)^2 + \{k_{+1}k_{-2}[(k_{+3}k_{+4} - k_{+1}k_{+4} - k_{+2}k_{+3})k_p k_q^2 + (k_{+3}k_{+4}k_{-1} - k_{+1}k_{+4}k_{-2} \\ & - k_{+2}k_{+3}k_{-1})k_q^2 + (k_{+3}k_{+4}k_{-4} - 3k_{+2}k_{+3}k_{-4} - k_{+2}k_{+3}k_{-1} - k_{+1}k_{+4}k_{-4} - k_{+1}k_{+4}k_{-3} - k_{+1}k_{+4}k_{-2} \\ & - k_{+3}k_{+4}k_{-3} - k_{+2}k_{+3}k_{-3})k_p k_q - (k_{+1}k_{+4}k_{-2}k_{-4} + 2k_{+1}k_{+4}k_{-2}k_{-3} + k_{+2}k_{+3}k_{-1}k_{-3})k_q \\ & - (2k_{+2}k_{+3}k_{-4}(k_{-1} + k_{-3} + k_{-4}) + k_{+2}k_{+3}k_{-1}k_{-3} + k_{+1}k_{+4}k_{-2}k_{-4})k_p\} + k_{+2}k_{+3}(k_{+1}k_{-4} - k_{+1}k_{-2} \\ & - k_{+3}k_{-4})k_p^2k_q + (k_{+1}k_{-3}k_{-4} - k_{+1}k_{-2}k_{-4} - k_{+1}k_{-2}k_{-3} - k_{+3}k_{-4}^2)k_p^2\}[M] \\ & + \{-k_{+2}^2k_{+3}^2k_p^2k_q - k_{+2}^2k_{+3}^2k_{-4}k_p^2 + k_{+1}k_{+4}(k_{+3}k_{+4}k_{-2} - k_{+1}k_{+4}k_{-2} - k_{+2}k_{+3}k_{-2})k_q^2 \\ & + k_{+2}k_{+3}(k_{+1}k_{+4}k_{-4} - 3k_{+1}k_{+4}k_{-2} - 2k_{+2}k_{+3}k_{-1} - k_{+2}k_{+3}k_{-4} - k_{+3}k_{+4}k_{-4})k_p k_q \\ & - (k_{+1}k_{+4}k_{-2}(2k_{+2}k_{+3}k_{-4} + 2k_{+2}k_{+3}k_{-1} + k_{+1}k_{+4}k_{-2} + 2k_{+2}k_{+3}k_{-3}) + k_{+2}^2k_{+3}^2k_{-1}^2)k_q \\ & - k_{+2}k_{+3}(2k_{+1}k_{+4}k_{-2}k_{-4} + 2k_{+1}k_{+4}k_{-2}k_{-3} + k_{+2}k_{+3}k_{-4}^2)k_p\}[M]^2 - \{k_{+2}k_{+3}k_{+4}(k_{+2}k_{+3}k_{-4}(k_p + k_q) \\ & + 2k_q(k_{+1}k_{+4}k_{-2} + k_{+2}k_{+3}k_{-1} + k_{+2}k_{+3}k_p))\}[M]^3 - k_{+2}^2k_{+3}^3k_{+4}^2k_q[M]^4 \\ -R_0^{2:2}([M]) = & (k_{+2}k_{+3}k_{-4}k_p - k_{+1}k_{+4}k_{-2}k_q)[M]\{(k_{+2}k_{+3}k_{+4}k_p k_q + k_{+4}(k_{+3}k_{+4} - k_{+2}k_{+3} - k_{+1}k_{+4})k_q^2)[M]^2 \\ & + [k_{+2}k_{+3}(k_{-4} + k_q)k_p^2 + (k_{+2}k_{+3}k_{-1} + 2k_{+3}k_{+4}k_{-4} + k_{+1}k_{+4}k_{-2} - k_{+2}k_{+3}k_{-4} - 2k_{+1}k_{+4}k_{-4} \\ & - k_{+1}k_{+4}k_{-3})k_p k_q + (k_{+3}k_{+4} - k_{+1}k_{+4} - k_{+2}k_{+3})k_q^2k_p + (k_{+3}k_{+4}k_{-1} - k_{+2}k_{+3}k_{-1} \\ & - k_{+1}k_{+4}k_{-2})k_q^2\}[M] + [(k_{+1}k_{-2}k_{-4} + k_{+1}k_{-2}k_{-3} + k_{+3}k_{-4}^2 - k_{+1}k_{-4}^2 - k_{+1}k_{-3}k_{-4} + (k_{+1}k_{-2} \\ & + k_{+3}k_{-4} - k_{+1}k_{-4})k_q)k_p + (k_{+3}k_{-1}k_{-4} - k_{+1}k_{-2}k_{-4} - k_{+1}k_{-2}k_{-3} - k_{+1}k_{-2}k_q)k_q\}k_p\} \end{aligned}$$

Note that $\phi_{21}([M])$ has only 0 or 2 positive zeros and, because of sign limitations, the Descartes rule of signs allows us to conclude that $\psi_{11}^{20}([M])$ has 0 or 2 positive zeros. Note that $R_0^{2:2}([M])$ has the factor $k_{+2}k_{+3}k_{-4}k_p - k_{+1}k_{-2}k_{-4}k_q$. Whitehead (1979) draws attention to the origin of this factor, showing why $R_0 = 0$ under the constraint of generalized microscopic reversibility, a result also noted by Botts (1958). Taking the limit as $[M] \rightarrow 0$ and $[M] \rightarrow \infty$, it is easy to see that the rate equation for mechanism 1(a) given above reduces to the Michaelis-Menten equation for the upper and lower nodes respectively,

$[M] \rightarrow \infty$ numerator and denominator of the rate equation have a common factor and yet the resultant does not seem to vanish. Actually, in the application of Sylvester's dialytic method of elimination, it is understood that the two polynomials in question do not both have infinite roots. The resultant of two polynomials with infinite roots vanishes identically, since at least one column has zero entries, and we shall reformulate the problem in such a way as to make it clear that this is what is happening with the modifier mechanism. First we re-write eqn. (1) as follows:

$$v = \frac{[E_0][S]\{(\alpha_{10}/[M]^{m+3} + \dots + \alpha_{1,m+3}) + (\alpha_{20}/[M]^{m+3} + \dots + \alpha_{2,m+2}/[M])[S]\}}{(\beta_{00}/[M]^{m+3} + \dots + \beta_{0,m+3}) + (\beta_{10}/[M]^{m+3} + \dots + \beta_{1,m+3})[S] + (\beta_{20}/[M]^{m+3} + \dots + \beta_{2,m+2}/[M])[S]^2} \quad (1')$$

and this conclusion follows, since $[M] = 0 \Rightarrow R_0 = 0$ and R_0 for all the schemes has been shown to have a single zero root. However, R_0 really has a double infinite root for all the mechanisms of Fig. 1. Hence, although R_0 does not vanish as $[M] \rightarrow \infty$, nevertheless numerator and denominator of eqn. (1) do have a common linear factor.

Infinite roots of Sylvester's resultant

We need to explain how it comes about that as

and from this it is obvious that both numerator and denominator of the rate equation have infinite roots as $[M] \rightarrow \infty$, since the coefficients of $[S]^2$ tend to zero. If now we calculate the resultant of numerator and denominator of eqn. (1'), it will be a polynomial in $1/[M]$ with a zero at $1/[M] = 0$ (i.e. $[M] = \infty$), since $1/[M]$ is a factor of the first row of the determinant. In other words, the resultant of numerator and denominator of eqn. (1) does vanish as $[M] \rightarrow \infty$, and it transpires that, whereas eqn. (1) is the appropriate

form to investigate behaviour as $[M] \rightarrow 0$, eqn. (1') is more convenient when interest centres on the case $[M] \rightarrow \infty$.

Checking the results in double precision

It might be argued that the low probabilities associated with more than two zeros of $v'([M])$ in the explicit formulation of the modifier mechanism arouse suspicion that the result could be due to rounding error, since this study was conducted in single precision, i.e. to 12 places. Frieden (1964), for instance, is typical of many authors who have argued that inclusion of extra steps does not change the form of the $v([S],[M])$ rate equation, and we have claimed that the degree of the rate equation does increase as a function of $[M]$. To settle this matter, we have chosen a set of integer values of k_i that give a non-vanishing Sylvester resultant, $R_0([S])$, and a $v([M])$ curve with two turning points when calculated by using double precision (24 places). Coefficients were calculated for mechanism 1(b) by using $k_{+1} = 10^6$, $k_{-1} = 10^2$, $k_{+2} = 10^2$, $k_{-2} = 10^3$, $k_{+3} = 10^2$, $k_{-3} = 1$, $k_{+4} = 1$, $k_{-4} = 10^3$,

$k_{+5} = 10^2$, $k_{-5} = 10^3$, $k_{+6} = 10^6$, $k_{-6} = 10$, $k_{+7} = 10^3$, $k_{-7} = 1$, $k_p = 10^2$, $k_q = 1$, $[S] = 0.1$. This gives $R_0([S]) = -1.99864 \times 10^{99}$ and gives a $v([M])$ curve with a minimum at $[M] = 70.3816$ and a maximum at $[M] = 837.792$ at the two positive zeros of $v'([M])$. The roots calculated in single precision agreed very closely with those calculated in double precision, and we concluded that the error associated with the probabilities in the Tables was very small. $v([M])$ for the explicit modifier mechanism allowing for detailed balancing is definitely degree 3:3, $R_0([S])$ does not vanish identically and there can be two positive zeros of $v'([M])$.

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