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Sigmoid Curves, Non-Linear Double-Reciprocal Plots and Allosterism

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1. The theory of plane curves was applied to the graphical methods used in enzyme kinetics and a mathematical analysis of the possible graph shapes is given. 2. The belief that allosterism can be inferred from steady-state data alone is subjected to criticism and the mathematical significance of sigmoid curves and non-linear double-reciprocal plots is explored. 3. It is suggested that the usual methods of interpreting steady-state kinetic data are often based on over-restrictive assumptions which prevent maximum utilization of the available data. 4. Methods for obtaining the degree of the rate equation from graph shapes obtained directly from initial-rate measurements and from replots of asymptotic behaviour as $x \to 0$ and $x \to \infty$ are discussed. 5. Detailed proofs of the theorems given in the text have been deposited as Supplementary Publication SUP 50049 (10 pages) at the British Library (Lending Division), Boston Spa, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in *Biochem. J.* (1975), **145**, 5.

Steady-state data are usually obtained in the form of initial-rate measurements with all variables constant except one of the substrate concentrations at a time. Since the steady-state velocity v is a function of the substrate concentrations (A, B, C etc.) and product concentrations (P, Q, R etc.) according to

$$v = f_1(A, B, C{-}{-}{-}{-}P, Q, R{-}{-}{-}{-})$$

at constant pH, ionic strength, temperature etc., the velocity will be a function of any one substrate concentration (A) with all the other substrate concentrations constant. This function must be of the form $v = 0$ when $A = 0$, i.e. $v = Af_2(A)$.

Now steady-state velocity equations are ratios of polynomials of the following type

$$v = \left(\frac{\alpha_1 A + \alpha_2 A^2 + {-}{-}{-}{-}\, \alpha_n A^n}{\beta_0 + \beta_1 A + \beta_2 A^2 {-}{-}{-}{-}\, \beta_m A^m}\right) E_0$$

where the coefficients are necessarily positive functions of the other variables (Cleland, 1963*a*,*b*,*c*; Wong & Hanes, 1962; Childs & Bardsley, 1975*b*). In the present paper we explore the possible information given by the graphical analysis of steady-state data for high-degree mechanisms.

General Properties of Polynomial Functions

(1) We shall, for convenience, consider y as a function of x only, and primes will always refer to differentiation with respect to x.

Consider the function

$$f(x) = \frac{N}{D} = \frac{\sum_0^n \alpha_i x^i}{\sum_0^m \beta_i x^i}$$

Subtraction of the quantity $f(0)$ will lead to a new function which will always go through the origin according to

$$f(x) - f(0) = \frac{N - \frac{\alpha_0}{\beta_0} D}{D} = \frac{\left(\alpha_1 - \frac{\alpha_0}{\beta_0}\beta_1\right) x + \left(\alpha_2 - \frac{\alpha_0}{\beta_0}\beta_2\right) x^2 + {-}{-}{-}{-}}{\beta_0 + \beta_1 x + \beta_2 x^2 + {-}{-}{-}{-}}$$

but the numerator coefficients are of the form

$$\left(\alpha_i - \frac{\alpha_0}{\beta_0}\beta_i\right)$$

and not necessarily finite or positive. The graphical interpretation of this procedure will be clear from Fig. 1, but, for our purposes, it is sufficient to realize that all the functions likely to be encountered can be reduced to the form

$$y = \frac{\sum_1^n \alpha_i x^i}{1 + \sum_1^m \beta_i x^i} \tag{1}$$

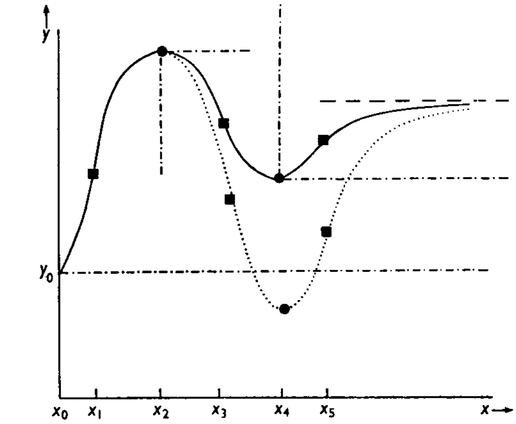

Fig. 1. *Typical graph of a rational polynomial*

The general features of a polynomial function of the type discussed in the text.

$$y(x)=\sum_{0}^{n}\alpha_i x^i\Big/\sum_{0}^{m}\beta_i x^i$$

$y'=0$, indicated by ● at x_2 and x_4 (turning point); $y''=0$, indicated by ■ at x_1, x_3 and x_5 (inflexion). These symbols will be used in all subsequent Figures. ——, Horizontal asymptote as $x\rightarrow\infty$; –·–·, shift of co-ordinate axes. Shift from $(x_0,0)$ to (x_0,y_0) is easily achieved by plotting $(y-y_0)$ against x. This would give a function of the type

$$y=\sum_{1}^{n}\alpha_i x^i\Big/\sum_{0}^{m}\beta_i x^i$$

which passes through the origin and is of the form in which steady-state data are obtained. In some circumstances, this could result in $(y-y_0)$ taking on negative values, as shown by the dotted line. Shift of origin to the minimum at (x_4,y_4) would give a straightforward sigmoid type of curve and this information could be replotted as $(1/y)/(1/x)$ or some other transformed plot. Shift of origin to the maximum at (x_2,y_2) and inversion of the ordinate would give a partial-substrate-inhibition type of curve. This approach may be a valuable analytical technique but does not seem to have been used by enzyme kineticists.

by subtraction of $y(0)$ where necessary, division by β_0 and redefinition of the coefficients.

We shall be concerned with polynomial functions with positive coefficients, which are zero at the origin, and which are defined and non-negative in the interval of concern to us, namely the first quadrant with $0<x<\infty$, which is the region accessible experimentally.

Usually, $n=m$ unless dead-end complexes are formed when $m>n$ (dead-end substrate inhibition). The physical situation dictates that we can never have $n>m$, and most of the following discussion will be for the case $n=m$. For an $n{:}n$ function $\lim_{x\rightarrow\infty} y=\alpha_n/\beta_n$ but when $m>n$ $\lim_{x\rightarrow\infty} y=0$. As will be shown, however, an $n{:}m$ function can have more inflexions than the corresponding $n{:}n$ function.

(2) It is often the case that rate equations result from the algebraic addition of two or more independent rate equations, as in the case of mixtures of isoenzymes.

Now it is readily shown from consideration of

$$y=\frac{\alpha_1 x+----+\alpha_n x^n}{1+\beta_1 x+----+\beta_n x^n}+\frac{\gamma_1 x+----+\gamma_m x^m}{1+\delta_1 x+----+\delta_m x^m}$$

that the condition for a sigmoid inflexion in y/x is

$$(\alpha_2-\alpha_1\beta_1)+(\gamma_2-\gamma_1\delta_1)>0$$

(Childs & Bardsley, 1975*b*)

Thus, if we add any two rational polynomial functions which are separately sigmoid (i.e. $\alpha_2-\alpha_1\beta_1>0$; $\gamma_2-\gamma_1\delta_1>0$), we find that the sum is necessarily sigmoid.

Similarly, the condition for a maximum in y/x is

$$\beta_n^2(\gamma_m\delta_{m-1}-\gamma_{m-1}\delta_m)+\delta_m^2(\alpha_n\beta_{n-1}-\alpha_{n-1}\beta_n)<0$$

whereas for each separately we require $\gamma_m\delta_{m-1}-\gamma_{m-1}\delta_m<0$; $\alpha_n\beta_{n-1}-\beta_n\alpha_{n-1}<0$.

(3) Common factors can arise in both the numerator and denominator of rational polynomial functions, cancellation then giving a function of lowered degree.

$$\text{e.g. in } y=\frac{\alpha_1 x+\alpha_2 x^2+-----+\alpha_n x^n}{1+\beta_1 x+----+\beta_n x^n}$$

if $(x+r_1)$ is to be a factor of the numerator $(r_1>0)$,

$$\alpha_n(-r_1)^n+\alpha_{n-1}(-r_1)^{n-1}+-----+\alpha_1(-r_1)=0 \quad (2)$$

Similarly, if the same $(x+r_1)$ is to be a factor of the denominator,

$$\beta_n(-r_1)^n+-----+\beta_1(-r_1)+1=0 \quad (3)$$

Thus eqns. (2) and (3) must hold simultaneously if a factor $(x+r_1)$ is to cancel top and bottom and so decrease the degree by 1.

For a quadratic factor to cancel, having positive coefficients but not necessarily real roots, we would take r_1 and r_2 as complex conjugates.

An obvious example of this decrease in degree is the case of a saturation function describing ligand binding to n distinct sites, which reduces in this way from $n{:}n$ to 1:1 eventually as the sites become identical and independent (Weber & Anderson, 1965; Monod *et al.*, 1965; Koshland *et al.*, 1966).

The Graphical Methods used in Enzyme Kinetics

(1) *The mathematical basis of the transformations*

The y/x data are frequently transformed by plotting derived functions and the following graphs have been widely employed:

$$y/x;\ \log y/\log x;\ \frac{1}{y}\Big/x;\ \frac{1}{y}\Big/\frac{1}{x};\ y\Big/\frac{y}{x};\ \frac{x}{y}\Big/x;\ \log y/x;$$

and $y/\log x$

To appreciate what is happening in these transformations, suppose that $y(x)$ is transformed into the two derived functions $F(x,y)$ and $G(x,y)$, which are then plotted as F/G. Since $y = y(x)$, we have

$$\begin{aligned} F(x,y) &= \theta(x) \\ G(x,y) &= \psi(x) \\ \frac{\mathrm{d}F}{\mathrm{d}G} &= \frac{\mathrm{d}\theta}{\mathrm{d}\psi} \\ &= \frac{\mathrm{d}\theta}{\mathrm{d}x}\left(\frac{\mathrm{d}\psi}{\mathrm{d}x}\right)^{-1} \end{aligned} \tag{4}$$

Also,

$$\begin{aligned} \frac{\mathrm{d}^2F}{\mathrm{d}G^2} &= \frac{\mathrm{d}^2\theta}{\mathrm{d}\psi^2} \\ &= \left(\frac{\mathrm{d}^2\theta}{\mathrm{d}x^2}\frac{\mathrm{d}\psi}{\mathrm{d}x} - \frac{\mathrm{d}^2\psi}{\mathrm{d}x^2}\frac{\mathrm{d}\theta}{\mathrm{d}x}\right)\left(\frac{\mathrm{d}\psi}{\mathrm{d}x}\right)^{-3} \end{aligned} \tag{5}$$

Eqns. (4) and (5) allow us to easily calculate derivatives for any F and G, but to choose a point of reference we will use the graph of y/x. This graph is the one which is intuitively easiest to interpret and represents the form in which rate equations are usually calculated and steady-state data obtained. We now relate the behaviour of y/x to F/G in order to explain what is happening in the 'mapping' operations used in enzyme kinetics.

We have

$$\frac{\mathrm{d}F}{\mathrm{d}G} = \frac{\left(\frac{\partial F}{\partial x} + \frac{\partial F}{\partial y}y'\right)}{\left(\frac{\partial G}{\partial x} + \frac{\partial G}{\partial y}y'\right)} \tag{6}$$

and so a turning point in y/x ($y' = 0, 0 < x < \infty$) implies a turning point in any function of y only, plotted against any function of x and y. Also, a turning point will occur in any function of x and y plotted against any other function of x and y when

$$y' + \frac{\partial F}{\partial x}\left(\frac{\partial F}{\partial y}\right)^{-1} = 0$$

provided that

$$y' + \frac{\partial G}{\partial x}\left(\frac{\partial G}{\partial y}\right)^{-1} \neq 0$$

To relate inflexions in y/x to F/G we require $\mathrm{d}^2F/\mathrm{d}G^2$ in terms of y' and y''.

$$\frac{\mathrm{d}^2F}{\mathrm{d}G^2} = \frac{\left\{\left(\frac{\partial G}{\partial x}\frac{\partial^2F}{\partial x^2} - \frac{\partial F}{\partial x}\frac{\partial^2G}{\partial x^2}\right) + \left[2\left(\frac{\partial G}{\partial x}\frac{\partial^2F}{\partial x\partial y} - \frac{\partial F}{\partial x}\frac{\partial^2G}{\partial x\partial y}\right) + \frac{\partial G}{\partial y}\frac{\partial^2F}{\partial x^2} - \frac{\partial F}{\partial y}\frac{\partial^2G}{\partial x^2}\right]y' + \left[2\left(\frac{\partial G}{\partial y}\frac{\partial^2F}{\partial x\partial y} - \frac{\partial F}{\partial y}\frac{\partial^2G}{\partial x\partial y}\right) + \frac{\partial G}{\partial x}\frac{\partial^2F}{\partial y^2} - \frac{\partial F}{\partial x}\frac{\partial^2G}{\partial y^2}\right]y'^2 + \left(\frac{\partial G}{\partial y}\frac{\partial^2F}{\partial y^2} - \frac{\partial F}{\partial y}\frac{\partial^2G}{\partial y^2}\right)y'^3 + \left(\frac{\partial F}{\partial y}\frac{\partial G}{\partial x} - \frac{\partial F}{\partial x}\frac{\partial G}{\partial y}\right)y''\right\}}{\left(\frac{\partial G}{\partial x} + \frac{\partial G}{\partial y}y'\right)^3} \tag{7}$$

From eqn. (7) we see that any function of y only plotted against any function of x and y will have a horizontal inflexion when y/x has a horizontal inflexion ($y = y'' = 0$; $0 < x < \infty$). An inflexion of positive or negative slope ($y'' = 0$; $|y'| > 0$) in y/x will not in general give an inflexion in F/G.

Table 1 gives the relationship between y, y' and y'' and the first and second derivatives of the usual plots,

Table 1. *Relationship between $y(x)/x$ and $F(x,y)/G(x,y)$ for graphical methods used in enzyme kinetics*

First and second derivatives are given in terms of y, y', y'' for the co-ordinate systems indicated.

Graph	First derivative	Second derivative
y/x	y'	y''
$\frac{1}{y}/x$	$\frac{-y'}{y^2}$	$\frac{1}{y^3}(2y'^2 - yy'')$
$\frac{1}{y}/\frac{1}{x}$	$\frac{x^2}{y^2}y'$	$\frac{x^3}{y^3}[2y'(xy'-y) - xyy'']$
$y/\frac{y}{x}$	$\frac{x^2y'}{(xy'-y)}$	$\frac{x^3}{(xy'-y)^3}[2y'(xy'-y) - xyy'']$
$\frac{x}{y}/x$	$\frac{-1}{y^2}(xy'-y)$	$\frac{1}{y^3}[2y'(xy'-y) - xyy'']$
$y/\log x$	xy'	$x(y' + xy'')$
$\log y/\log x$	$\frac{xy'}{y}$	$\frac{-x}{y^2}[y'(xy'-y) - xyy'']$
$\log y/x$	$\frac{y'}{y}$	$\frac{1}{y^2}(yy'' - y'^2)$

and we note that a turning point in y/x implies a turning point in all the other graphs except for $(x/y)/x$. An inflexion of positive or negative slope in y/x will not give an inflexion in any other plot, but a horizontal inflexion in y/x implies a horizontal inflexion in all the derived plots. The consequences of $(xy'-y)=0$ should be noted. $(xy'-y)=0$ is true at the origin and at discrete points for $0<x<\infty$ where $y'\neq 0$ for higher-degree functions. This produces a $y/(y/x)$ plot which will 'double back on itself' at each point where $y'=y/x$, giving an infinite gradient and also a $(x/y)/x$ plot with a turning point.

The graphs of y/x and $(1/y)/(1/x)$ will now receive further attention. Some authors recommend the plot of $y/(y/x)$, which gives a better spread of experimental data and which may show greater curvature making it easier to spot complex mechanisms (Walter, 1974; Frieden, 1967). However, the plot of $(1/y)/(1/x)$ is still the most widely used and has the advantage that the function can always be written explicitly, and asymptotic equations can be easily calculated.

(2) *Replots of asymptotic behaviour for multisubstrate enzymes*

One example of a complex two-substrate enzyme mechanism studied by the authors is that of horseradish peroxidase (EC 1.11.1.7) (Childs & Bardsley, 1975*a*). Some interesting and useful features were found to follow from the rate equation and are discussed here together with suggestions for possible generalizations.

The rate equation for peroxidase was found to be 4:4 in one substrate (A) and 3:3 in the other (B), and may be written:

$$v=\frac{AB(\alpha_{11}+\alpha_{12}B+\alpha_{13}B^2+\alpha_{21}A+\alpha_{22}AB+\alpha_{23}AB^2+\alpha_{31}A^2+\alpha_{32}A^2B+\alpha_{41}A^3)E_0}{(\beta_{00}+\beta_{01}B+\beta_{02}B^2+\beta_{03}B^3+\beta_{10}A+\beta_{11}AB+\beta_{12}AB^2+\beta_{13}AB^3+\beta_{20}A^2+\beta_{21}A^2B+\beta_{22}A^2B^2+\beta_{23}A^2B^3+\beta_{30}A^3+\beta_{31}A^3B+\beta_{32}A^3B^2+\beta_{40}A^4+\beta_{41}A^4B)} \tag{8}$$

As $A\to\infty$,

$$v(A,B)\to\frac{\alpha_{41}B}{\beta_{40}+\beta_{41}B}E_0 \tag{9}$$

and similarly, as $B\to\infty$,

$$v(A,B)\to\frac{(\alpha_{13}A+\alpha_{23}A^2)}{\beta_{03}+\beta_{13}A+\beta_{23}A^2}E_0 \tag{10}$$

Thus, although the rate equation is 3:3 in B, the horizontal asymptote approached as $A\to\infty$ is 1:1 in B (eqn. 9), considerably decreased in degree and easily tested by a double-reciprocal plot. Similarly, the behaviour with respect to A is of decreased degree (eqn. 10) and so of diagnostic significance also.

Analysis of the above rate eqn. (8) shows that the straight-line asymptote for $[1/v(A,B)]/(1/A)$ will have both slope and intercept with the $1/v$ axis dependent on the second substrate B as follows:

$$\text{Slope}=\frac{\beta_{00}+\beta_{01}B+\beta_{02}B^2+\beta_{03}B^3}{\alpha_{11}B+\alpha_{12}B^2+\alpha_{13}B^3}$$

$$\text{Intercept}=\frac{[(\beta_{10}+\beta_{11}B+\beta_{12}B^2+\beta_{13}B^3)(\alpha_{11}B+\alpha_{12}B^2+\alpha_{13}B^3)-(\beta_{00}+\beta_{01}B+\beta_{02}B^2+\beta_{03}B^3)(\alpha_{21}B+\alpha_{22}B^2+\alpha_{23}B^3)]}{(\alpha_{11}B+\alpha_{12}B^2+\alpha_{13}B^3)^2}$$

It is obvious that the intercept replot is of higher degree than the initial rate equation and hence of little or no use in discriminating between possible mechanisms, whereas the slope replot is of some use for those mechanisms which are 3:3 or less in substrate concentration.

Similar analysis applies to slope and intercept replots as functions of A, and is easily extended to other multisubstrate cases, e.g. the random Bi Bi has horizontal asymptotes which are 1:1 in the second substrate.

Now the success of this method for determining the degree of a rate equation with respect to several substrates depends on the fact that, though we might have a rate equation of the form $v=f(A^n,B^m)$, i.e. $n{:}n$ in A and $m{:}m$ in B, nevertheless the rate equation will not include terms of the form A^nB^m.

(3) *Graphical methods of value when $m>n$*

(*a*) *The plot of $x^\lambda y/x$.* A process of some use is one for determining the difference in degree $(m-n)$ for an $n{:}m$ function when $m>n$.

Since $y\to 0$ as $x\to\infty$, then a plot of $x^\lambda y$ against x will still approach zero as long as $\lambda<(m-n)$. If $\lambda=(m-n)$, the graph $x^\lambda y$ against x will approach a finite positive value α_n/β_m, and for $\lambda>(m-n)$ the plot will rise at the end $x\to\infty$ and approach a straight line, parabola etc. depending on the value of $\lambda-(m-n)$.

Hence it is theoretically possible to determine $(m-n)$ by finding that value of λ which gives either a finite positive horizontal asymptote as $x\to\infty$ or gives a straight line of positive slope as $x\to\infty$, whichever is most easily distinguished in the plot of $x^\lambda y$ against x.

Experimentally obtained $y(x)$ data as $x\to\infty$ are then systematically replotted as $x^\lambda y$ against x for $\lambda=0, 1, 2$ etc. until eventually a constant, line, parabola etc. results when $(m-n)$ is determined.

(*b*) *Symmetry of $y/\log x$ functions.* To discover whether there is some value (or values) of $u=\log x$ about which the function $y(\log x)$ is symmetrical, we suppose the function is symmetrical about the line $u=\log x$. Then $y(u+\varepsilon)=y(u-\varepsilon)$ for all ε and detailed analysis shows that this is always true for the 1:2 function. (Detailed proofs of theorems have been deposited as Supplementary Publication SUP 50049 under the headings 'Section A. The graph of y against $\log x$' and 'Section B. Doubly-sigmoid $y/\log x$ curves'.) Higher-degree functions may be symmetrical but not the 2:3 because common factors cancel between numerator and denominator to give a function that is decreased in degree to 1:2. Thus when dead-end substrate inhibition is suspected, a plot of $v/\log A$ should be inspected, and if this is perfectly symmetrical about the maximum at $\frac{1}{2}\log(\beta_0/\beta_2)$ then the rate equation is 1:2. Any deviation from symmetry implies a higher-degree rate equation, since these are usually unsymmetrical.

(*c*) *The slope of* $\log y/\log x$. This graph has a slope that is always less than n (Endrenyi *et al.*, 1971), but it is also of interest in that the limiting gradient as $x \to \infty$ is $(n-m)$.

(*d*) *The case* $m = n+1$. Here the graph of $(x/y)/x$ is asymptotic to a parabola, but the graph of $(1/y)/x$ is asymptotic to the line

$$\left(\frac{\beta_{n+1}}{\alpha_n}\right)x+\frac{(\alpha_n\beta_n-\alpha_{n-1}\beta_{n+1})}{\alpha_n^2}$$

and this line is approached from below for

$$\left(\frac{\alpha_{n-1}}{\alpha_n}\right)^2\beta_{n+1}-\beta_n\left(\frac{\alpha_{n-1}}{\alpha_n}\right)+\beta_{n-1}<\frac{\alpha_{n-2}\beta_{n+1}}{\alpha_n}$$

(4) *Doubly sigmoid* $y(\log x)$ *curves*

Doubly sigmoid log plots (with three inflexions) have been reported (Koshland *et al.*, 1966; Dixon & Tipton, 1973), but the mathematical significance of this feature has never been given.

The 2:2 function

$$y=\frac{\alpha_1x+\alpha_2x^2}{1+\beta_1x+\beta_2x^2}$$

can be written in a form dependent on just two parameters σ and ρ. $\alpha_2=\sigma\alpha_1\beta_1$; $\beta_2=\rho\sigma\beta_1^2$; $t=\beta_1x$; $\omega=\beta_1y/\alpha_1$ giving:

$$\omega=(t+\sigma t^2)/(1+t+\rho\sigma t^2).$$

To be doubly sigmoid requires three real values of $\log t$ for which $d^2\omega/d(\log t)^2$ is zero, i.e. three positive roots t_1, t_2, $t_3>0$ of the quartic

$$1+(4\sigma-1)t-3\sigma(2\rho-1)t^2-\sigma(4\sigma\rho+\rho-1)t^3+\rho\sigma^2(\rho-1)t^4=0$$

From this, a necessary set of conditions for doubly sigmoid ω $(\log t)$ is

$$\begin{pmatrix}\rho<\frac{1}{2}\\ \sigma<\frac{1}{4}\end{pmatrix}\quad\text{or}\quad\begin{pmatrix}\frac{1}{2}<\rho<1\\ \sigma<\left(\frac{1-\rho}{4\rho}\right)\end{pmatrix}$$

The application of Sturm's Theorem to the quartic shows these two conditions to be sufficient. Hence doubly sigmoid curves are possible under these conditions. We note also that either a maximum or a sigmoid inflexion in the $\omega(t)$ function precludes a doubly sigmoid $\omega(\log t)$ function in the 2:2 case.

The Local Behaviour of the Graphs of y/x and $(1/y)/(1/x)$

(1) *When* $x \to 0$

From eqn. (1) with $\beta_0=1$, we have $y'(0)=\alpha_1$, and the function y/x can have a continuously decreasing gradient if $\alpha_2-\alpha_1\beta_1<0$, or a continuously increasing gradient, implying a sigmoid inflexion, if $\alpha_2-\alpha_1\beta_2>0$ for all n, m (Childs & Bardsley, 1975*b*). Also, the function when $\alpha_1=0$ has zero gradient at the origin and must therefore have a sigmoid inflexion.

The graph (see Fig. 2) of $(1/y)/(1/x)$ as $x \to 0$ will become asymptotic to a straight line of slope $1/\alpha_1$ and intercept $\alpha_1\beta_1-\alpha_2$. When a sigmoid inflexion occurs in y/x, this will always be associated with a negative intercept for the asymptote of $(1/y)/(1/x)$ and this feature will be much easier to spot experimentally than a sigmoid inflexion. An exception to this rule is the case when $\alpha_1=0$, for then the $(1/y)/(1/x)$ asymptote will be parabolic rather than linear and this provides a useful diagnostic feature. When $\alpha_1>0$, the function $(1/y)/(1/x)$ can approach the asymptote from above or below depending on whether

$$\left(\frac{\alpha_2}{\alpha_1}\right)^2-\frac{\beta_1}{\beta_0}\left(\frac{\alpha_2}{\alpha_1}\right)+\frac{\beta_2}{\beta_0}>\frac{\alpha_3}{\alpha_1}\quad\text{or}\quad<\frac{\alpha_3}{\alpha_1}$$

respectively.

(2) *When* $x \to \infty$

Case (*a*): $m>n$, $\lim_{x\to\infty} y=0$, and so y/x approaches zero, but $(1/y)/(1/x)$ is undefined at the origin (dead-end substrate inhibition).

Case (*b*): $m=n$, $\lim_{x\to\infty} y=\alpha_n/\beta_n$, but this asymptote will be approached from above if $\alpha_n\beta_{n-1}<\alpha_{n-1}\beta_n$, implying at least one maximum in the y/x curve (partial substrate inhibition), whereas if $\alpha_n\beta_{n-1}>\alpha_{n-1}\beta_n$ then the asymptote is approached from below and this is an ambiguous case as regards maxima.

Note that $\alpha_n\beta_{n-1}<\alpha_{n-1}\beta_n$ implies

$$\frac{d\left(\frac{1}{y}\right)}{d\left(\frac{1}{x}\right)}<0,$$

$\alpha_n\beta_{n-1}=\alpha_{n-1}\beta_n$ implies

$$\frac{d\left(\frac{1}{y}\right)}{d\left(\frac{1}{x}\right)}=0$$

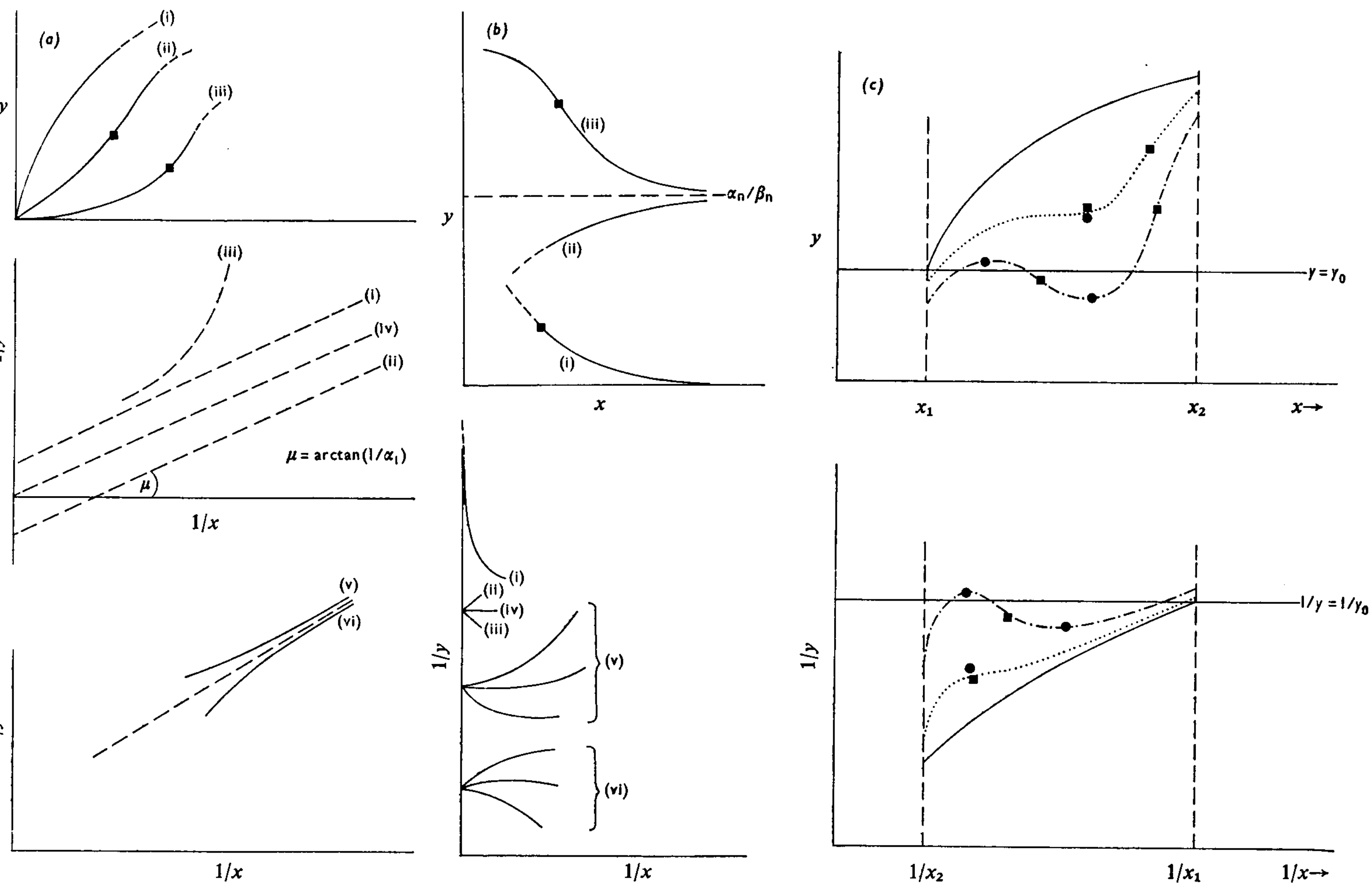

Fig. 2. *Local features of graphs of rational polynomials*

The local behaviour of polynomial functions of the type

$$y = \sum_{1}^{n} \alpha_i x^i \Big/ \left(1 + \sum_{1}^{m} \beta_i x^i\right)$$

(*a*) The behaviour as $x \to 0$. (i) $\alpha_2 < \alpha_1 \beta_1$; the curve gives a non-sigmoid y/x plot and the $(1/y)/(1/x)$ asymptote has a positive intercept. (ii) $\alpha_2 > \alpha_1 \beta_1$; y/x has a sigmoid inflexion and the $(1/y)/(1/x)$ asymptote has a negative intercept. (iii) $\alpha_1 = 0$; y/x has $y' = 0$ at $x = 0$ and must be sigmoid, but $(1/y)/(1/x)$ is now parabolic (2:2) or

approaches a parabolic asymptote ($n>2$). For higher-degree functions $\alpha_1 = \alpha_2 = 0$ would give a cubic $(1/y)/(1/x)$ plot, and so on. (iv) $\alpha_2 = \alpha_1\beta_1$; y/x has $y''(0) = 0$ and the $(1/y)/(1/x)$ asymptote passes through the origin. (v) $(\alpha_2/\alpha_1)^2 - \beta_1(\alpha_2/\alpha_1) + \beta_2 > \alpha_3/\alpha_1$; the $(1/y)/(1/x)$ asymptote is approached from above. (vi) $(\alpha_2/\alpha_1)^2 - \beta_1(\alpha_2/\alpha_1) + \beta_2 < \alpha_3/\alpha_1$; the $(1/y)/(1/x)$ asymptote is approached from below. $(\alpha_2/\alpha_1)^2 - \beta_1(\alpha_2/\alpha_1) + (\beta_2 - \alpha_3/\alpha_1) = 0$ is ambiguous and the next $(1/x)$ term must be considered.
(*b*) The behaviour as $x \to \infty$. (i) $m>n$,

$$\lim_{x\to\infty} y = 0, \qquad \lim_{x\to\infty} 1/y = \infty.$$

(ii) $m = n$, $\alpha_n\beta_{n-1} > \alpha_{n-1}\beta_n$. The horizontal asymptote α_n/β_n in y/x is approached from below and $(1/y)/(1/x)$ has a positive gradient at the origin. (iii) $m = n$, $\alpha_n\beta_{n-1} < \alpha_{n-1}\beta_n$. The horizontal y/x asymptote is approached from above and the curve must have at least one maximum. $(1/y)/(1/x)$ has negative gradient at the origin and must have at least one minimum. (iv) $m = n$, $\alpha_n\beta_{n-1} = \alpha_{n-1}\beta_n$. y/x is ambiguous and the next lower x term must be considered in order to discover the mode of approach to the horizontal asymptote. $(1/y)/(1/x)$ has zero gradient at the origin. (v) The curve can be concave up or (vi) concave down, depending on

$$\left(\frac{\alpha_{n-1}}{\alpha_n}\right)^2 \beta_n - \beta_{n-1}\left(\frac{\alpha_{n-1}}{\alpha_n}\right) + \beta_{n-2} > \frac{\alpha_{n-2}\beta_n}{\alpha_n} \quad \text{as in (v) or} \quad < \frac{\alpha_{n-2}\beta_n}{\alpha_n} \quad \text{as in (vi)}$$

(*c*) The behaviour for $0<x<\infty$. A smooth curve in y/x becomes a shallow curve or line in $(1/y)/(1/x)$. A horizontal inflexion in y/x maps into a horizontal inflexion in $(1/y)/(1/x)$ and maxima and minima in y/x become minima and maxima respectively. A horizontal line $y = y_0$ intersects the original $y(x)$ curve at most m times and so the example illustrated is at least 3:3. A similar rule applies to $(1/y)/(1/x)$. Symbols are defined in the legend to Fig. 1.

and $\alpha_n\beta_{n-1} > \alpha_{n-1}\beta_n$ implies a positive gradient at the origin of $(1/y)/(1/x)$. The condition for the curve to be concave up or down at the $1/y$ intercept is found from

$$\left(\frac{\mathrm{d}\left(\frac{1}{y}\right)}{\mathrm{d}\left(\frac{1}{x}\right)}\right)_{\frac{1}{x}=0} = (\alpha_n\beta_{n-1} - \alpha_{n-1}\beta_n)/\alpha_n^2$$

and

$$\left(\frac{\mathrm{d}^2\left(\frac{1}{y}\right)}{\mathrm{d}\left(\frac{1}{x}\right)^2}\right)_{\frac{1}{x}=0} = 2\left[\left(\frac{\alpha_{n-1}}{\alpha_n}\right)^2 \beta_n - \beta_{n-1}\left(\frac{\alpha_{n-1}}{\alpha_n}\right) + \beta_{n-2} - \frac{\alpha_{n-2}\beta_n}{\alpha_n}\right] \Big/ \alpha_n$$

(3) *Intermediate values of x*

A maximum in y/x results in a minimum in $(1/y)/(1/x)$ and vice versa. Also, the number of maxima in y/x must exceed by one the number of minima or be equal to it, since it is impossible to have more minima than maxima. The converse holds for $(1/y)/(1/x)$. A horizontal inflexion in y/x will produce a horizontal inflexion in $(1/y)/(1/x)$. Setting $y = y_0$ in eqn. (1) generates a straightforward mth degree polynomial in standard form. Hence a horizontal line in y/x can intersect the curve a maximum of m times corresponding to m roots. A similar horizontal intersection rule applies to $(1/y)/(1/x)$.

Some Representative *n*:*m* Polynomial Functions

The 1:1 and 1:2 functions are trivial and have been analysed many times. Credit for first applying the principles of calculus to the analysis of enzyme-kinetic graphs must go to Botts (1958), who gave an analysis of the 2:2 function by application of Descartes' rule of signs to the numerator of the first and second derivatives, the denominator being finite and positive in the first quadrant. Her findings have been used several times by other workers (Walter, 1974; Endrenyi *et al.*, 1971; Ainsworth, 1968; Ferdinand, 1966; Henderson, 1968; Pettersson, 1969; Ricard *et al.*, 1974) and we have shown how this type of analysis can be extended to some aspects of higher-degree functions where the derivatives rapidly become of high degree (Childs & Bardsley, 1975*b*). The behaviour of asymptotes from non-linear double-reciprocal plots has been treated for the 2:2 case (Ainslie *et al.*, 1972; Pettersson, 1970; Neal, 1972; Moraal, 1972) and we have extended this treatment to 4:4 functions (Childs & Bardsley, 1975*a*). However, for completeness, it seems appropriate now to gather together all the information that is available about functions likely to be encountered experimentally. The analysis is extended as far as 4:4 functions, which should cover most cases, but subsequently general formulae will be given to facilitate analysis of higher-degree functions should the need arise.

Definitions

$$\phi_{ir} = \alpha_i \beta_r - \alpha_r \beta_i$$
$$\phi = \alpha_2 - \alpha_1 \beta_1$$

We define

$$\sum_0^n \alpha_i x^i \Big/ \sum_0^m \beta_i x^i$$

as an $n{:}m$ function. Other authors define functions on the double-reciprocal form of the rate equation (Plowman, 1972). This seems confusing, since initial-rate data are obtained in y/x form, rate equations are calculated in y/x form and many workers do not use double-reciprocal plots but prefer the more discriminatory $y/(y/x)$ plot.

The 1:1 *function* (*Fig.* 3)

$$y = \frac{\alpha_1 x}{1+\beta_1 x}$$
$$= \frac{\alpha_1}{\beta_1} - \frac{\alpha_1}{\beta_1(1+\beta_1 x)}$$
$$y' = \alpha_1(1+\beta_1 x)^{-2}$$
$$y'' = -2\alpha_1\beta_1(1+\beta_1 x)^{-3}$$
$$\frac{1}{y} = \frac{1}{\alpha_1}\left(\frac{1}{x}\right) + \frac{\beta_1}{\alpha_1}$$
$$\frac{d^2\left(\frac{1}{y}\right)}{d\left(\frac{1}{x}\right)^2} = 0$$

The 2:2 *function*

$$y = \frac{\alpha_1 x + \alpha_2 x^2}{1+\beta_1 x + \beta_2 x^2}$$
$$= \frac{\alpha_2}{\beta_2} + \left[\frac{\phi_{12} x - \alpha_2}{\beta_2(1+\beta_1 x+\beta_2 x^2)}\right]$$
$$y' = (\alpha_1 + 2\alpha_2 x - \phi_{12} x^2)(1+\beta_1 x + \beta_2 x^2)^{-2}$$
$$y'' = 2(\phi - 3\alpha_1\beta_2 x - 3\alpha_2\beta_2 x^2 + \beta_2\phi_{12} x^3)(1+\beta_1 x + \beta_2 x^2)^{-3}$$
$$\frac{1}{y} = \frac{\left(\frac{1}{x}\right)^2 + \beta_1\left(\frac{1}{x}\right) + \beta_2}{\alpha_1\left(\frac{1}{x}\right) + \alpha_2}$$
$$= \frac{1}{\alpha_1}\left(\frac{1}{x}\right) - \frac{\phi}{\alpha_1^2} + \left\{\frac{\alpha_1^2\beta_2 + \alpha_2\phi}{\alpha_1^2\left[\alpha_1\left(\frac{1}{x}\right)+\alpha_2\right]}\right\}$$
$$\frac{d^2\left(\frac{1}{y}\right)}{d\left(\frac{1}{x}\right)^2} = 2(\alpha_2^2 + \phi_{12})\left[\alpha_1\left(\frac{1}{x}\right) + \alpha_2\right]^{-3}$$

When

$$\beta_2\left(\frac{\alpha_1}{\alpha_2}\right)^2 - \beta_1\left(\frac{\alpha_1}{\alpha_2}\right) + 1 = 0$$

then cancellation produces the 1:1 function

$$y = \frac{\alpha_1\alpha_2 x}{\alpha_2 + \alpha_1\beta_2 x}$$

$\alpha_2 - \alpha_1\beta_1 > 0$ implies a sigmoid inflexion in y/x and negative intercept in $(1/x)/(1/y)$, but it also leads to $(xy'-y) = 0$ at one discrete point in the first quadrant other than the origin. Consequently, $y/(y/x)$ will have a point with infinite gradient and $(x/y)/x$ will have just one turning point.

The features of this function have been fully discussed (Childs & Bardsley, 1975*b*) and the possible graph shapes are given in Fig. 4.

The 3:3 *function*

$$y = \frac{\alpha_1 x + \alpha_2 x^2 + \alpha_3 x^3}{1+\beta_1 x + \beta_2 x^2 + \beta_3 x^3}$$
$$= \frac{\alpha_3}{\beta_3} + \left[\frac{\phi_{23} x^2 + \phi_{13} x - \alpha_3}{\beta_3(1+\beta_1 x + \beta_2 x^2 + \beta_3 x^3)}\right]$$
$$y' = [\alpha_1 + 2\alpha_2 x + (3\alpha_3 - \phi_{12})x^2 - 2\phi_{13} x^3 - \phi_{23} x^4](1+\beta_1 x + \beta_2 x^2 + \beta_3 x^3)^{-2}$$

Although there are three possible sign changes, further analysis shows that there can be only two turning

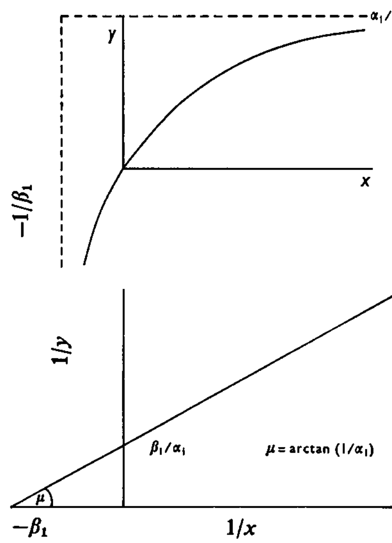

Fig. 3. *The* 1:1 *function*

$$y = \frac{(\alpha_1 x)}{(1+\beta_1 x)}$$

Dashed line indicates asymptotes.

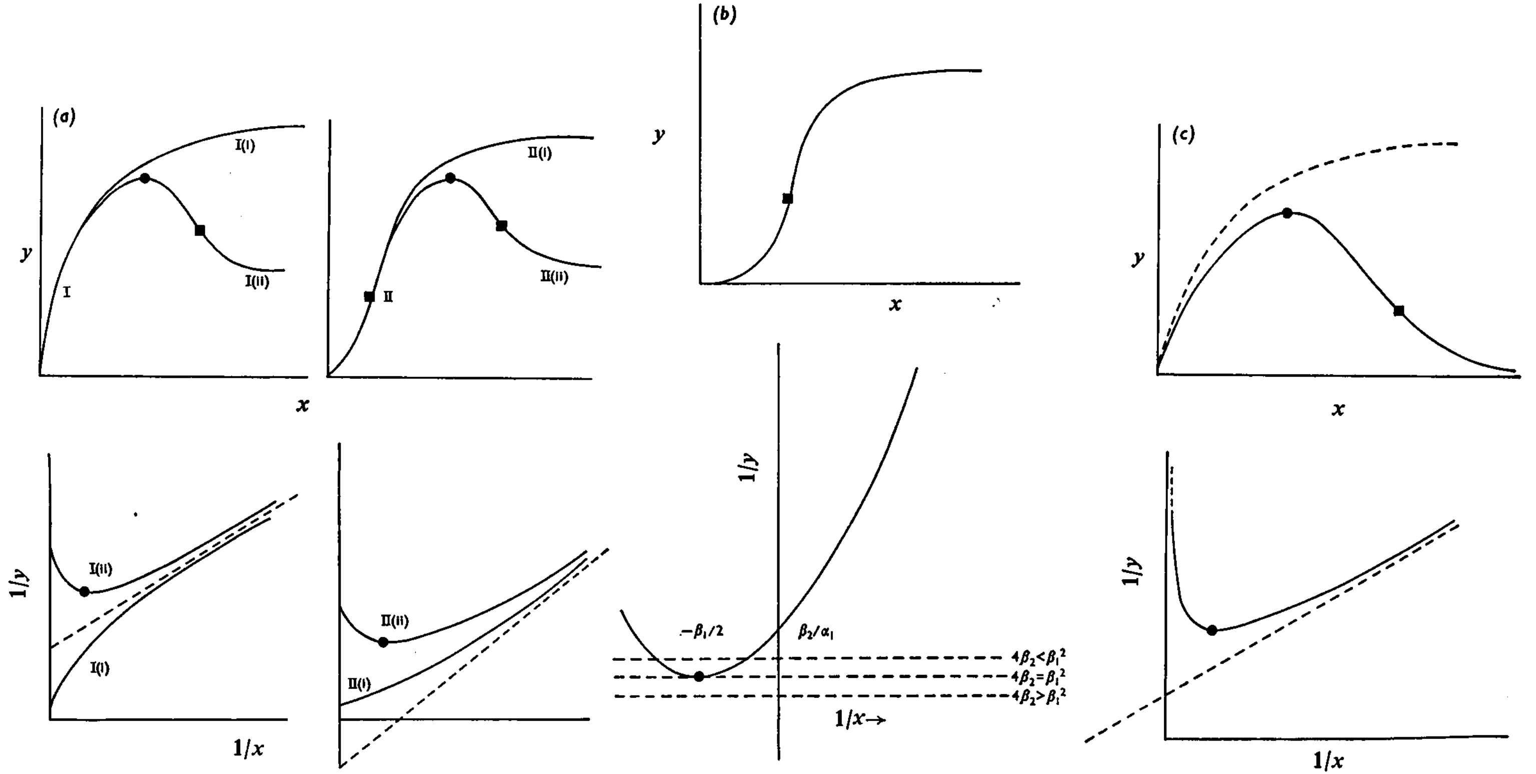

Fig. 4. *The* 2:2 *function*

$$y = \frac{(\alpha_1 x + \alpha_2 x^2)}{(1+\beta_1 x + \beta_2 x^2)}$$

(*a*) I, $\alpha_2 < \alpha_1\beta_1$: I (i) $\alpha_1\beta_2 < \alpha_2\beta_1$; (ii) $\alpha_1\beta_2 > \alpha_2\beta_1$. II, $\alpha_2 > \alpha_1\beta_1$: II (i) $\alpha_1\beta_2 < \alpha_2\beta_1$; (ii) $\alpha_1\beta_2 > \alpha_2\beta_1$. Note that when $\alpha_1\beta_2 = \alpha_2\beta_1$, y/x has no maximum and $(1/y)/(1/x)$ has zero gradient at the origin. Also, when $\alpha_2 = \alpha_1\beta_1$, the y/x behaviour is as for case I but the $(1/y)/(1/x)$ asymptote passes through the origin. (*b*) $\alpha_2, \beta_1, \beta_2 > 0$ but $\alpha_1 = 0$. y/x must be sigmoid ($y' = 0$ at the origin) and can have no maxima. $(1/y)/(1/x)$ is a parabola as indicated. (*c*) $\alpha_1, \beta_1, \beta_2 > 0$, but $\alpha_2 = 0$. The dashed line is the same function but with $\beta_2 = 0$ also. This is the equation of dead-end substrate inhibition (1:2 function), which looks superficially like the 2:2 function with a maximum [partial substrate inhibition, see case I (ii) above]. They can be distinguished since $y/\log x$ is symmetrical for the 1:2 case but asymmetrical for the 2:2 case. Also, $(1/y)/x$ has no inflexion for the 1:2 case but can have one inflexion for the 2:2 case. Further graphical procedures for finding the difference in degree between numerator and denominator in the general *n*:*m* case are described in the text. Symbols are defined in the legend to Fig. 1.

points for $x>0$. y'' is a VI-degree polynomial with a maximum of six positive roots.

$$\frac{1}{y}=\frac{\left(\frac{1}{x}\right)^3+\beta_1\left(\frac{1}{x}\right)^2+\beta_2\left(\frac{1}{x}\right)+\beta_3}{\alpha_1\left(\frac{1}{x}\right)^2+\alpha_2\left(\frac{1}{x}\right)+\alpha_3}$$

$$=\frac{1}{\alpha_1}\left(\frac{1}{x}\right)-\frac{\phi}{\alpha_1^2}+\left[\frac{(\alpha_1\phi_{12}+\alpha_2^2-\alpha_1\alpha_3)\left(\frac{1}{x}\right)+\alpha_1^2\beta_3+\alpha_3\phi}{\alpha_1^2\alpha_1\left(\frac{1}{x}\right)^2+\alpha_2\left(\frac{1}{x}\right)+\alpha_3}\right]$$

$[d^2(1/y)]/[d(1/x)^2]$ is of degree III and can have a maximum of three positive roots.

The graph can have any of the shapes given by the 2:2 function but, in addition, y/x can have one maximum and a minimum or any of the shapes intermediate between these extremes. $(1/y)/(1/x)$ can only have three inflexions.

Setting $\alpha_3=0$ generates a 2:3 function which is the lowest-degree rate equation which can have multiple inflexions in y/x or any inflexion at all in $(1/y)/(1/x)$. The extra shapes possible are illustrated in Fig. 5.

The 4:4 *function*

$$y=\frac{\alpha_1x+\alpha_2x^2+\alpha_3x^3+\alpha_4x^4}{1+\beta_1x+\beta_2x^2+\beta_3x^3+\beta_4x^4}$$

$$=\frac{\alpha_4}{\beta_4}+\left[\frac{\phi_{34}x^3+\phi_{24}x^2+\phi_{14}x-\alpha_4}{\beta_4(1+\beta_1x+\beta_2x^2+\beta_3x^3+\beta_4x^4)}\right]$$

$$y'=[\alpha_1+2\alpha_2x+(3\alpha_3-\phi_{12})x^2+(4\alpha_4-2\phi_{13})x^3-(3\phi_{14}+\phi_{23})x^4-2\phi_{24}x^5)-\phi_{34}x^6](1+\beta_1x+\beta_2x^2+\beta_3x^3+\beta_4x^4)^{-2}$$

y'' is IX degree and can have a maximum of nine positive roots.

$$\frac{1}{y}=\frac{\left(\frac{1}{x}\right)^4+\beta_1\left(\frac{1}{x}\right)^3+\beta_2\left(\frac{1}{x}\right)^2+\beta_3\left(\frac{1}{x}\right)+\beta_4}{\alpha_1\left(\frac{1}{x}\right)^3+\alpha_2\left(\frac{1}{x}\right)^2+\alpha_3\left(\frac{1}{x}\right)+\alpha_4}$$

$$=\frac{1}{\alpha_1}\left(\frac{1}{x}\right)-\frac{\phi}{\alpha_1^2}+\frac{(\alpha_1\phi_{12}+\alpha_2^2-\alpha_1\alpha_3)\left(\frac{1}{x}\right)^2+(\alpha_1\phi_{13}+\alpha_2\alpha_3-\alpha_1\alpha_4)\left(\frac{1}{x}\right)+\alpha_1^2\beta_4+\alpha_4\phi}{\alpha_1^2\left[\alpha_1\left(\frac{1}{x}\right)^3+\alpha_2\left(\frac{1}{x}\right)^2+\alpha_3\left(\frac{1}{x}\right)+\alpha_4\right]}$$

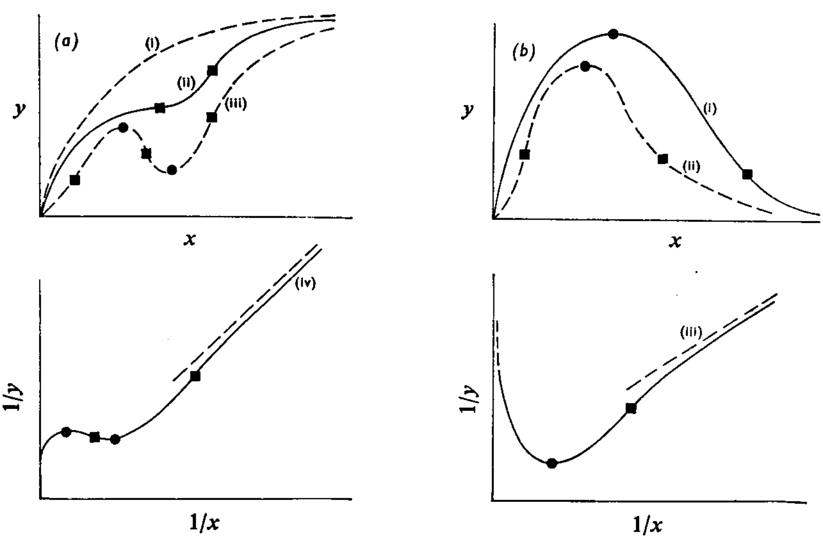

Fig. 5. *The* 3:3 *function*

$$y=\frac{\alpha_1x+\alpha_2x^2+\alpha_3x^3}{1+\beta_1x+\beta_2x^2+\beta_3x^3}$$

(*a*) $\alpha_1, \alpha_2, \alpha_3, \beta_1, \beta_2, \beta_3>0$. (i) The simplest case indistinguishable from the 2:2 case in y/x form. (ii) Stair-step curve frequently encountered in enzyme studies. (iii) All the shapes in Fig. 4(*a*) for the 2:2 function are possible, but the most complex curve possible has two turning points and a maximum of six inflexions (three illustrated). (iv) The maximum complexity possible in $(1/y)/(1/x)$ form has three inflexions (two illustrated) and two turning points. (*b*) $\alpha_1, \alpha_2, \beta_1, \beta_2, \beta_3>0$, but $\alpha_3=0$. (i) The simplest shape is indistinguishable in y/x form from the 1:2 function. (ii) A maximum of two inflexions is possible but a horizontal inflexion is not possible. (iii) The 2:3 function is common in enzyme kinetics and is important as the lowest-degree function having an inflected double-reciprocal curve. Symbols are defined in the legend to Fig. 1.

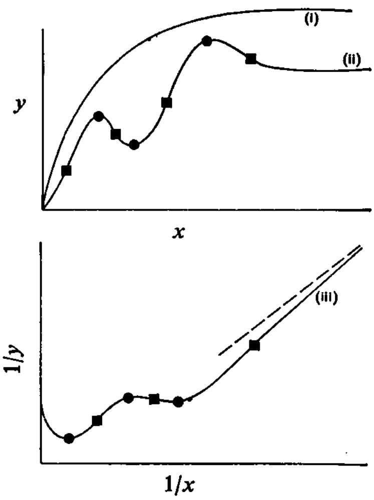

Fig. 6. *The* 4:4 *function*

$$y=\frac{\alpha_1 x+\alpha_2 x^2+\alpha_3 x^3+\alpha_4 x^4}{1+\beta_1 x+\beta_2 x^2+\beta_3 x^3+\beta_4 x^4}$$

All the shapes shown in Fig. 5 for the 3:3 function are possible and, in addition, the extremely complex curve (ii) with a sigmoid inflexion and three further inflexions together with two maxima and one minimum is an example of the shapes possible. (iii) illustrates the degree of complexity possible in double-reciprocal form, e.g. with three turning points and three inflexions. Symbols are defined in the legend to Fig. 1.

$d^2(1/y)/d(1/x)^2$ is of degree VI with a maximum of six possible positive roots. All the shapes possible for 3:3 are also possible, but some of the additional complexities are indicated in Fig. 6.

It could be argued that such complex curves as Figs. 5 and 6 are not often encountered in enzyme studies. However, the fact that some examples are known (Engel & Dalziel, 1969; Tanner, 1974; Lee *et al.*, 1973; Ziegler, 1974) suggests that many more await discovery. In fact, since realistic allosteric steady-state equations are of extremely high degree, we would agree with Teipel & Koshland (1969) that experimental workers would have previously dismissed such bumpy curves assuming them to be due to experimental error. Also Atkinson (1966) noted that, until 1956, all enzymes had been found to follow 1:1 functions, whereas, subsequently, sigmoid curves had become common and suggested that reinvestigation would reveal many more deviations from hyperbolas than had been suspected. Extension of this argument to even more complex curves is obvious. Hopefully, future studies will be enriched by paying particular attention to inflexions and turning points and thus gaining greater insight into the catalytic mechanism rather than unintentionally suppressing valuable experimental evidence.

Allosteric Fuctions

The Adair–Koshland–Némethy–Filmer (AKNF) (Koshland *et al.*, 1966) and Monod–Wyman–Changeux (MWC) (Monod *et al.*, 1965) allosteric models are actually extreme treatments of a more general scheme which takes into account all possible combinations of site occupancy, subunit symmetry and co-operativity. These particular models which, being saturation functions, must have $\bar{y}(0)=0$ and $\bar{y}(\infty)=1$ with no intervening turning points. If it is presumed that the initial velocity is merely proportional to the number of occupied sites, then this forces considerable restrictions on the velocity profile possible. Now realistic steady-state models can, especially with random mechanisms, give v/A curves with maxima (partial substrate inhibition) or with $\lim_{A\to\infty} v = 0$ (dead-end substrate inhibition) and so these commonly encountered features of experimental enzyme kinetics are not possible with the MWC or AKNF models. This criticism applies in addition to the usual one about the assumption that ligand-induced transitions have to be much more rapid than the catalytic event in order to use a ligand-binding model instead of a steady-state one.

Some properties of the Monod–Wyman–Changeux model for allosterism

Here

$$\bar{y}(\alpha)=\frac{LC\alpha(1+C\alpha)^{n-1}+\alpha(1+\alpha)^{n-1}}{L(1+C\alpha)^n+(1+\alpha)^n}$$

with $n>1$, $L,C>0$, $\alpha=F/K_R$, $T_0/R_0=L$ and $C=K_R/K_T$.

(*a*) $\bar{y}(\alpha)$ can have no final maximum, since this would require

$$(LC^n+1)(LC^{n-1}+1)<0$$

(*b*) Such curves $\bar{y}(\alpha)$ are sigmoid if

$$n>\left(\frac{L+1}{L}\right)\cdot\frac{(LC^2+1)}{(C-1)^2}$$

or $\quad L(n-L-1)\cdot C^2-2nLC+(nL-L-1)>0$

[See Fig. 7(*a*).]

(*c*) In the non-sigmoid case, the linear asymptote of the double-reciprocal form will intersect the $1/\bar{y}$ axis at some positive value and hence there is the possibility of so-called positive or negative co-operativity. The minimum 'valid' experimental criterion for negative co-operativity is that

$$\left(\frac{d\left(\frac{1}{\bar{y}}\right)}{d\left(\frac{1}{\alpha}\right)}\right)_{\frac{1}{\alpha}=0}>\left(\frac{d\left(\frac{1}{\bar{y}}\right)}{d\left(\frac{1}{\alpha}\right)}\right)_{\frac{1}{\alpha}\to\infty}$$

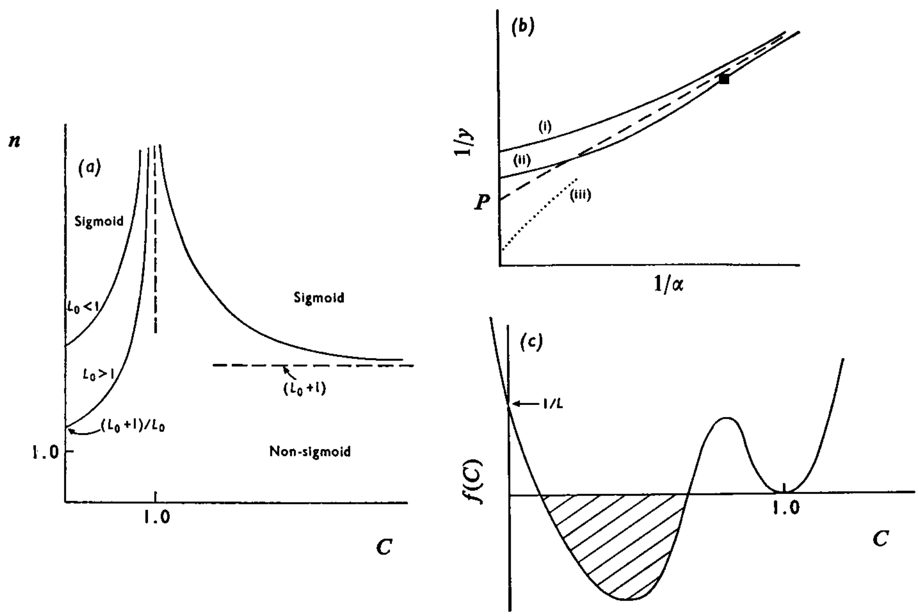

Fig. 7. *Graphical behaviour of the MWC saturation function* $\bar{y}(\alpha)$

(*a*) The graph of $\bar{y}(\alpha)$ is sigmoid in regions for n, C, L lying above the curve, i.e. for

$$n > \left(\frac{L+1}{L}\right)\frac{(LC^2+1)}{(C-1)^2}$$

$n(C)$ is plotted for $L = L_0$. (*b*) The graph of $(1/\bar{y})/(1/\alpha)$ with a positive intercept at P, i.e. for a non-sigmoid $\bar{y}(\alpha)$ curve. The slope at the origin is always concave up for $1/\alpha = 0$ and in cases (i) and (ii) less than the limiting gradient as $1/\alpha \to \infty$, but the curve can be concave upward (i), i.e. 'positive co-operativity', despite $\bar{y}(\alpha)$ being non-sigmoid, or (ii) have one or more inflexions. There can be no maxima or minima and a concave-downward curve (iii), i.e. 'negative co-operativity', is not possible. (See the Discussion section in the text.) (*c*) The asymptotic slope in $(1/\bar{y})/(1/\alpha)$ is reached from below for C giving negative $f(C)$ as indicated by the shaded section. Approach from below will occur for $n > 2$ when $n(n-1)L^2 f(C) < 0$ where

$$f(C) = (C-1)^2\left[C^2 + \frac{(2-n)(L+1)C}{nL} + \frac{1}{L}\right]$$

Only one of several possible shapes of this quartic are shown and it is also possible to have a negative region for $C > 1$ or no negative region at all. Symbols are defined in the legend to Fig. 1.

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i.e. a 'concave downward' double-reciprocal plot with

$$\left(\frac{LC^{n-1}+1}{LC^n+1}\right) - \left(\frac{L+1}{LC+1}\right) = \frac{-L(C-1)(C^{n-1}-1)}{(LC+1)(LC^n+1)} > 0$$

for negative co-operativity.

Thus there can never be 'negative co-operativity' in the Monod–Wyman–Changeux model for any combination of n, C and L. Also $(1/\bar{y})/(1/\alpha)$ is seen to be always concave up for $1/\alpha = 0$, since

$$\left(\frac{d^2\left(\frac{1}{\bar{y}}\right)}{d\left(\frac{1}{\alpha}\right)^2}\right)_{\frac{1}{\alpha}=0} = 2(n-1)LC^{n-2}(C-1)^2(1+LC^n)^{-2}$$

The condition for approach to the asymptote from below requires

$$\left[\frac{(n-1)(LC^2+1)}{LC+1}\right]^2 < \frac{(n-1)(n-2)(LC^3+1)}{2(LC+1)} + \frac{n(n-1)(LC^2+1)}{2(L+1)}$$

which leads to:

$$L(n-1)\{(nLC^4 + [(2-3n)L+2-n]C^3 + (3n-4)(L+1)C^2 + [(2-n)L+2-3n]C+n)\} < 0$$

We conclude that for $n = 2$, a 2:2 MWC function cannot approach its double-reciprocal asymptote from below, otherwise for $n > 2$ approach from below will occur, as indicated in Figs. 7(*b*) and 7(*c*), giving at least one inflexion.

The preceding analysis shows that the MWC model can give a family of sigmoid and a family of non-sigmoid $\bar{y}(\alpha)$ curves. In double-reciprocal form, all the curves are concave up at the origin and have a gradient at the origin which is less than the limiting slope as $1/\alpha \to \infty$, but some will approach this asymptote from above and some from below. $(1/\bar{y})/(1/\alpha)$ curves with inflexions are thus possible and the difference between the sigmoid and non-sigmoid curves in $(1/\bar{y})/(1/\alpha)$ form is that a sigmoid $\bar{y}(\alpha)$ curve has an asymptotic line in double-reciprocal form which has a negative $1/\bar{y}$ intercept and vice versa. Also, a straightforward concave-downward double-reciprocal form is not possible.

It should now be clear that the reason for the widespread confusion over the operational definition (Conway & Koshland, 1968) as opposed to the graphical tests (Hammes & Wu, 1974) for positive or negative co-operativity is due to a complete misunderstanding of the relationship between the y/x and $(1/y)/(1/x)$ graphs. In fact, there is a mistaken belief that there are basically two types of curves in addition to the 1:1 type, i.e. a family of curves moved to the right of a rectangular hyperbola (positive co-operativity with y/x sigmoid and $(1/y)/(1/x)$ concave upward) and a family of curves moved to the left of a rectangular hyperbola (negative co-operativity with y/x non-sigmoid and $(1/y)/(1/x)$ concave downward). We have demonstrated that realistic allosteric mechanisms have an enormous range of possible curve shapes owing to the high degree of the rate equations.

Finally, we would like to comment on the degree of sigmoidicity of any y/x curve. Laidler & Bunting (1973), following Monod *et al.* (1965), state that increase in the allosteric constant leads to more marked co-operativity and that $C = 1$ or $L \to 0$ reduces $\bar{y}(\alpha)$ to a hyperbola. Also, Atkinson (1966) states that the MWC model predicts sigmoid relationships only when C is small. Actually, we have now replaced this qualitative discussion by stating the quantitative relationships between n, C and L that must be satisfied for sigmoidicity to appear. This requires $C<1$ as $L \to \infty$ and $C<1$ and appropriate n as $L \to 0$ for sigmoidicity. Also, we contest the statement that co-operativity is more marked when L is large (Monod *et al.*, 1965). Actually increasing L merely pulls the curve over to the right without displacement of the horizontal asymptote and although this may have some mechanistic significance at the molecular level, it has nothing to do with sigmoidicity. Sigmoidicity is not related to the slope at the inflexion (Koshland *et al.*, 1966), nor is it related to the curvature $y''(1+y'^2)^{-\frac{3}{2}} = 0$ at the inflexion and, in fact, a curve is either sigmoid or not and, where a sigmoid inflexion is a feature of a curve, there is so far no acceptable mathematical definition of sigmoidicity.

Probably the best quantitative measure of 'sigmoidicity' is the magnitude of the curvature of y/x for $x = 0$ [i.e. $K(0)$], which gives an estimate of how concave upwards the plot is at the origin. This is calculated from $y'(0) = \alpha_1/\beta_0$ and $y''(0) = 2(\alpha_2\beta_0 - \alpha_1\beta_1)/\beta_0^2$ to be $K(0) = 2(\alpha_2\beta_0 - \alpha_1\beta_1)\beta_0/(\alpha_1^2+\beta_0^2)^{\frac{3}{2}}$. Alternatively, the $1/y$ intercept when $1/x = 0$ could be used, and this is

$$\left(\frac{1}{y}\right)_{\frac{1}{x}=0} = (\alpha_1\beta_1 - \alpha_2\beta_0)/\alpha_1^2$$

General Formulae

(1) *The functions*

A number of general results concerning rational polynomials are of use in the work discussed in this paper and are collected here and used to obtain the results in Table 2.

The general $n{:}n$ polynomial is:

$$y = \sum_1^n \alpha_i x^i \Big/ \sum_0^n \beta_i x^i$$

which may be written

$$y = \frac{\alpha_n}{\beta_n} + \frac{1}{\beta_n}\sum_{i=0}^{n-1} \phi_{in} x^i \Big/ \sum_{i=0}^{n} \beta_i x^i$$

where ϕ_{in} is as previously defined.

The double-reciprocal form of y/x is

$$\frac{1}{y} = \frac{\beta_0}{\alpha_1}\left(\frac{1}{x}\right) + \frac{1}{\alpha_1^2}(\alpha_1\beta_1 - \alpha_2\beta_0) + \frac{\sum_{r=2}^{n}[\beta_r\alpha_1^2 - \beta_0\alpha_1\alpha_{r+1} + \alpha_r(\alpha_2\beta_0 - \alpha_1\beta_1)]\left(\frac{1}{x}\right)^{n-r}}{\alpha_1^2\sum_1^n \alpha_i\left(\frac{1}{x}\right)^{n-i}}$$

which if we let $1/x \to \infty$ becomes:

$$\frac{1}{y} \approx \frac{\beta_0}{\alpha_1}\left(\frac{1}{x}\right) + \frac{1}{\alpha_1^2}(\alpha_1\beta_1 - \alpha_2\beta_0) + \frac{1}{\alpha_1^3}(\alpha_1^2\beta_2 - \alpha_1\alpha_3\beta_0 + \alpha_2^2\beta_0 - \alpha_1\alpha_2\beta_1)x$$

From this approximation we see that the curve $(1/y)/(1/x)$ will approach the asymptotic line

$$\frac{\beta_0}{\alpha_1}\left(\frac{1}{x}\right) + \frac{1}{\alpha_1^2}(\alpha_1\beta_1 - \beta_0\alpha_2)$$

from above or below depending on

$$\beta_2\alpha_1^2 + \beta_0\alpha_2^2 - \beta_0\alpha_1\alpha_3 - \beta_1\alpha_1\alpha_2 > 0$$

or <0 respectively,

i.e. as

$$\left(\frac{\alpha_2}{\alpha_1}\right)^2 - \frac{\beta_1}{\beta_0}\left(\frac{\alpha_2}{\alpha_1}\right) + \frac{\beta_2}{\beta_0} \gtrless \frac{\alpha_3}{\alpha_1}$$

Table 2. *Degree of the numerator for y', y'' and $[d^2(1/y)]/[d(1/x)^2]$ and the maximum possible number of positive roots*

Note that Descartes' rule of signs gives a necessary, but not sufficient condition for positive roots of polynomials and further analysis shows that in most cases high degree functions will have fewer inflexions and turning points than those allowed by the sign rule, e.g. a 3:3 function can have only two turning points in y/x despite the 3 allowed, and a 2:3 function can have only two inflexions.

	1:1	1:2	2:2	2:3	3:3	3:4	4:4	4:5	5:5	n:m
y' or $\dfrac{d\left(\frac{1}{y}\right)}{d\left(\frac{1}{x}\right)}$ degree ...	0	II	II	IV	IV	VI	VI	VIII	VIII	$2m-2$
Maximum positive roots ...	0	1	1	1	3	3	5	5	7	$2n-3$ $m, n\geqslant 2$
y'' degree ...	0	III	III	VI	VI	IX	IX	XII	XII	$3m-3$
Maximum positive roots ...	0	1	2	4	6	8	9	11	12	$2m+n-3$ $n, m\geqslant 3$
$\dfrac{d^2\left(\frac{1}{y}\right)}{d\left(\frac{1}{x}\right)^2}$ ...	0	III	0	III	III	VI	VI	IX	IX	$3m-6$
Maximum positive roots ...	0	0	0	1	3	4	6	7	9	$2n+m-6$ $n, m\geqslant 3$

Note that this contains the quadratic inequality of the 2:2 case ($\alpha_3=0$) previously discussed.

(2) *The derivatives*

We find that:

$$y' = \sum_{k=0}^{2n-1} f_k x^k \Big/ \left(\sum_0^n \beta_i x^i\right)^2 = \frac{y^2}{x^2}\frac{d\left(\frac{1}{y}\right)}{d\left(\frac{1}{x}\right)}$$

$$= \left\{\sum_1^n i\alpha_i x^{i-1} + \sum_{r=1}^{n-1}\left[\sum_{i=r+1}^{n}(i-r)\phi_{ir}x^{i+r-1}\right]\right\}\Big/\left(\sum_0^n \beta_i x^i\right)^2 \qquad (11)$$

as alternate forms of y' where:

$$f_k = \begin{cases} \sum_{s=0}^{k+1}(k+1-2s)\beta_s\alpha_{k+1-s}; & k<n \\ \sum_{s=k+1-n}^{n}(k+1-2s)\beta_s\alpha_{k+1-s}; & k\geqslant n \end{cases}$$

and

$$\phi_{ir} = -\phi_{ri} = \alpha_i\beta_r - \alpha_r\beta_i$$

In the case where the degree of the numerator is less than, or equal to, that of the denominator, we can proceed as follows:

Let

α_1 ---- α_p be finite positive

and

α_{p+1} ---- α_n be zero for $2\leqslant p\leqslant n$.

Then ϕ_{ir} may be zero ($i>r>p$), negative ($-\alpha_r\beta_i$; $i>p\geqslant r$) or positive or negative ($p\geqslant i\geqslant r$).

The number of possible changes of sign in the numerator and hence the maximum number of possible turning points for a p:n function is just one if $p=1$, and $2p-3$ otherwise.

(3) *The second derivatives*

$$y'' = \left(\sum_0^n \beta_i x^i \sum_0^{2n-2} g_k x^k - 2\sum_1^n i\beta_i x^{i-1}\sum_0^{2n-1} f_k x^k\right)\Big/\left(\sum_0^n \beta_i x^i\right)^3 \qquad (12)$$

where f_x is as before and

$$g_k = \begin{cases} \sum_{r=0}^{k+1}(k+1)(k+2-2r)\beta_r\alpha_{k-r+2}; & k<n-1 \\ \sum_{r=k+2-n}^{n}(k+1)(k+2-2r)\beta_r\alpha_{k+2-r}; & k\geqslant n-1 \end{cases}$$

Also, from

$$\frac{d\left(\frac{1}{y}\right)}{d\left(\frac{1}{x}\right)} = \frac{x^2}{y^2}y'$$

and

$$\frac{d^2\left(\frac{1}{y}\right)}{d\left(\frac{1}{x}\right)^2} = \frac{x^3}{y^3}[2y'(xy'-y)-xyy'']$$

it is possible to derive general formulae as in eqn. (12) for these derivatives also.

Conclusion

We have attempted to present a definitive mathematical analysis of sigmoid and other non-hyperbolic curves and non-linear double-reciprocal plots, and to argue that steady-state data alone can never give unambiguous evidence for allosterism. However, allosterism undoubtedly seems to exist and where independent evidence indicates co-operative phenomena, then steady-state data may still be of some limited value in deciding between possible models.

Also, we would like to suggest that very few enzymes actually follow Michaelis–Menten kinetics. If experimental workers would extend the concentration ranges used, it would, in all probability, be discovered that most mechanisms are actually random or at least of degree greater than 1. Most of the enzymes studied so far that have been found to obey 1:1 functions only do so because one pathway is favoured rather than obligatory. Obviously, the same criticism could be made of isotope-exchange studies and partial reactions at the molecular level, and in any given case it may be extremely difficult to decide between random and compulsory order or preferred as opposed to exclusive mechanism. In defence of this statement, we would point out that many enzymes have been thought to be ordered until closer study has revealed this random character.

Finally, the following set of rules and procedures should prove useful in the interpretation of complex curves of the type

$$y=\frac{\alpha_1 x+\alpha_2 x^2+----\alpha_n x^n}{\beta_0+\beta_1 x+\beta_2 x^2----\beta_m x^m}$$

(1) If y/x reaches zero asymptotically $(m>n)$, plot $y/\log x$ which is symmetrical only for the 1:2 function. Otherwise, plot $x^\lambda y/x$ for $\lambda=1, 2, 3$ etc. and discover $(m-n)$. The graph $\log y/\log x$ also reaches a limiting slope of $(n-m)$ and where $m=n+1$, $(1/y)/x$ or $(x/y)/x$ may be valuable, as discussed in the text.

(2) If there are insufficient data to decide whether a zero or horizontal non-zero asymptote is reached as $x\to\infty$, plot $(1/y)/x$ and an inflexion will rule out the 1:2 function.

(3) If y/x reaches a horizontal non-zero asymptote $(m=n)$, plot the horizontal asymptotes against second substrate (B) and asymptote^{-1} against B^{-1}. A rectangular hyperbola and line respectively imply the degree of B to be 2:2 or 3:3. Higher-degree curves imply 4:4 or higher degree in B and can be analysed by the methods described in this paper. Now repeat for v against B and discover the degree with respect to A, etc.

(4) y/x has a sigmoid inflexion implying minimum degree 2:2 and $\alpha_2\beta_0>\alpha_1\beta_1$. The graph of $y/(y/x)$ has a positive gradient at $y=0$, giving a doubled-back type of curve, and $(x/y)/x$ has a negative gradient at $x=0$ implying a minimum. Note that a sigmoid curve usually implies a double-reciprocal plot characterized by an asymptotic line giving a negative apparent $V_{max.}$ and apparent K_m when extrapolated. Also a y/x curve with no turning points or inflexions giving a doubly sigmoid $y/\log x$ curve implies minimum degree 2:2, but a doubly sigmoid $y/\log x$ curve resulting from y/x with a sigmoid inflexion or maximum implies degree>2:2. When $\alpha_2\beta_0=\alpha_1\beta_1$ the next term, $\alpha_3\beta_0-\alpha_1\beta_2$, must be considered for sigmoidicity.

(5) Non-linear double-reciprocal plots imply minimum degree 2:2 and are either parabolic concave upward $(\alpha_1=0)$ or else approach asymptotically the line

$$\frac{\beta_0}{\alpha_1}\left(\frac{1}{x}\right)+\left(\frac{\alpha_1\beta_1-\alpha_2\beta_0}{\alpha_1{}^2}\right)$$

This line is approached from above (concave upwards) for

$$\left(\frac{\alpha_2}{\alpha_1}\right)^2\beta_0-\beta_1\left(\frac{\alpha_2}{\alpha_1}\right)+\beta_2>\left(\frac{\alpha_3}{\alpha_1}\right)\beta_0$$

(6) A single maximum in y/x implies minimum degree 2:2 and requires $\alpha_{n-1}\beta_n>\alpha_n\beta_{n-1}$. It is important to note that there is a certain ambiguity with higher-degree functions in the inequalities dictating curve shape. For instance, in the unlikely event that $\alpha_{n-1}\beta_n=\alpha_n\beta_{n-1}$, then it will be necessary to consider the next lower degree term $\alpha_{n-2}\beta_n-\alpha_n\beta_{n-2}$. A final maximum in any y/x plot usually requires $\alpha_{n-1}\beta_n>\alpha_n\beta_{n-1}$ and this gives a double-reciprocal plot which has negative gradient for $1/x=0$. However, the $(1/y)/(1/x)$ curve will be concave up at $1/x=0$ for

$$\left(\frac{\alpha_{n-1}}{\alpha_n}\right)^2\beta_n-\beta_{n-1}\left(\frac{\alpha_{n-1}}{\alpha_n}\right)+\beta_{n-2}>\left(\frac{\alpha_{n-2}\beta_n}{\alpha_n}\right)$$

(7) A minimum in y/x implies minimum degree 3:3 and requires $\alpha_{n-1}\beta_n<\alpha_n\beta_{n-1}$ for approach to the horizontal asymptote from below.

(8) Multiple inflexions in y/x and any inflexion at all in $(1/y)/(1/x)$ require minimum degree 2:3.

(9) y/x with a horizontal non-zero asymptote and a horizontal plateau implies minimum degree 3:3.

(10) y/x with one maximum and one minimum implies minimum degree 3:3, whereas two maxima and one minimum imply minimum degree 4:4. It is, however, possible for high-degree functions to give y/x curves with no inflexions or turning points. In general, a minimum estimate of the degree of the rate equation can be obtained from the number of times a horizontal line cuts y/x curves, from the maximum positive slope of $\log y/\log x$ or from counting inflexions and turning points and referring to Table 2. Sometimes other graphical methods are useful, e.g. $y/(y/x)$ curves with any inflexion at all require degree >2:2, since the 2:2 function gives conic sections in this plot, as it does in $(1/y)/(1/x)$ and $(x/y)/x$.

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