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The Quantitative Analysis of Ligand Binding and Initial-Rate Data for Allosteric and other Complex Enzyme Mechanisms

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1. The eight methods for plotting enzyme kinetic data are classified and analysed, and it is shown how, in each case, it is only possible to obtain quantitative data on the coefficients of the lowest- and highest-degree terms in the rate equation. 2. The combinations of coefficients that are accessible experimentally from limiting slopes and intercepts at both low and high substrate concentration are stated for all the graphical methods and the precise effects of these on curve shape in different spaces is discussed. 3. Ambiguities arising in the analysis of complex curves and certain special features are also investigated. 4. Four special ordering functions are defined and investigated and it is shown how knowledge of these allows a complete description of all possible complex curve shapes.

Childs & Bardsley (1975) have discussed the existing literature on the structure and degree of ligand-binding and steady-state rate equations and, in particular, have given a mathematical analysis of the conditions necessary for velocity versus substrate concentration curves to be sigmoid or to have turning points. Also, they have developed a comprehensive theory of the graphical techniques used in the analysis of experimental binding or velocity data, investigated the relationship between the various methods and suggested several new techniques for elucidating the degree and form of rate and binding equations (Bardsley & Childs, 1975). Details must be sought in the two publications cited but basically it has been shown that analysis of all ligand-binding and initial-rate data can be reduced to a common pursuit, namely, that of fitting experimentally observed data (y, say) to a rational polynomial function in the concentration variable (x, say) of the following type:

$$y = N/D$$

where

$$N = \alpha_1 x + \alpha_2 x^2 + \cdots + \alpha_n x^n$$

and

$$D = \beta_0 + \beta_1 x + \beta_2 x^2 + \cdots + \beta_m x^m$$

This will be referred to as an n to m (n:m) function and although it will be the case that the coefficients α_i and β_i are always zero or positive and that the degree m will be greater than or equal to the degree n, the powers n and m and the magnitude of the coefficients are the subject of experimental investigation. In fact, n and m will depend on the number of enzyme species reacting with the substrate and the coefficients will be complex functions of the rate constants and other concentration variables (second substrates, modifiers, etc.), and the practice of steady-state enzyme kinetics might be defined as the attempt to discover n, m, α_i, β_i and then to interpret this information mechanistically. Since curves of y against x are rather featureless for this purpose, the usual experimental procedure is to generate two new functions of dependent and independent variable $F(x, y)$ and $G(x, y)$ say, which are then plotted against each other to extract quantitative parameters.

Thus, for instance, eight graphical methods which evolved during the historical development of enzyme kinetics have been generally used, particularly the double-reciprocal plot which was the first linearization of the Michaelis–Menten hyperbola. Considerable confusion exists, however, when double-reciprocal plots are non-linear, which is probably the rule rather than the exception. For instance, where an enzyme-catalysed reaction involves addition of only one substrate to one site, the steady-state equation will be a 1:1 function and can be completely described by the two experimentally determined parameters V and K. If other substrates are involved in the reaction and the order of addition is obligatory, then the resulting rate equation will still be 1:1 in all substrates, provided that there is no consecutive addition of more than one molecule of the same substrate. The kinetic constants describing such a system can still be obtained by replotting slopes and intercepts, but all other mechanisms give higher-degree rate equations. For instance, two kinetically distinct isoenzymes catalysing the same reaction are described, in the absence of product, by

$$v = \frac{(V_1 K_2 + V_2 K_1) S + (V_1 + V_2) S^2}{K_1 K_2 + (K_1 + K_2) S + S^2}$$

which gives a concave-down hyperbola in double-reciprocal space from which the constants can be approximately obtained by extrapolation.

All other mechanisms (random, multi-sited, allosteric, etc.) give high-degree rate equations which are characterized experimentally by complex curves or non-linear double-reciprocal plots and a number of questions seem important and relevant here. (i) What quantitative data can be readily extracted from complex curves that will be of permanent value irrespective of the degree of the rate equation or any speculation about interpretation? (ii) What happens to intuitively obvious features such as maxima and sigmoid inflexions in the various graphical methods? (iii) To what extent can kinetic constants or binding constants be obtained from complex curves? (iv) Which are the best methods to use in analysing complex kinetics? (v) What are the mathematical features of rate equations giving rise to complex curves? (vi) To what extent do contemporary ideas about enzyme mechanisms lead to predictions about the relationship between curve shape and interpretation in molecular terms? The purpose of this paper is to answer questions (i), (ii), (iii) (iv) and (v). Detailed proofs of the conclusions in the text have been given previously (Childs & Bardsley, 1975; Bardsley & Childs, 1975), are straightforward calculations or are included in the Appendix to the present paper.

Theoretical

We adopt the notation $y(x)$ to describe initial reaction rate (y) with all experimental conditions constant except for the varied substrate (x) and note that

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

$$= N/D \qquad (1)$$

where $m = (n+r)$, r being the number of substrate molecules adding consecutively to form a possible dead-end complex. Some simplification of eqn. (1) can be achieved by using dimensionless parameters when $\beta_0 = \beta_1 = 1$ and $\alpha_n = \beta_n$ for $n = m$, but for convenience we shall allow α_i, β_i to be finite for all i values since rate equations are calculated and experimental data plotted in this form.

It is common practice to take $y(x)$ data and transform it into $F(x, y)$ and $G(x, y)$ which are then plotted as F/G (Bardsley & Childs, 1975), and it is the aim of the present study to show that this process invariably leads to similar relationships involving the lowest- or highest-degree coefficients.

The importance of certain numerator/denominator cross products will be shown subsequently (see the Appendix) and we propose the following notation for the lowest-degree terms

$$\psi_{kl}^{ij} = \alpha_i \beta_j - \alpha_k \beta_l \qquad (2)$$

e.g. $$\psi_{11}^{20} = \alpha_2 \beta_0 - \alpha_1 \beta_1$$

and $$\psi_{12}^{30} = \alpha_3 \beta_0 - \alpha_1 \beta_2, \text{ etc.}$$

Symmetry allows a simple definition for the highest-degree cross products, namely

$$\phi_{ij} = \alpha_i \beta_j - \alpha_j \beta_i \qquad (3)$$

Now the importance of these low-degree and high-degree cross products is that the local behaviour of the curve will be dictated by the first and second derivatives which will be dominated in the extreme range of independent variable by either the low-degree or high-degree terms. These will be either positive or negative depending on the difference in magnitude between cross products of comparable degree. The denominator of these derivatives will be positive definite in the first quadrant and so inflexions and turning points will in all cases be determined by sign changes in a polynomial with coefficients consisting of ψ values and ϕ values as defined by eqns. (1), (2) and (3). Similar expressions hold for all other graphical methods usually employed in addition to y/x and we would anticipate the curve shape to be dictated by α_i, β_i; $i = 1, 2, 3$; ψ_{11}^{20} and ψ_{12}^{30} as $x \to 0$ and by α_i, β_i; $i = n$, $n-1$, $n-2$; $\phi_{n,n-1}$ and $\phi_{n,n-2}$ as $x \to \infty$. For intermediate values of x, the curve shape will be determined for high-degree curves by a much larger number of coefficients as equal degree terms are contributed by a larger number of cross product combinations and, in consequence, it seems unlikely that any graphical method could give unambiguous quantitative information about this region of experimental values, i.e. the intermediate range of substrate concentration.

A further point concerns the behaviour when the extreme coefficients are zero. When $\phi_{n,n-1} = 0$ or $\psi_{11}^{20} = 0$ then the curve shapes are to a degree ambiguous, being then determined by the next ϕ values and ψ values and in this instance, special features appear in all graphical methods. Of even greater interest is the graphical behaviour when the extreme α_i values are zero. Successive addition of two molecules of the same substrate uninterrupted by product release gives a rate equation with $\alpha_1 = 0$, and formation of a dead-end complex between enzyme and substrate gives a rate equation with $m = n+1$ which is mathematically equivalent to setting $\alpha_n = 0$ in a $n{:}n$ function. When α_1 or $\alpha_n = 0$ dramatic features are produced in all graphs as will be discussed.

The transformations $y/x \to F/G$ used by kineticists are algebraic or logarithmic and can be classified into three distinct groups: Class I which are functions of

y only, plotted against functions of x only; Class II which are functions of y only, plotted against functions of x and y only; Class III which are functions of x only, plotted against functions of x and y only. It will be systematically demonstrated that in all cases analysis of F/G can only give information about n, m, α_1/β_0, α_n/β_n and relationships involving α_i, β_i, ψ_{kl}^{ij} and ϕ_{ij} and to see this we first consider an arbitrary graph of F/G shown in Fig. 1.

Behaviour as $G \rightarrow 0$

Determination of accurate data for small values of G could lead to the determination of the intercept and gradient at $G = 0$ and, in some cases, the second derivative. As seen from Fig. 1, (i) the first derivative could be positive, zero or negative and the curve could be concave-down (ii), concave-up (iii) or, in exceptional circumstances, could have an inflexion as in (iv) and (v). Quantitative information is only available from extrapolated slopes and intercepts when the first derivative is non-zero, but zero first and second derivatives have diagnostic value as will be shown.

Behaviour for intermediate values of G

The presence of inflexions (vi), (viii) or turning points (vii), (ix) is only of diagnostic significance as regards the degree of the rate equation and mechanism and is of no quantitative value except as source of experimental points for curve fitting after n, m and the extreme coefficients have been determined.

Behaviour as $G \rightarrow \infty$

An asymptotic line can be reached from above (x) or below (xi) and this depends qualitatively on the sign of ψ or ϕ, but quantitative information is only accurately obtained from the slope (xiii) and intercept (xii) of the asymptotic line.

We now proceed to the analysis of the usual graphical procedures and, in all cases, the extreme regions $G \rightarrow 0$ and $G \rightarrow \infty$ give the quantitative information.

The Graphs Used in Enzyme Kinetic Studies

Class I graphs: functions of y only plotted against functions of x only

$$\frac{dF}{dG} = F_y y'/G_x$$

where subscripts denote partial derivatives.

$$\frac{d^2F}{dG^2} = [y'(F_{yy}G_x y' - F_y G_{xx}) + F_y G_x y'']/G_x^3$$

(*a*) *Graph of y/x*. (i) As $x \rightarrow 0$. From eqn. (1) we can investigate y/x near the origin and find, omitting higher powers of x

$$y' \approx [\alpha_1\beta_0 + 2\alpha_2\beta_0 x + (3\alpha_3\beta_0 + \phi_{21})x^2 + \cdots]/D^2$$

$$y'' \approx 2\beta_0[\psi_{11}^{20} + 3\psi_{12}^{30}x + 3(2\psi_{13}^{40} + \psi_{22}^{31})x^2 + \cdots]/D^3$$

When $\alpha_1 = 0$ we have $y'(0) = 0$ and $y''(0) > 0$, i.e. the curve must be sigmoid. It is also sigmoid for $\psi_{11}^{20} > 0$ but when $\psi_{11}^{20} = 0$ y/x has an inflexion at the origin and resembles a straight line there. Considerable interest attaches to curves being concave-up at the origin (sigmoid curves) as discussed previously (Childs & Bardsley, 1975) and we see that even with $\psi_{11}^{20} = 0$ y/x may still be sigmoid if $\psi_{12}^{30} > 0$. However, this ambiguity will be resolved by inspecting other graphs since, as will become clear, $\psi_{11}^{20} = 0$ gives rise to well defined features in all other graphical methods.

(ii) As $x \rightarrow \infty$. In the case $m = n$, we find, omitting lower powers of x

$$y' \approx [\cdots(3\phi_{n,n-3} + \phi_{n-1,n-2})x^{2n-4} + 2\phi_{n,n-2}x^{2n-3} + \phi_{n,n-1}x^{2n-2}]/D^2$$

$$y'' \approx 2\beta_n[\cdots 3(2\phi_{n-3,n} + \phi_{n-2,n-1})x^{3n-5} + 3\phi_{n-2,n}x^{3n-4} + \phi_{n-1,n}x^{3n-3}]/D^3$$

The horizontal asymptote α_n/β_n is approached from above (concave-up) for $\phi_{n,n-1} < 0$, i.e. $\phi_{n-1,n} > 0$. When $\phi_{n,n-1} = 0$ approach to the asymptote will depend on $\phi_{n,n-2} \gtrless 0$, but this will lead to exceptional behaviour in other plots. When $m > n$ then $\lim_{x \to \infty} y = 0$.

In conclusion, we note that the only reliable quantitative parameters available from a plot of y/x are the gradient $[y'(0) = \alpha_1/\beta_0]$ and concavity at the origin $[y''(0) = 2\psi_{11}^{20}/\beta_0^2]$, the horizontal asymptote α_n/β_n as $x \rightarrow \infty$ and an approximate expression for the gradient there, $y'(x \gg 1) \approx \phi_{n,n-1}/\beta_n^2 x^2$.

(*b*) *Graph of* $(1/y)/(1/x)$. (i) as $1/x \rightarrow 0$. When $m > n$, the curve is undefined at the origin and we only consider the case $m = n$. The $1/y$ intercept is β_n/α_n and the derivatives, neglecting higher powers of $(1/x)$ are

$$\frac{d\left(\frac{1}{y}\right)}{d\left(\frac{1}{x}\right)} \approx \left[\phi_{n,n-1} + 2\phi_{n,n-2}\left(\frac{1}{x}\right) + (3\phi_{n,n-3} + \phi_{n-1,n-2})\left(\frac{1}{x}\right)^2 + \cdots\right] \Big/ N^2\left(\frac{1}{x}\right)^{2n}$$

$$\frac{d^2\left(\frac{1}{y}\right)}{d\left(\frac{1}{x}\right)^2} \approx 2\left\{[\alpha_n\phi_{n,n-2} - \alpha_{n-1}\phi_{n,n-1}] + 3[\alpha_n\phi_{n,n-3} - \alpha_{n-2}\phi_{n,n-1}]\left(\frac{1}{x}\right) + 3[2(\alpha_n\phi_{n,n-4} - \alpha_{n-3}\phi_{n,n-1}) + \alpha_{n-1}\phi_{n,n-3} - \alpha_{n-2}\phi_{n,n-2}]\left(\frac{1}{x}\right)^2 + \cdots\right\} \Big/ N^3\left(\frac{1}{x}\right)^{3n}$$

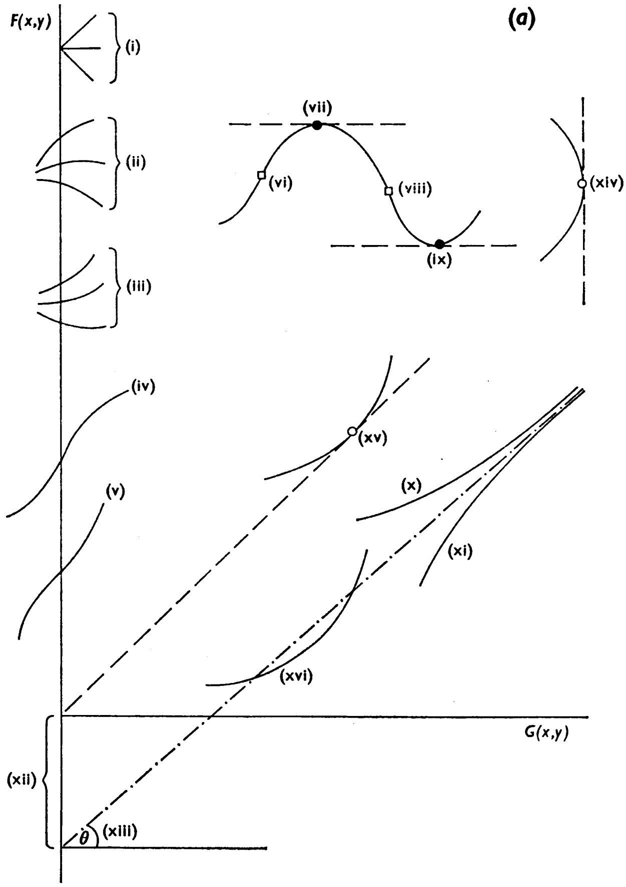
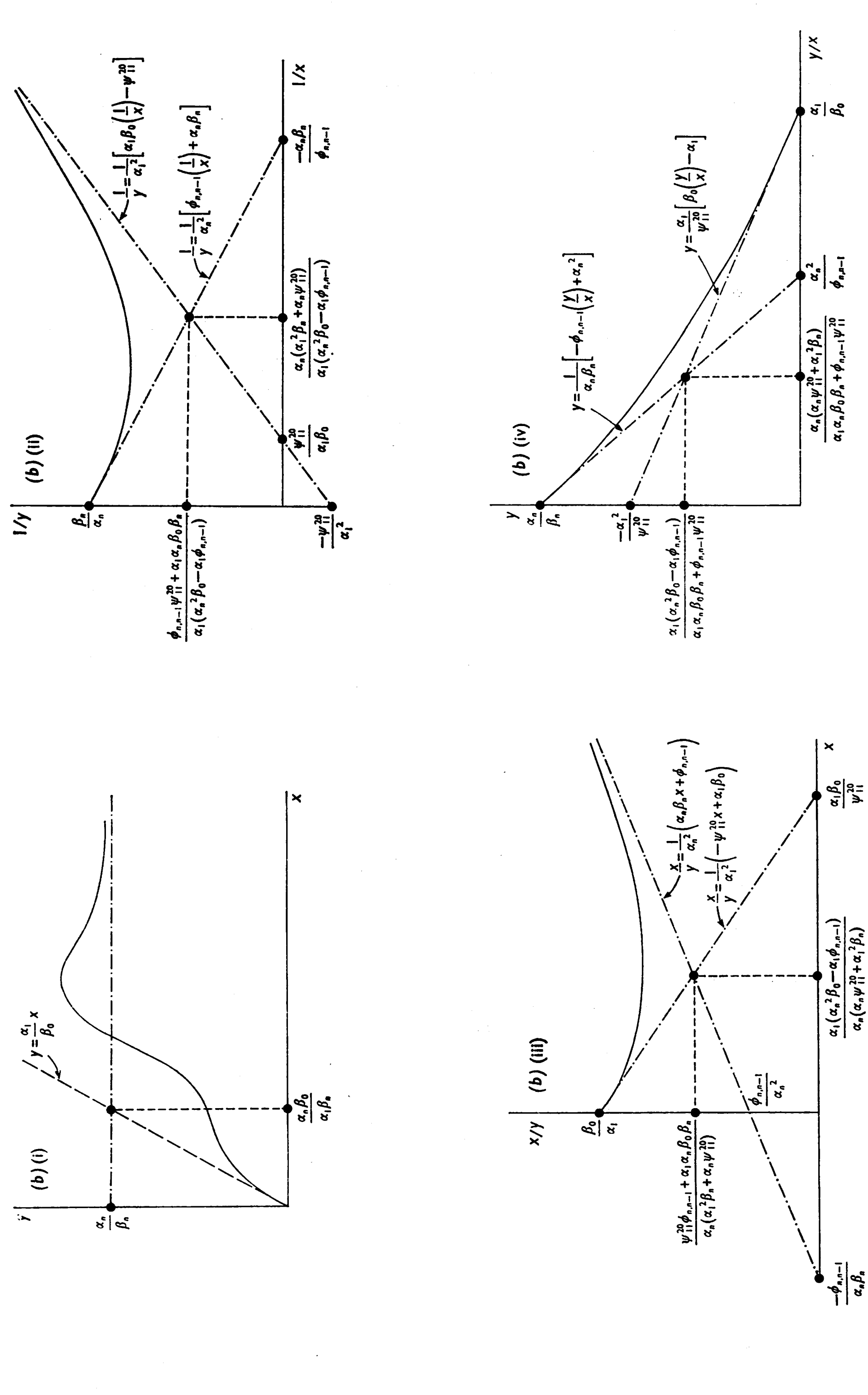


Fig. 1. *Quantitative information available experimentally and local features concerning the graphical analysis of complex steady-state data*

(*a*) An arbitrary graph of F/G is shown containing all the features likely to be encountered. (i) The gradient and F axis intercept are accessible and these yield quantitative parameters. (ii) A curve can be locally concave-down or, as in (iii) concave-up and this is not related to the gradient. With adequate experimental data, this may be quantitatively useful in some instances. (iv) An inflexion may, in unusual circumstances, be present at the origin and the resulting curve may still be locally concave-down or, as in (v) concave-up. This sort of rare ambiguity is fully discussed in the text. (vi) Inflexions of positive slope, (vii) maxima, (viii) inflexions of negative slope and (ix) local minima may be useful in deciding about degree of the rate equation or mechanism and in applying curve-fitting techniques but do not yield any reliable quantitative parameters. (x) Concave-up or (xi) concave-down approach to an asymptotic line with an experimentally accessible intercept (xii) and slope (xiii) provides useful quantitative parameters. (xiv) Tangents to the curve may be vertical, horizontal (vii; ix) or radiate from the origin (xv), and a curve may intersect its own asymptote (xvi), or an arbitrary line. These features may have diagnostic value, and are discussed in the text. (*b*) Slopes and intercepts available from the four principal algebraic transformations. (i) y/x; (ii) $(1/y)/(1/x)$; (iii) $(x/y)/x$; (iv) $y/(y/x)$. All these graphs have limiting intercepts, gradients, and points of intersection of asymptotic lines, which can yield information on the α_i and β_i. Four arbitrary examples are shown for purposes of illustration, but it should be noted that since negative or positive gradients and intercepts can occur, the point of intersection of the asymptotic lines can be in any of the four quadrants (except for y/x).


(b) (i)
(b) (ii)
(b) (iii)
(b) (iv)
1/y
1/x
x/y
x
y
y/x

Notice how $\phi_{n,n-1}=0$ leads to zero gradient at the origin of this plot, thus clearing up ambiguity in approach to the horizontal asymptote in y/x discussed previously. In the extremely unlikely event that $(\alpha_n\phi_{n,n-2}-\alpha_{n-1}\phi_{n,n-1})=0$, concavity at the origin of $(1/y)/(1/x)$ will be determined by $(\alpha_n\phi_{n,n-3}-\alpha_{n-2}\phi_{n,n-1})$ and a similar situation exists in all other plots.

(ii) As $1/x\to\infty$. Neglecting lower powers of $1/x$ the derivatives are

$$\frac{d\left(\frac{1}{y}\right)}{d\left(\frac{1}{x}\right)}\approx\left[\alpha_1\beta_0\left(\frac{1}{x}\right)^{2m-2}+2\alpha_2\beta_0\left(\frac{1}{x}\right)^{2m-3}+(3\alpha_3\beta_0+\phi_{21})\left(\frac{1}{x}\right)^{2m-4}+\cdots\right]\bigg/N^2\left(\frac{1}{x}\right)^{2m}$$

$$\frac{d^2\left(\frac{1}{y}\right)}{d\left(\frac{1}{x}\right)^2}\approx 2\left\{[\alpha_2\psi^{20}_{11}-\alpha_1\psi^{30}_{12}]\left(\frac{1}{x}\right)^{3m-6}+3[\alpha_3\psi^{20}_{11}-\alpha_1\psi^{40}_{13}]\left(\frac{1}{x}\right)^{3m-7}+3[2(\alpha_4\psi^{20}_{11}-\alpha_1\psi^{50}_{14})+\alpha_3\psi^{30}_{12}-\alpha_2\psi^{40}_{13}]\left(\frac{1}{x}\right)^{3m-8}+\cdots\right\}\bigg/N^3\left(\frac{1}{x}\right)^{3m}$$

In conclusion, we note that the only quantitative parameters available from the graph of $(1/y)/(1/x)$ are the slope $\phi_{n,n-1}/\alpha_n^2$ and concavity $2(\alpha_n\phi_{n,n-2}-\alpha_{n-1}\phi_{n,n-1})/\alpha_n^3$ at the intercept β_n/α_n as $1/x\to 0$ and the asymptotic line $1/y\approx\beta_0/\alpha_1(1/x)-\psi^{20}_{11}/\alpha_1$ and concavity $2(\alpha_2\psi^{20}_{11}-\alpha_1\psi^{30}_{12})/\alpha_1^3(1/x)^3$ as $1/x\to\infty$.

Note that $\alpha_1=0$ gives a parabolic instead of straight-line asymptote and that the ambiguity $\psi^{20}_{11}=0$ gives an asymptotic line through the origin. If now y/x is sigmoid because $\psi^{20}_{11}=0$; $\psi^{30}_{12}>0$ then the asymptotic double-reciprocal line is reached from below.

(c) *Graph of* $(1/y)/x$. For the case $m=n$, this graph has no special properties, being merely the inverse of y/x, but the graph has considerable importance in the case $m=n+1$ since it is asymptotic to a straight line (or a parabola for $m=n+2$ and so on) as $x\to\infty$ with

$$\left(\frac{1}{y}\right)'\approx[\alpha_n\beta_{n+1}x^{2n}+2\alpha_{n-1}\beta_{n+1}x^{2n-1}+(3\alpha_{n-2}\beta_{n+1}+\phi_{n-1,n})x^{2n-2}+\cdots]/N^2$$

and the concavity can be estimated from

$$\left(\frac{1}{y}\right)''(x\gg 1)=2(\alpha_{n-1}\psi^{n-1,n+1}_{n,n}-\alpha_n\psi^{n-2,n+1}_{n,n-1})/\alpha_n^3x^3$$

which indicates the mode of approach to the asymptotic line

$$\frac{1}{y}\approx(\beta_{n+1}/\alpha_n)x+\psi^{n,n}_{n-1,n+1}/\alpha_n^2$$

An important point with this graph concerns the possibility of an inflexion. It has been pointed out that a 1:2 function can have no inflexion in $(1/y)/x$ whereas a y/x curve with a final maximum gives an inflexion and approach to the horizontal asymptote from below and this has been proposed as a method for distinguishing dead-end from partial substrate inhibition (Childs & Bardsley, 1975). Note that the above analysis indicates that a 2:3 function can approach the asymptotic line in $(1/y)/x$ from below implying at least one inflexion when $\alpha_1\alpha_2\beta_2>\alpha_2^2\beta_1+\alpha_1^2\beta_3$.

(d) *Graph of y/log x*. A sigmoid inflexion in y/x produces no special feature in this plot and turning points are as for y/x. The curve cuts the y axis at $y(\log x=0)=\sum_1^n\alpha_i\Big/\sum_0^m\beta_i$ and has gradient and concavity there given by $y'(1)$ and $[y'(1)+y''(1)]$ respectively. Otherwise the graph is of little value for obtaining quantitative parameters.

(e) *Graph of log y/x*. The curve is undefined for $x=0$, but from $\log y=\log N-\log D$ we see that at the point of intersection (x_0) of the curve with the x axis $\sum_{i=0}^m(\beta_i-\alpha_i)x_0^i=0$ $(\log y=0)$ and since $(\log y)'=N'/N-D'/D$ and $N=D$ the gradient at the intersection is $\sum_1^m i(\alpha_i-\beta_i)x_0^{i-1}/N(x_0)$. As $x\to\infty$ the graph approaches a limiting slope given by $(\log y)'\approx(n-m)x^{-1}$.

Again, as with the previous semi-logarithmic plot, there is little of value for estimating quantitative parameters.

(f) *Graph of log y/log x*. From the gradient

$$\frac{d\log y}{d\log x}=\sum_1^n i\alpha_i x^{i-1}\Big/\sum_1^n\alpha_i x^{i-1}-\sum_1^m i\beta_i x^i\Big/\sum_0^m\beta_i x^i$$

$$=n-\sum_1^{n-1}(n-i)\alpha_i x^{i-1}\Big/\sum_1^n\alpha_i x^{i-1}-\sum_1^m i\beta_i x^i\Big/\sum_0^m\beta_i x^i$$

we see that the graph starts in the third quadrant with a limiting slope of 1.0 (or 2.0 if $\alpha_1=0$, 3.0 if $\alpha_1=\alpha_2=0$ and so on) and cuts the log y axis at $\log\left(\sum_1^n\alpha_i\Big/\sum_0^m\beta_i\right)$ with a gradient of

$$\left[\left(\sum_1^n i\alpha_i\right)\left(\sum_0^m\beta_i\right)-\left(\sum_1^n\alpha_i\right)\left(\sum_1^m i\beta_i\right)\right]\Big/\left(\sum_1^n\alpha_i\right)\left(\sum_0^m\beta_i\right)$$

and the log x axis at $\log x_0$ with a gradient of $\sum_1^m i(\alpha_i-\beta_i)x_0^i/N(x_0)$. Thereafter the curve has a maximum gradient of less than n and reaches a limiting final slope of $(n-m)$.

An interesting point concerns the mode of approach to these limiting slopes. As $x \to 0$, $\log y$ and $\log x \to -\infty$ and the local concavity is seen from

$$\frac{d\log y}{d\log x}-1 \quad (0<x\ll 1) \approx \frac{\psi_{11}^{20}x+2\psi_{12}^{30}x^2}{\alpha_1\beta_0+(\alpha_1\beta_1+\alpha_2\beta_0)x+(\alpha_1\beta_2+\alpha_2\beta_1+\alpha_3\beta_0)x^2}$$

Hence, a sigmoid y/x curve is always associated with approach from above to the limiting asymptote in the third quadrant. When $m=n$ the horizontal asymptote is approached as for y/x but when $m=n+1$ the local concavity as $x\to\infty$ is discovered from

$$-1-\frac{d\log y}{d\log x} \approx \frac{\psi_{n,n}^{n-1,n+1}x+2\psi_{n,n-1}^{n-2,n+1}}{\alpha_n\beta_{n+1}x^2+(\alpha_n\beta_n+\alpha_{n-1}\beta_{n+1})x} \quad (x\gg 1)$$

i.e. approach from above for

$$\alpha_{n-1}\beta_{n+1}>\alpha_n\beta_n$$

Class II graphs: functions of y only plotted against functions of x and y only

$$\frac{dF}{dG}=F_y y'/(G_x+G_y y')$$

$$\frac{d^2F}{dG^2}=\{y'[(F_{yy}G_y-F_yG_{yy})y'^2+(F_{yy}G_x-2F_yG_{xy})y'-F_yG_{xx}]+F_yG_xy''\}/(G_x+G_yy')^3$$

The most familiar graph in this category is $y/(y/x)$ and the only quantitative parameters obtainable experimentally are the y intercept α_n/β_n ($m=n$) with gradient $\phi_{n-1,n}/\alpha_n\beta_n$ and second derivative $2(\alpha_n\phi_{n-2,n}-\alpha_{n-1}\phi_{n-1,n})/\alpha_n^3$ and the y/x intercept α_1/β_0 with gradient $\alpha_1\beta_0/\psi_{11}^{20}$ and second derivative $2\beta_0^3(\alpha_2\psi_{11}^{20}-\alpha_1\psi_{12}^{30})/(\psi_{11}^{20})^3$. Apart from the qualitative value in exaggerating sigmoidicity, this particular graphical method is of little quantitative value. However, one situation giving a unique type of plot by this graphical method is the case of an enzyme mechanism involving successive addition of the same substrate twice uninterrupted by product release ($\alpha_1=0$) and also involving a dead-end complex ($m>n$). Then this graph gives an interesting curve starting from the origin and turning back completely into the origin to form a closed loop.

Class III graphs: functions of x only plotted against functions of x and y only

$$\frac{dF}{dG}=F_x/(G_x+G_yy')$$

$$\frac{d^2F}{dG^2}=[(F_{xx}G_x-F_xG_{xx})+(F_{xx}G_y-2F_xG_{xy})y'-F_xG_{yy}y'^2-F_xG_yy'']/(G_x+G_yy')^3$$

The most frequently encountered graph in this group is $x/(x/y)$ which is more conveniently discussed as G/F. If $m=n+r$, then the graph approaches a linear asymptote for $r=0$ or a quadratic for $r=1$, cubic for $r=2$, etc., and subsequent discussion is for the case $r=0$, i.e. $m=n$.

The asymptotic line is easily seen by synthetic division to be

$$\frac{x}{y}\approx(\beta_n/\alpha_n)x+\phi_{n,n-1}/\alpha_n^2 \quad \text{for} \quad x\gg 1$$

and the condition for approach to this asymptote from above or below is readily found from the remainder or from

$$\left(\frac{x}{y}\right)''\approx 2(\alpha_{n-1}\phi_{n-1,n}-\alpha_n\phi_{n-2,n})/\alpha_n^3x^3 \quad \text{for} \quad x\gg 1$$

The x/y intercept is β_0/α_1 and the gradient and concavity there are easily seen from

$$\left(\frac{x}{y}\right)'=-\psi_{11}^{20}/\alpha_1^2$$

$$\left(\frac{x}{y}\right)''=2(\alpha_2\psi_{11}^{20}-\alpha_1\psi_{12}^{30})/\alpha_1^3 \quad \text{for} \quad x=0$$

Table 1. *Quantitative parameters accessible experimentally for the graphical methods used in enzyme kinetic studies (for $m=n$, $\alpha_1>0$)*

Graph		Independent variable → 0	Independent variable → ∞
y/x	for $x=0$	$y=0$ $y'=\alpha_1/\beta_0$ $y''=2\psi_{11}^{20}/\beta_0^2$	$y=\alpha_n/\beta_n$ at $x=\infty$ $y'\approx\phi_{n,n-1}/\beta_n^2x^2$ for $x\gg 1$
$\frac{1}{y}\Big/\frac{1}{x}$	for $\frac{1}{x}=0$	$\frac{1}{y}=\beta_n/\alpha_n$ 1st derivative $=\phi_{n,n-1}/\alpha_n^2$ 2nd derivative $=2(\alpha_n\phi_{n,n-2}-\alpha_{n-1}\phi_{n,n-1})/\alpha_n^3$	Asymptotic line as $\frac{1}{x}\to\infty$ is $(\beta_0/\alpha_1)\frac{1}{x}-\psi_{11}^{20}/\alpha_1^2$ and 2nd derivative $\approx 2(\alpha_2\psi_{11}^{20}-\alpha_1\psi_{12}^{30})/\alpha_1^3\left(\frac{1}{x}\right)^3$
$y\Big/\frac{y}{x}$	for $\frac{y}{x}=0$	$y=\alpha_n/\beta_n$ 1st derivative $=\phi_{n-1,n}/\alpha_n\beta_n$ 2nd derivative $=2(\alpha_n\phi_{n-2,n}-\alpha_{n-1}\phi_{n-1,n})/\alpha_n^3$	for $y=0$ $\frac{y}{x}=\alpha_1/\beta_0$ 1st derivative $=\alpha_1\beta_0/\psi_{11}^{20}$ 2nd derivative $=2\beta_0^3(\alpha_2\psi_{11}^{20}-\alpha_1\psi_{12}^{30})/(\psi_{11}^{20})^3$
$\frac{x}{y}\Big/x$	for $x=0$	$\frac{x}{y}=\beta_0/\alpha_1$ 1st derivative $=-\psi_{11}^{20}/\alpha_1^2$ 2nd derivative $=2(\alpha_2\psi_{11}^{20}-\alpha_1\psi_{12}^{30})/\alpha_1^3$	Asymptotic line as $x\to\infty$ is $(\beta_n/\alpha_n)x+\phi_{n,n-1}/\alpha_n^2$ and 2nd derivative $\approx 2(\alpha_n\phi_{n,n-2}-\alpha_{n-1}\phi_{n,n-1})/\alpha_n^3x_x^3$

Information Available from Limiting Slopes and Intercepts

The quantitative information available from asymptotic slopes and intercepts resulting from the four principal algebraic graphing procedures together with deductions about concavity are shown in Fig. 1(*b*) and Tables 1 and 2. A further important point concerns the use of extrapolated asymptotic lines in determining the degree of the rate equation. Analysis shows that the degree of a $n:n$ rate equation is two greater than the maximum number of times the actual graph can cut the extrapolated asymptote in $(1/y)/(1/x)$ (asymptote as $1/x \to \infty$) or $(x/y)/x$ (asymptote as $x \to \infty$). Hence a 2:2 function cannot cut the asymptote at all, whereas a 3:3 function can cut it only once, and a 4:4 function up to two times, etc. Many experimental workers extrapolate data from Scatchard $[y/(y/x)]$ plots to obtain information on ligand binding, the y intercept being taken as n, the number of independent binding sites. Curved plots are interpreted as an indication of co-operativity, and despite the absence of a rigorous theory, data is sometimes extrapolated from low ligand concentration to obtain a y intercept, instead of high ligand concentration to give α_n/β_n, which might be a better approximation to n. Fig. 1(*b*) gives the actual value of all possible intercepts and co-ordinates of intersection of asymptotes. Analysis of such plots in terms of low- and high-affinity sites is extremely unreliable, and for further details concerning non-linear Scatchard plots see Childs & Bardsley (1976).

Experimental Demonstration of Sigmoid Inflexions and Final Maxima

It is presumed that difficulty can arise in the experimental determination of a sigmoid inflexion because reaction rate is too low to be accurately measured at low substrate concentration and that difficulty or inaccuracy can arise in determining a final maximum with high substrate concentration owing to solubility or other experimental limitations. In these circumstances, an experimental worker would wish to know which graphical methods would be likely to accentuate such characteristics as sigmoid inflexions and maxima and the present discussion concerns the mapping of points in the four principal algebraic transformations since, as we have indicated, logarithmic methods are of little value. Curvature is also of no value in settling this sort of question since curvature ratios between the graphical methods cannot be reduced to simple relationships but are complex functions of the independent variable in question and can show large multiple inversions of magnitude. However, there are certain important analytical features that should be indicated at this point and which are further developed in the Appendix.

Table 2. *Mapping of sigmoid inflexions, turning points, local concavities and special features into various spaces*

	Low-degree inequalities ($m=n$, $\alpha_1>0$)		High-degree inequalities ($m=n$, $\alpha_1>0$)		Special features	
Graph	$\psi_{11}^{20}>0$	$\alpha_2\psi_{11}^{20}-\alpha_1\psi_{12}^{30}>0$	$\phi_{n-1,n}>0$	$\alpha_n\phi_{n-2,n}-\alpha_{n-1}\phi_{n-1,n}>0$	$m>n$ (i.e. $\alpha_n=0$)	$\alpha_1=0$
y/x	Sigmoid inflexion (concave-up at $x=0$)	—	Final maximum	—	$\lim y=0$ $x\to\infty$	$y'(0)=0$, curve must be sigmoid
$\frac{1}{y}\Big/\frac{1}{x}$	Negative intercept from asymptotic line as $\frac{1}{x}\to\infty$	Concave-up as $\frac{1}{x}\to\infty$	Negative gradient at $\frac{1}{y}$ intercept (initial minimum)	Concave-down at the $\frac{1}{y}$ intercept $\left(\frac{1}{x}=0\right)$	Undefined at the $\frac{1}{y}$ axis	Asymptotic to a parabola instead of a line as $\frac{1}{x}\to\infty$
$y\Big/\frac{y}{x}$	Positive gradient at $\frac{y}{x}$ intercept (curve doubles back with infinite gradient at at least one point)	Concave-up if $\psi_{11}^{20}>0$ Concave-down if $\psi_{11}^{20}<0$ at the $\frac{y}{x}$ intercept	Positive gradient at the y intercept (initial maximum)	Concave-up at the y intercept $\left(\frac{y}{x}=0\right)$	Lim $y=0$ $x\to\infty$ with infinite slope and concave-down shape at the origin	$\lim \frac{y}{x}=0$ $x\to 0$ with zero gradient and concave-up shape at the origin
$\frac{x}{y}\Big/x$	Negative gradient at $\frac{x}{y}$ intercept (initial minimum)	Concave-up at the $\frac{x}{y}$ intercept	Negative intercept for asymptotic line as $x\to\infty$	Concave-down as $x\to\infty$	Asymptote to higher degree curve	Undefined at the $\frac{x}{y}$ axis

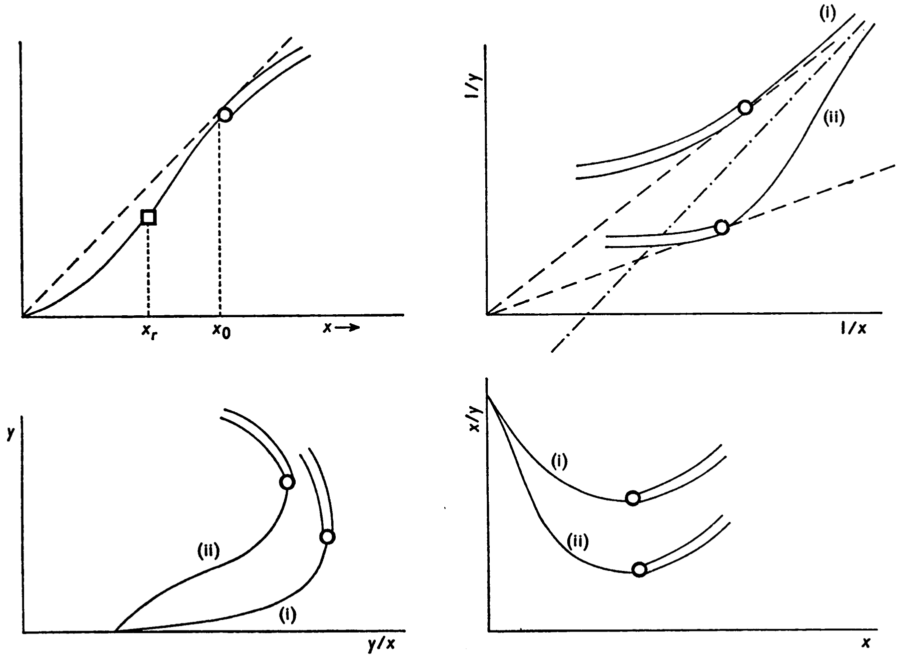


Fig. 2. *Graphical behaviour at low substrate concentration*

The curve shapes indicated are all for a sigmoid curve ($\psi_{11}^{20}>0$) and either (i) $\alpha_2\psi_{11}^{20}-\alpha_1\psi_{12}^{30}>0$ or (ii) $\alpha_2\psi_{11}^{20}-\alpha_1\psi_{12}^{30}<0$. The point □ is at x_r the first positive root of y''. The point ○ is at x_0 the first positive root of $xy'-y$. –·–·, Asymptote; ––––, tangents to the curves from the origin. For purposes of illustration the curves are divided into two hypothetical sections. ═══○, The region readily accessible experimentally; ——○, the region of greatest experimental difficulty. Important features of the individual graphs are as follows: y/x; $x_0>x_r$ and at x_0 the curve is concave-down. $(1/y)/(1/x)$; the curve is always concave-up at x_0 and a tangent there passes through the origin. $y/(y/x)$; a vertical section occurs at x_0. $(x/y)/x$; a horizontal section occurs at x_0. Features (i) and (ii) are indistinguishable in y/x but in the other graphs we see that (i) is always concave-up for $x<x_0$ whereas (ii) undergoes a change in concavity for $x<x_0$.

Sigmoid inflexions

The condition that a curve be sigmoid in y/x is that there by a positive root of y'' and $xy'-y$ and denoting the smallest positive root of $(xy'-y)$ as x_0 and of y'' as x_r then invariably $x_0>x_r$ (see the Appendix). Suppose that experimental data are only available as far down as x_0, then this lowest experimental point will occur as indicated in Fig. 2. To locate the point in $(1/y)/(1/x)$ we note that a line joining the origin with x_0^{-1}, $[y(x_0)]^{-1}$ has the equation

$$\frac{1}{y}=\frac{x_0}{y(x_0)}\left(\frac{1}{x}\right)$$

and from

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

x_0/y_0 is also the gradient of the curve at x_0^{-1}. In other words, a tangent drawn through the curve at the point x_0^{-1} will pass through the origin irrespective of whether the asymptotic line has a positive or negative intercept. Also, from

$$\frac{\mathrm{d}^2\left(\frac{1}{y}\right)}{\mathrm{d}\left(\frac{1}{x}\right)^2}=-\frac{x^4y''}{y^2}$$

at x_0 and since $y''(x_0)<0$ for $x_0>x_r$ the double-reciprocal plot will be concave-up at this point. Hence equal ambiguity is shown by all plots.

Final maxima

The condition $\phi_{n-1,n}>0$ leading to a final maximum at $x=x_f$ in y/x, an initial minimum in $(1/y)/(1/x)$ and initial maximum in $y/(y/x)$ is also ambiguous

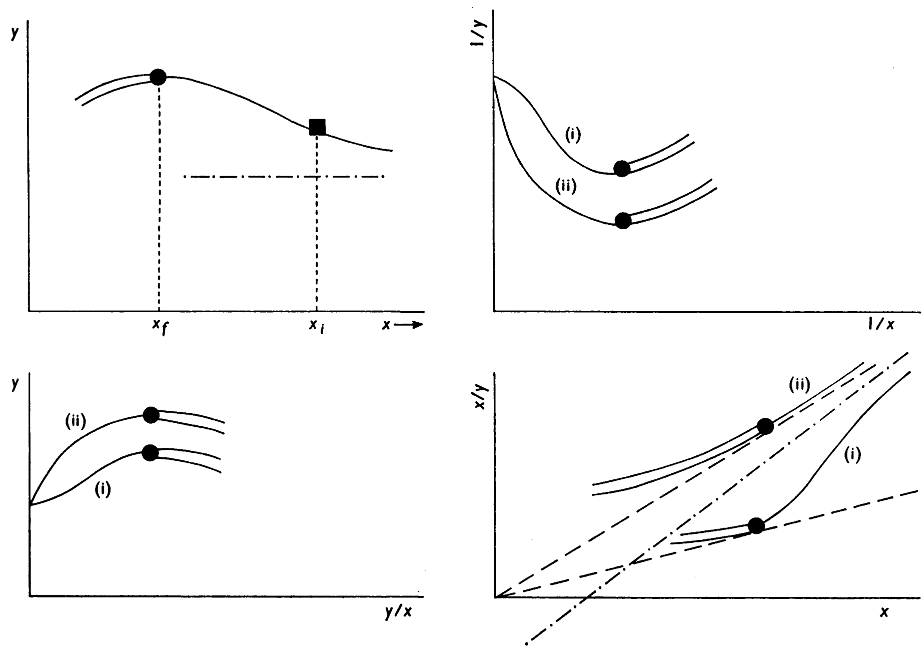


Fig. 3. *Graphical behaviour at high substrate concentration*

The curve shapes shown are all for a $n\!:\!n$ curve with a final maximum ($\phi_{n-1,n}>0$) and either (i) $\alpha_n\phi_{n,n-2}-\alpha_{n-1}\phi_{n,n-1}<0$ or (ii) $\alpha_n\phi_{n,n-2}-\alpha_{n-1}\phi_{n,n-1}>0$. The point ● is at x_f the largest positive root of y'. The point ■ is at x_i the largest positive root of y''. –·–· Asymptote; ––––, tangents to the curve from the origin. For purposes of illustration, the curves are divided into two hypothetical sections. ═══●, The region readily accessible experimentally; ——●, the region of greatest experimental difficulty. Important features of the individual graphs are as follows: y/x; x_f where the curve is concave-down is followed by x_i where concavity changes as the curve approaches α_n/β_n. $(1/y)/(1/x)$; x_f is at a minimum. $y/(y/x)$; x_f is at a maximum. $(x/y)/x$; the curve is always concave-up at x_f and a tangent there passes through the origin. Features (i) and (ii) are indistinguishable in y/x but in the other graphs we see that (i) undergoes a change in concavity for $x>x_f$ whereas (ii) is always concave-up in $(1/y)/(1/x)$, $(x/y)/x$ but concave-down in $y/(y/x)$.

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as regards $(x/y)/x$ where the asymptotic line has a negative intercept. The equation of the line joining the origin to the point x_f, $x_f/y(x_f)$ is

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

and from

$$\left(\frac{x}{y}\right)'=(y-xy')/y^2$$

we see that this is also the expression for the derivative at x_f and a similar ambiguity arises as illustrated in Fig. 3. Since at the final maximum $y'=0$ and $y''<0$, it follows that y/x and $y/(y/x)$ are always concave-down, whereas $(1/y)/(1/x)$ and (x/y) are always concave-up at the point x_f.

Fig. 4 illustrates the resolution of ambiguities resulting from $\psi^{20}_{11}=0$ and $\phi_{n-1,n}=0$ previously discussed.

Conclusion

The aim of this study has been to decide what quantitative parameters are available from the eight graphical methods used in enzyme studies that will be completely independent of the degree of the rate equation and free from any assumptions as to mechanism. In any given case, there is, of course, no substitute for the careful determination of experimental data and statistical processing to obtain the coefficients in the usual way, but the aim here has been that of mathematical analysis of the relationship between the coefficients of the rate or binding equation in order to extract a set of rules relating the intercepts and limiting gradients of the various graphs to the coefficients. From this point of view, it has been proved that accumulating data at extreme ranges of substrate concentration to the neglect of the intermediate range is the best procedure for obtaining kinetic or binding constants by extrapolation

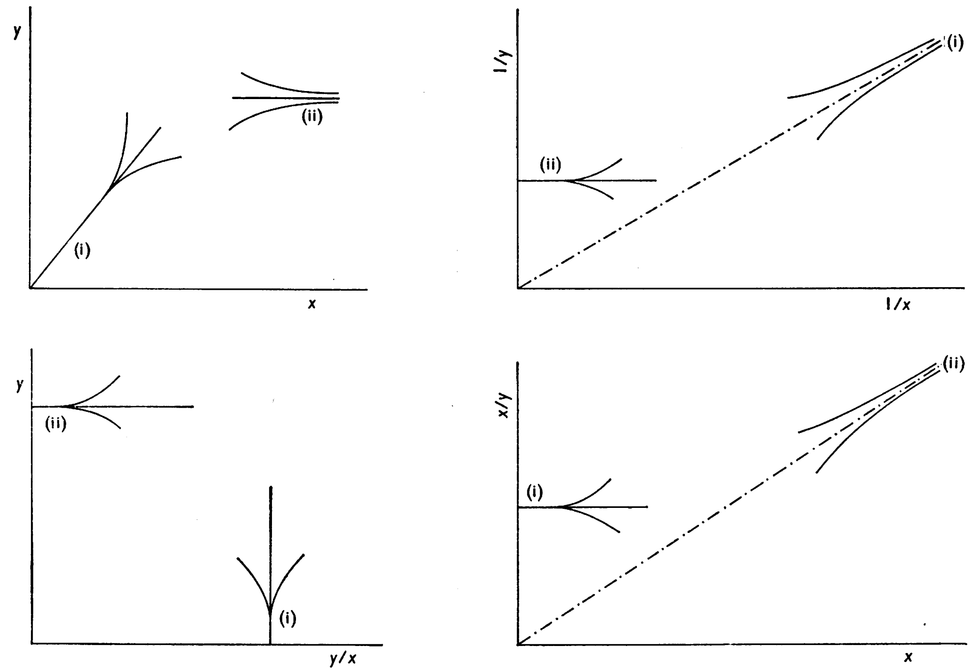

Fig. 4. *Experimental determination of ambiguities in sigmoid inflexions and final maxima*

Where ψ_{11}^{20} or $\phi_{n-1,n}$ are zero, then the curves can still show all possibilities of local concavities due to the next terms ψ_{12}^{30} and $\phi_{n-2,n}$ as discussed in the text. This behaviour is unlikely but can occur with high-degree functions and spotting this type of ambiguity is of some importance. The special features produced in the graphs under these circumstances which are unaffected by all other parameters and which therefore have diagnostic value are illustrated. (i) When $\psi_{11}^{20}=0$, y/x; $y''(0)=0$ and the graph leaves the origin much like a straight line. $(1/y)/(1/x)$; the asymptotic line goes through the origin. $y/(y/x)$; y/x intercept vertical. $(x/y)/x$; x/y intercept horizontal: (ii) When $\phi_{n-1,n}=0$, y/x, no special feature. $(1/y)/(1/x)$; $1/y$ intercept horizontal. $y/(y/x)$; y intercept horizontal. $(x/y)/x$; the asymptotic line passes through the origin.

procedures and will always, irrespective of the method used, give the same information. This will be apparent from Tables 1 and 2.

The interpretation of these limiting intercepts and gradients is more controversial. Ligand-binding functions have $n=m=$ number of non-identical binding sites and have a very limited range of possible curve shapes, e.g. maxima are not possible, whereas steady-state equations can have $n=m$ or $n<m$ and may have $n>$ number of binding sites and in addition may have turning points. For this reason, interpretation of saturation functions will always be less ambiguous than steady-state equations. Now α_i, β_i will usually vary in magnitude more for steady-state equations than saturation functions and further analysis of curves with ψ and ϕ values near zero shows that these give smooth featureless curves in all spaces and, in these circumstances, the double log plot or Hill plot gives a useful approximation to n. However, when α_i, β_i are of differing magnitude as in steady-state mechanisms, then the ψ and ϕ values are not zero and complex curve shapes result, and, under these circumstances, double log or Hill plots are completely worthless. This criticism is in addition to the usual one that no enzyme-catalysed reaction even approximately obeys the Hill equation.

It seems appropriate to conclude with an examination of possible answers to the six questions formulated in the introduction.

(i) Table 1 and Fig. 1 contain a summary of all the information available from limiting intercepts and gradients in terms of α_i, β_i, ψ and ϕ.

(ii) Table 2 and Figs. 2 and 3 describe the relationship between curve shape in various spaces and Figs. 4 and 5 illustrate the ambiguous cases and special features discussed in the text.

(iii) Interpretation of α_i, β_i in terms of kinetic or binding constants will always be ambiguous unless alternative experimental evidence is available. For instance, extrapolation of curved reciprocal plots does not always give K_1, K_2, V_1, V_2 for a two-sited enzyme, but only for the special case of two

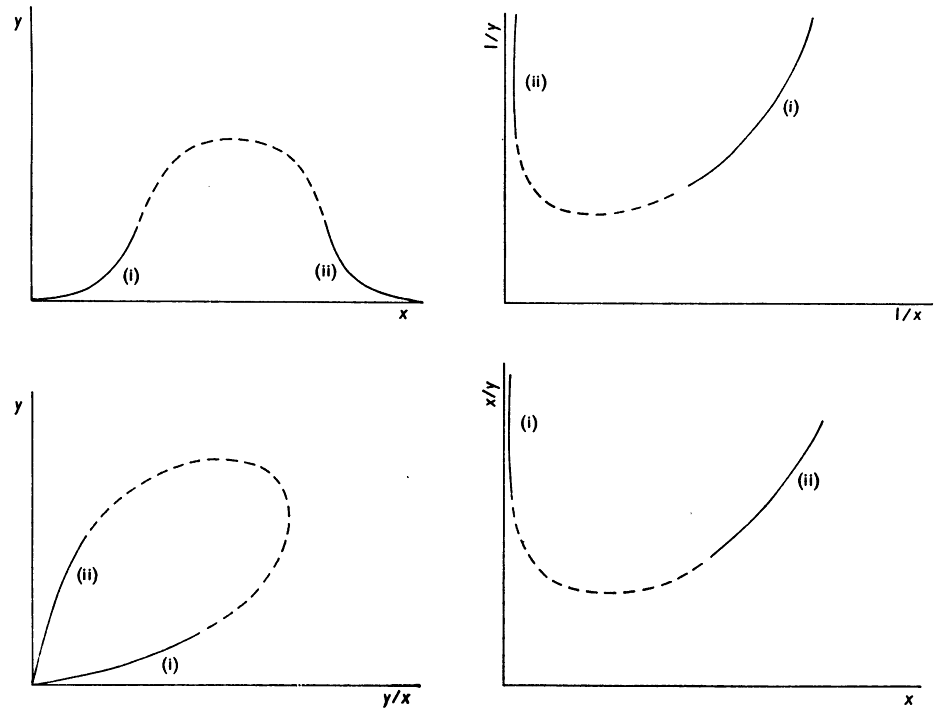

Fig. 5. *Graphical manifestations of special features in the graphs of rational polynomial functions*

Certain mechanisms discussed in the text lead to rate equations in which either $\alpha_1 = 0$ or $\alpha_n = 0$ and this gives rise to dramatic features in all graphs. ——, The special feature; ----, the rest of the curve which may have additional complexities. (i) When $\alpha_1 = 0$, y/x; $y'(0) = 0$ and the curve must be sigmoid. $(1/y)/(1/x)$; parabolic asymptote as $1/x \rightarrow \infty$. $y/(y/x)$; curve is concave-up at the origin with zero gradient. $(x/y)/x$; curve undefined at the origin. (ii) When $\alpha_n = 0$ i.e., an $(n-1)\colon n$ function (or, as discussed in the text an $n\colon m$ function with $m > n$). y/x; $y \rightarrow 0$ as $x \rightarrow \infty$. $(1/y)/(1/x)$; curve undefined at the origin. $y/(y/x)$; curve is concave down at the origin and has infinite gradient. $(x/y)/x$; parabolic asymptote as $x \rightarrow \infty$.

kinetically distinct isoenzymes, i.e. fully independent sites, each obeying a 1:1 function. Further, a large number of mechanisms give curved reciprocal plots and independent evidence for two sites is required before extrapolation is justified. Any inflexion at all in a double-reciprocal plot requires at least a 2:3 function which excludes two independent sites.

(iv) y/x, $(1/y)/(1/x)$ and the log plots are useful for curves with turning points but not very good for spotting 'sigmoid inflexions'. $y/(y/x)$ is extremely valuable for demonstrating 'sigmoid inflexions' and for spotting α_1 or $\alpha_n = 0$ $(m > n)$ but not, in general, useful quantitatively. $(1/y)/(1/x)$ gives β_0/α_1 and $(x/y)/x$ gives β_n/α_n as the gradient of asymptotic lines, but there may be considerable difficulty in deciding from experimental points the precise location of the asymptotic line. Also, these methods are generally inferior to $y/(y/x)$ for demonstrating non-linearity, but this method does exaggerate experimental error at low substrate concentration. There are no simple formulae connecting curvature, $y''(1+y'^2)^{-3/2}$ for these graphical methods, i.e. no one graph is more curved than another as such but this will depend on the particular function and x value. The Hill plot has some value for ligand-binding curves $[\bar{y}(\infty) = 1]$ and for smooth 'well behaved' v/s graphs $[v(\infty) = V_{max.} = \alpha_n/\beta_n]$, but is completely worthless for analysing complex curves with sigmoid inflexions (where extrapolated $V_{max.}$ and K_m values are negative) or maxima (where $V_{max.}$ has no meaning) that is to say, in precisely those circumstances where it is most used. Fuller details of special uses of the various graphical methods will be found in Bardsley & Childs (1975).

(v) The relationship between n, m, α_i, β_i and curve shape is now well understood (Childs & Bardsley, 1975; Bardsley & Childs, 1975) for the existing graphical methods but there may be much better transformations for plotting complex curves. After all, the methods currently employed are not mathematically calculated procedures but originated accidentally as historical attempts to transform or linearize the Michaelis–Menten equation, which probably has more limited applicability than has hitherto been thought.

(vi) Terminology such as substrate inhibition, substrate activation, positive, negative co-operativity, etc. has been introduced into the interpretation of enzyme kinetics on an insufficiently secure theoretical basis. If the terms are merely adjectives to describe curve shape, then it is surely better to say that a curve is concave-up in a certain region and concave-down in another which implies no mechanistic conclusion, rather than to state that a curve indicates a region of positive co-operativity followed by a region of negative co-operativity and so on, with all the associated interpretation in terms of molecular gymnastics. In fact, the whole transfer of allosteric theory from static binding systems to dynamic catalytic systems has been carried out uncritically because of insufficient knowledge of the mathematical description of curve shape and because of the enormous complexity of realistic allosteric rate equations. The former deficiency is now remedied and preliminary investigations of allosteric rate equations shows that positive and negative co-operativity do not invariably lead to the effects on curve shape predicted.

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APPENDIX

(1) Relationship between Curve Shape and ϕ and ψ Values as Defined in the Text (Eqns. 1, 2, 3)

At this stage we augment the discussion in the text and it will be found necessary to consult a previous publication [Bardsley & Childs (1975), especially Table 1]. The aim will be to show that, for the principal graphical methods, local behaviour will be determined by four special ordering functions which can largely be written in terms of ϕ and ψ, particularly for extreme ranges of independent variable. The function $y(x)$ can be thought of as a ratio of two polynomials, N which is continuous and monotonically increasing and D which is also always concave-up for $x>0$. As the two graphs of N/x and D/x approach, separate or cross-over, etc., then the graph of y/x will show inflexions, turning points etc., which will be reflected in all other derived graphs and we now seek the connexion between concavity and gradient in all spaces. To do this, we first consider six primary functions which give rise to four special ordering functions and subsequently we investigate the geometric consequences of the behaviour of the ordering functions as touching on the underestimation or exaggeration of sigmoidicity, say by extrapolation of data points at low substrate concentration.

(2) Primary Functions

Denoting differentiation with respect to x by primes, we find that the following six primary polynomials are required.

$$N=\sum_{1}^{n}\alpha_i x^i$$

$$N'=\sum_{1}^{n}i\alpha_i x^{i-1}$$

$$N''=\sum_{2}^{n}i(i-1)\alpha_i x^{i-2}$$

$$D=\sum_{0}^{m}\beta_i x^i$$

$$D'=\sum_{1}^{m}i\beta_i x^{i-1}$$

$$D''=\sum_{2}^{m}i(i-1)x^{i-2}$$

Now all these polynomials are positive for $x\geqslant 0$ except for N which has $N(0)=0$. Thus denominators consisting of D functions, being positive, need not be considered when analysing derivatives for roots and further analysis of derivatives with indeterminate forms (N in denominator at $x=0$) by l'Hospital's rule establishes continuity in all cases except for α_1 or $\alpha_n=0$ as discussed previously.

(3) Origins and Definition of the Ordering Functions

Considering differentiation of y, we find $y=N/D$ leads to $y'=(N'D-D'N)/D^2$ and $y''=[(N''D-D''N)D-2D'(N'D-D'N)]/D^3$, and similar expressions hold for all derivatives.

In fact, for the graphs of y/x, $(1/y)/(1/x)$, $y/(y/x)$ and $(x/y)/x$ to decide about inflexions and turning points there are just four polynomials required, which we refer to as ordering functions, namely:

$$F_1=N'D-D'N$$

$$F_2=(N''D-D''N)D-2D'(N'D-D'N)$$

$$F_3=(N'D-D'N)x-ND$$

$$F_4=[2N'(N'D-D'N)-N(N''D-D''N)]x-2N(N'D-D'N)$$

It will be obvious that the coefficients of these polynomials will consist of differences of cross products between coefficients of N and D and these can be arranged in several different ways. The ϕ, ψ nomenclature introduced in the text arises naturally in the consideration of the structure of these ordering functions.

(4) Significance of the Ordering Functions

(*i*) F_1

From the relationships

$$F_1 = D^2 y' = N^2 x^{-2} \frac{d\left(\frac{1}{y}\right)}{d\left(\frac{1}{x}\right)} = F_3 x^{-2} \frac{dy}{d\left(\frac{y}{x}\right)}$$

it will be clear that roots of y' occur at the intersection of the graphs of $N'D$ and $D'N$ and produce turning points in y/x, $(1/y)/(1/x)$ and $y/(y/x)$, but not $(x/y)/x$. A general formula for F_1 in terms of ϕ has been given (Bardsley & Childs, 1975).

(*ii*) F_2

Since $F_2 = D^3 y''$ then roots produce inflexions in y/x and have a more subtle effect on curve shape in other spaces as will be discussed. A general formula for F_2 in terms of ϕ has been given (Bardsley & Childs, 1975), but it is also possible to write the first terms entirely as ψ as shown in the main text.

(iii) F_3

We find the following relationships

$$F_3 = -N^2 \left(\frac{x}{y}\right)' = D^2 (xy' - y) = D^2 x^2 \left(\frac{y}{x}\right)' = D^2 x^2 y' \bigg/ \frac{dy}{d\left(\frac{y}{x}\right)}$$

indicating that roots of F_3 produce turning points in $(x/y)/x$ and correspond to points of infinite gradient in $y/(y/x)$ and as discussed in the text, correspond to points on the $(1/y)/(1/x)$ curve where a line joining that point to the origin is actually at a tangent to the curve. This important feature will shortly be exploited and now we merely record the general formulae for F_3 which can be written entirely in ψ as follows:

$$F_3 = \sum_{k=0}^{n-1} \sum_{1}^{n-k} i\,\psi_{(k+1),(i+k)}^{(i+k+1),k}\, x^{(2k+i+1)}$$

(iv) F_4

This function relates to the other graphs as follows:

$$F_4 = N^3 \left(\frac{x}{y}\right)'' = D^3 [2y'(xy'-y) - xyy''] = x^3 N^{-3} \frac{d^2\left(\frac{1}{y}\right)}{d\left(\frac{1}{x}\right)^2} = \left(\frac{F_3}{Dx}\right)^3 \frac{d^2 y}{d\left(\frac{y}{x}\right)^2}$$

Hence, roots of F_4 imply inflexions in $(1/y)/(1/x)$, $(x/y)/x$ and $y/(y/x)$. $F_4 > 0$ implies that $(1/y)/(1/x)$ and $(x/y)/x$ are concave-up and so is $y/(y/x)$ for $F_3 > 0$.

(4) Roots of the Ordering Functions

The roots of F_1 and F_2 have been extensively discussed (Bardsley & Childs, 1975) and it only remains to discuss F_3 and F_4.

Since a sigmoid curve has the first term in F_3 positive and since the last two terms are always negative, it follows that, in this circumstance, there must be at least one positive root. However, multiple roots can occur in complex high-degree curves and these might be thought of as points of intersection of the graphs of y and xy'. Consider a curve of y/x as in Appendix Fig. 1(*a*). A tangent drawn from the origin to the curve has the equation $y = xy'$ and so intersects the curve at the root of F_3. As shown, this must occur at least once for a sigmoid curve.

Positive roots of F_4 occur at the intersection of $2y'(xy'-y)$ and xyy'' and to discover these points it is necessary to first discover the order of occurrence of the roots of $xy'-y$ and y''.

(5) Order of Occurrence of the Roots of the Ordering Functions

Attention is now confined to the behaviour of curves for small value of x in order to illuminate the problem of the overestimation or exaggeration of sigmoidicity by extrapolation from data points for small x. Consider the first terms in the four functions where we have:

$$F_1 = \alpha_1 \beta_0 + 2\alpha_2 \beta_0 x + (3\alpha_3 \beta_0 + \phi_{21}) x^2 + (4\alpha_4 \beta_0 + \phi_{31}) x^3 + \cdots$$

$$F_2 = 2\beta_0 \{\psi_{11}^{20} + 3\psi_{12}^{30} x + (6\psi_{13}^{40} + 3\psi_{22}^{31}) x^2 + [10\psi_{14}^{50} + 8\psi_{33}^{41} + \phi_{23} + (\beta_1 \phi_{31} + \beta_2 \phi_{12})/\beta_0] x^3 + \cdots\}$$

$$F_3 = x^2 [\psi_{11}^{20} + 2\psi_{12}^{30} x + (3\psi_{13}^{40} + \psi_{22}^{31}) x^2 + (4\psi_{14}^{50} + 2\psi_{33}^{41}) x^3 + \cdots]$$

$$F_4 = 2x^3 \{[\alpha_2 \psi_{11}^{20} - \alpha_1 \psi_{12}^{30}] + 3[\alpha_3 \psi_{11}^{20} - \alpha_1 \psi_{13}^{40}] x + 3[2(\alpha_4 \psi_{11}^{20} - \alpha_1 \psi_{14}^{50}) + \alpha_3 \psi_{12}^{30} - \alpha_2 \psi_{13}^{40}] x^2 + \cdots\}$$

Now, although we know that $\psi_{11}^{20}>0$ leads to at least one positive root of F_2 and F_3, that $\phi_{n-1,n}>0$ leads to at least one positive root of F_1 and F_2 and that $\psi_{11}^{20}>0$ and $\alpha_2\psi_{11}^{20}-\alpha_1\psi_{12}^{30}<0$ leads to at least one root of F_4, it is clear that complete algebraic analysis of such high-degree polynomials is not possible for the general case.

For this reason, we first turn attention to a low-degree function, namely the 2:2 which we take for convenience in the dimensionless forms:

$$y=(Ax+Bx^2)/(1+x+Bx^2)$$

$$y'=[A+2Bx+B(1-A)x^2]/(1+x+Bx^2)^2$$

$$y''=2[(B-A)-3ABx-3B^2x^2-B^2(1-A)x^3]/(1+x+Bx^2)^3$$

$$xy'-y=x^2[(B-A)-2ABx-B^2x^2]/(1+x+Bx^2)^2$$

$$\left(\frac{x}{y}\right)''=B[B-A(1-A)]/(A+Bx)^3$$

The four ordering functions after removing common factors and roots at 0 are:

$$f_1=A+2Bx+B(1-A)x^2$$

$$f_2=(B-A)-3ABx-3B^2x^2-B^2(1-A)x^2$$

$$f_3=(B-A)-2ABx-B^2x^2$$

$$f_4=B-A(1-A)$$

Now f_1 must have a root for $A>1$ leading to a maximum in y/x, and f_2 has one root for $B>A$ (a sigmoid inflexion in y/x) and one root for $A>1$ (following the final maximum in y/x). Further, f_4 can have no sign changes being positive for $B>A$, or $A>1$ (i.e. 2:2 sigmoid y/x curves or y/x curves with maxima always give concave-up double-reciprocal plots), otherwise f_4 is negative (double-reciprocal plots concave-down) or zero corresponding to factorization giving y/x as a 1:1 function. To locate the relative positions of the roots of f_2 and f_1 we note that

$$f_2=f_3-BxR$$

where

$$R=A+2Bx+B(1-A)x^2$$

For the case of interest ($B>A$), we observe that the first root of y'' is always less than that of $xy'-y$ for $A\leqslant 1$ and it is only necessary to consider the relative magnitude of the positive roots of f_3 (q, say) and R (r, say) for $B>A$, $A>1$. Since $q=(A-\sqrt{A^2+B-A})/-B$ and $r=(B+\sqrt{B^2+AB(1-A)})/B(A-1)$ and $r>q$ implies that the positive root of y'' is smaller than the root of $xy'-y$ we find the condition for this to be the case is $A^2+B-A+(1-A)\sqrt{A^2+B-A}+\sqrt{B^2-AB(1-A)}>0$ and this is always true since $A^2+B-A>A\sqrt{A^2+B-A}$.

(5) Topological Considerations about the Roots of the Ordering Functions

In considering the extrapolation of data points for determination of sigmoidicity, it is of some interest to know the order of occurrence of the roots of F_1, F_2, F_3, F_4. For the sake of illustration, the argument will be based on the double-reciprocal plot and it is presumed that the problem is: given a sigmoid curve or stair-step curve, then extrapolation of data points in double-reciprocal space will overestimate sigmoidicity (as judged by a negative intercept for the extrapolated asymptote) for a curve that is concave-down and will underestimate it for a curve that is concave-up in approach to the asymptote. Our approach will be to treat x as a parameter and trace out the curve from $x=0$ and since we are dealing with small values of x, we assume no roots of y', i.e. $F_1>0$ in the interval of concern.

Case 1: stair-step curves (Appendix Fig. 1a)

These functions have $\psi_{11}^{20}>0$ if sigmoid, but otherwise the first positive root of y'' produces a stair-step rather than sigmoid inflexion. Geometric arguments lead to the conclusion that the double-reciprocal plot must be concave-up at a root of F_3 for $\alpha_2\psi_{11}^{20}-\alpha_1\psi_{12}^{30}>0$ and also for $\alpha_2\psi_{11}^{20}-\alpha_1\psi_{12}^{30}<0$ only in this case a root of F_4 has also occurred.

Case 2: a sigmoid curve approaching the double-reciprocal asymptote from below (Appendix Fig. 1b)

The condition on this curve is $\psi_{11}^{20}>0$ and $\alpha_2\psi_{11}^{20}-\alpha_1\psi_{12}^{30}<0$ and for complex high-degree functions several inflexions are possible as shown in Appendix Fig. 1(*b*) for three roots of F_3. Now as we trace the curve from $x=0$ onwards, we find that the first tangent that can possibly be drawn from the origin to the curve is at a point where the curve is concave-up. At this point $F_3=0$, $F_4>0$ (although as $x\rightarrow 0$ it was originally negative) and so y'' must have changed sign after F_4, i.e. $2y'(xy'-y)-xyy''$ changed sign. Hence, the roots of F_2, F_3 and F_4 must occur in the order indicated in Appendix Fig. 1(*b*) and the practical significance of this is that data extrapolated from $x=0$ to x_1 will overestimate sigmoidicity, from x_1 to x_3 will depend on the range and from the section x_3 to x_6 will actually lead to the erroneous conclusion that y/x is non-sigmoid.

Case 3: a sigmoid curve approaching the double-reciprocal plot from above (Appendix Fig. 1c)

The condition for this is $\psi_{11}^{20}>0$ and $\alpha_2\psi_{11}^{20}-\alpha_1\psi_{12}^{30}>0$ and a possible shape for three roots of F_3 is shown in Appendix Fig. 1(*c*). In this case, the curve must again be concave-up at the first tangent implying that the first positive root of y'' is smaller than the

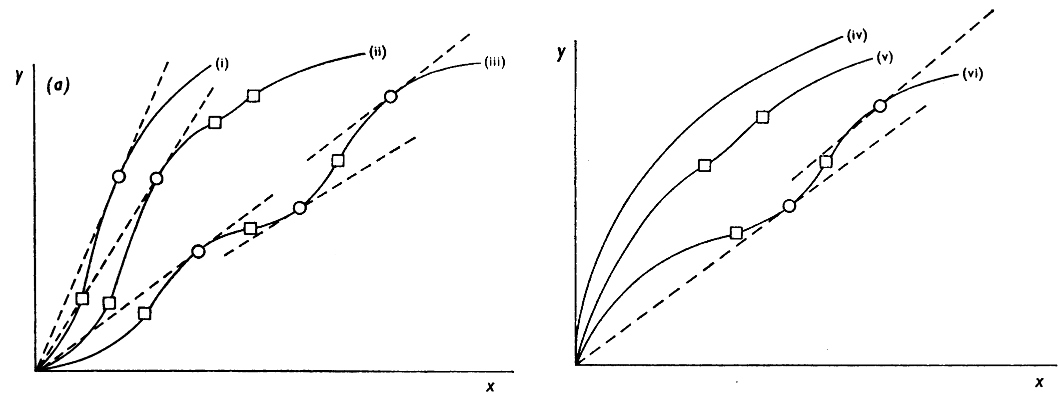


Fig. 1. *Roots of the ordering functions and the experimental demonstration of sigmoidicity with high-degree functions*

Positive roots occur as indicated: ○, $F_3 = 0(xy'-y = 0)$; △, $F_4 = 0[2y'(xy'-y)-xyy'' = 0$; □, $F_2 = 0(y'' = 0)$. It is assumed that $F_1 > 0(y' > 0)$ throughout. –·–·, Asymptote; ––––, successive tangents to the curve from the origin. (*a*) Stair-step curves. The sigmoid case (i), (ii), and (iii). These represent a family of increasingly exaggerated sigmoid stair-step curves, with $\psi_{11}^{20} > 0$. The curves differ in the number of roots of F_3 [tangents from the origin to the curve in y/x and $(1/y)(1/x)$, but vertical sections in $y/(y/x)$ and horizontal sections in $(x/y)/x$]. Geometric considerations lead to the following conclusions: (1) The first root of F_2 is always followed by a root of F_3 in a sigmoid curve. (2) F_2 and F_4 can have successive roots with no intervening roots of F_3. Roots of F_2 produce inflexions uniquely in y/x and roots of F_4 produce inflexions uniquely in $(1/y)/(1/x)$, $(x/y)/x$ and $y(y/x)$. Hence inversions of concavity can occur in y/x with no corresponding feature in the other graphs and vice versa. (3) Two successive roots of F_3 always have a root of F_2 and F_4 intervening. (4) After the first root of F_3 in the sigmoid case, successive roots occur in pairs, and the same is also true for F_2 up to a maximum point, which requires at least one further root of F_2. The non-sigmoid case (iv), (v), and (vi). These represent a family of increasingly exaggerated non-sigmoid curves with $\psi_{11}^{20} < 0$. Geometric considerations reinforce rules (2) and (3) above and also require: (5) a root of F_2 must occur in a non-sigmoid curve before any root of F_3; (6) a non-sigmoid curve can have no roots of F_3 at all, or an even number, and the same is true for roots of F_2 under the conditions referred to in (4) above. (*b*) Estimation of sigmoidicity for $\psi_{11}^{20} > 0$ and $\alpha_2\psi_{11}^{20} - \alpha_1\psi_{12}^{30} < 0$ with three roots of F_3. Extrapolation of experimental data points to determine sigmoidicity (intercept of asymptotic line) would result in the conclusions indicated.

Range of x	Conclusion concerning 'sigmoidicity'
0–1	Overestimation
1–3	Overestimation at lower end but underestimation at higher end of range
3–6	Erroneous conclusion that y/x is non-sigmoid
6–9	Correct conclusion as regards sigmoidicity but with variable quantitative estimation.

(*c*) Estimation of sigmoidicity for $\psi_{11}^{20} > 0$ and $\alpha_2\psi_{11}^{20} - \alpha_1\psi_{12}^{30} > 0$ with three roots of F_3. In this curve no root of F_4 precedes the first root of F_2 and F_3 and in the range of at least $0 - x_2$ sigmoidicity will be consistently underestimated. In the range $x_2 - x_5$ the erroneous conclusion would be drawn that the y/x is non-sigmoid.

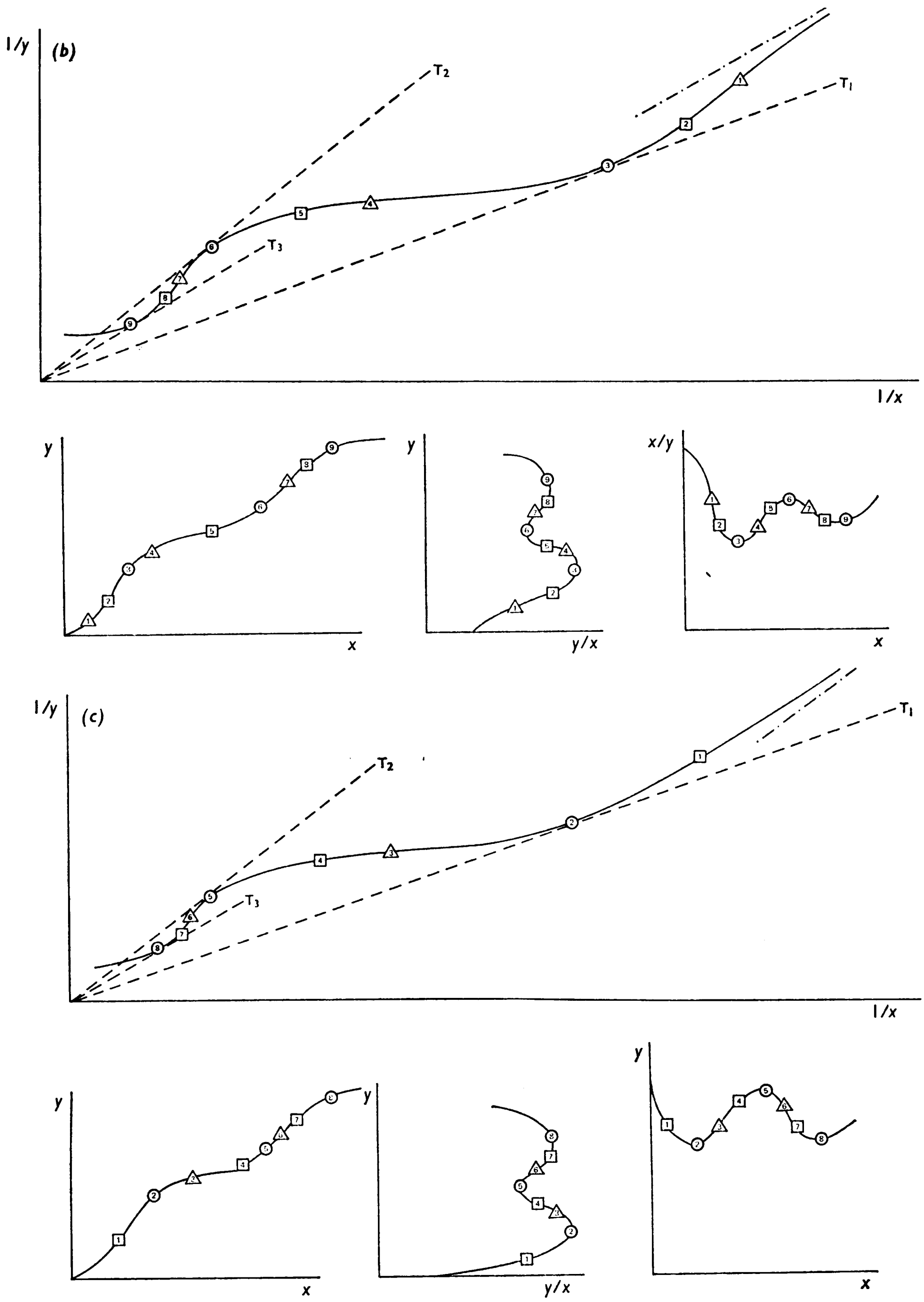
first root of $xy'-y$, but in this case there has been no change in sign of $2y'(xy'-y)-xyy''$ intervening between $x = 0$ and x_2.

Conclusion

For all algebraic curves a set of four ordering functions has been defined and knowledge of these functions allows a complete description of all possible curve shapes. In all cases, the first positive root of F_2 occurs before that of F_3 and geometric arguments lead to the conclusion that although successive roots of F_2 and F_4 can occur with no intervening roots of F_3, successive roots of F_3 must have at least one intervening root of both F_4 and F_2 in that order.

Roots of F_1 and F_3 map into all algebraic spaces producing characteristic geometric features, but F_2 uniquely determines the sign of curvature of y/x, and F_4 uniquely determines that of the graphs of $(1/y)/(1/x)$ and $(x/y)/x$, which therefore always have the same sign of curvature for any given positive x. A combination of F_3 and F_4 are needed to specify concavity in $y/(y/x)$ except that as $x \to \infty$ F_3 becomes negative and $y/(y/x)$ has concavity opposite to that of $(1/y)/(1/x)$. A sigmoid curve must have at least one root of F_3 and an odd number overall. A non-sigmoid curve can have no roots or only an even number of roots of F_3.

Thus, a y/x curve can have multiple inflexions with no corresponding inflexions at all in $(1/y)/(1/x)$, e.g. a



stair-step curve (negative co-operativity followed by a region of positive co-operativity) could give a uniformly concave double-reciprocal plot, which would be interpreted as no change in apparent co-operativity. This is not so surprising when it is considered that a 2:2 sigmoid y/x curve with a maximum has two inflexions, but gives a concave-up $(1/y)/(1/x)$ plot with no inflexions.

Reference

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