A normalized plot as a novel and time-saving tool in complex enzyme kinetic analysis

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A new data treatment is described for designing kinetic experiments and analysing kinetic results for multi-substrate enzymes. Normalized velocities are plotted against normalized substrate concentrations. Data are grouped into $n+1$ families across the range of substrate or product tested, n being the number of substrates plus products assayed. It has the following advantages over traditional methods: (1) it reduces to less than a half the amount of data necessary for a proper description of the system; (2) it introduces a self-consistency checking parameter that ensures the 'scientific reliability' of the mathematical output; (3) it eliminates the need for a prior knowledge of V_{max}; (4) the normalization of data allows the use of robust and fuzzy methods suitable for managing really 'noisy' data; (5) it is appropriate for analysing complex systems, as the complete general equation is used, and the actual influence of effectors can be typified; (6) it is amenable to being implemented as a software that incorporates testing and electing among rival kinetic models.

Key words: data normalization, enzymology, model–fitting, parameter estimation, rate equations.

INTRODUCTION

Graphic and numerical analysis of the experimental data from enzyme-catalysed reactions provides information about the mechanism and the kinetic parameters of the system [1]. Graphic analysis has been the classic tool for enzyme classification and numerical description. The basic Michaelis–Menten equation is a rectangular hyperbola, and this remained fairly unapproachable for direct analysis until non-linear fitting methods had matured and become widespread. It was avoided by resorting to data transformations such as logarithmic, Lineweaver–Burk double-reciprocal, Hanes, Eadie–Hofstee or Eisenthal–Cornish-Bowden direct linear plots [2]. All of these linearize the behaviour of the system and allow its description through a simple linear regression. Although there are differences between the statistical validity of these methods, proper analysis using data weighting and computer methods avoids this problem [2–4].

However, the relationship between the experimental observables and enzymic information is non-linear, and the appropriate methodology for non-linear data management is non-linear least-squares regression [5]. This methodology is less sensitive to the spacing and number of the data, and is a versatile and general curve-fitting procedure [6,7].

It has been proposed that the availability of pre-packaged non-linear data-analysis software should relegate classic linear transformations to only data plotting, while analysis should be performed in a non-linear fashion on the actual untransformed data [5]. However, visual inspection of the data supplies intuitive information about trends in the behaviour of the system and allows one to evaluate the statistical analysis. Indeed, it remains a useful tool in biochemical research and teaching [1,8,9]. It is important to bear in mind that statistical analysis is a more solid tool than experimentation; however, it is the experimental data that in fact reflect reality, and not vice versa, and hence mathematics cannot replace common sense [3]. Emphasis should therefore be placed on the acquisition of good data more than on improving the analysis of poor data [3,6]. In this sense, we here describe a graphic and analytical approach to the study of multi-substrate enzyme kinetics that reduces the amount of data necessary for a proper description of the system to less than half, while at the same time introducing a self-consistency checking parameter that ensures the reliability of the output. This method normalizes the experimental data without the need for prior knowledge of V_{max}, thus allowing the use of tougher methods such as artificial neural networks [10], or robust parameter estimation [11], suitable for managing real normalized noisy data. Moreover, it eliminates the need to replot slopes and/or intersects for characterizing the system.

THEORY

The behaviour of multi-substrate and/or multi-product enzyme-catalysed reactions can be described by using multivariate functions. The usual approach for system characterization requires the determination of velocity values in the presence of all substrates, varying their concentrations sequentially. A system with m substrates therefore requires the construction of an m-dimensional matrix with $\Pi\ n_i$ data, n_i being the number of concentration values at which the i-th substrate is assayed. Taking as an example a Bi Bi system, the assay of six different concentrations for either substrate generates a two-dimensional matrix formed by $6^2 = 36$ experimental data cells. These determinations must be repeated separately for each introduction of each effector to be studied. Thus each introduction of product, inhibitor, analogue, etc. gives rise to an $m+1$ dimensional matrix with $m_j\ \Pi\ n_i$ data, where m_j is the number of concentration values at which the j-th effector is assayed. Fromm [12] has outlined a method for building a five five-by-five substrate concentration matrix, covering a range wide enough to determine rate data. In the Bi Bi system example, the assay of six concentrations for each of both products yields two matrices (one for each product) formed by $6 \times 6^2 = 216$ experimental data cells. Finally, the study of enzyme behaviour in the reverse catalytic sense requires the same treatment.

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Normalization of velocity and substrate concentration: a Bi Bi ordered system as an example

In the absence of products, the velocity equation in the steady-state is given by:

$$v = \frac{V_{max} AB}{K_{iA}K_{mB} + K_{mB}A + K_{mA}B + AB} \quad (1)$$

where, following the Enzyme Commission nomenclature, A is the concentration of substrate A, B is concentration of substrate B, K_{mA} is the Michaelis constant for A, K_{mB} is the Michaelis constant for B, K_{iA} is the inhibition constant for A and V_{max} is the limiting maximum velocity.

We define any A' and B' concentration as a function of two arbitrary values, A and B:

$$A' = aA$$

$$B' = bB$$

This will be the normalized substrate concentration. The velocity in the presence of a times A (arbitrarily fixed) and b times B (arbitrarily fixed), which will be denoted as $V_{a,b}$, is expressed as:

$$V_{a,b} = \frac{V_{max} aAbB}{K_{iA}K_{mB} + K_{mB}aA + K_{mA}bB + aAbB} \quad (2)$$

The velocity when both $a = 1$ and $b = 1$ will be:

$$V_{1,1} = \frac{V_{max} AB}{K_{iA}K_{mB} + K_{mB}A + K_{mA}B + AB} \quad (3)$$

The normalized velocity in the presence of a times A and b times B will be denoted by $\bar{V}_{a,b}$, defined as:

$$\bar{V}_{a,b} = \frac{V_{1,1}}{V_{a,b}} = \frac{K_{iA}K_{mB} + K_{mB}aA + K_{mA}bB + aAbB}{ab \cdot den1} \quad (4)$$

where the parameter *den1* is a combination of the unknown parameters K_{iA}, K_{mA} and K_{mB} and the fixed values A and B, as follows:

$$den1 = K_{iA}K_{mB} + K_{mB}A + K_{mA}B + AB$$

Eqn (4) will be used in the plotting, fitting and interpretation of the results. By means of a proper experimental design it defines a quadratic dependence of the normalized velocity on $1/a$, if $a = b$ and the first term is non-negligible. This fact will differentiate between Bi Bi mechanisms, as the behaviour in a Bi Bi Ping Pong enzyme is given by a linear dependence of the normalized velocity on $1/a$ (see eqn 11 below).

Other authors have already used data normalization before either representation and/or treatment. Hunter and Downs defined fractional activities, when dealing with enzyme inhibitors, as the fraction of activity obtained in the presence of an inhibitor, v, related to the activity found in its absence, v_0 [13]. The values for v_0 are easy to determine. However, this procedure increases data scattering, since two experimental values are combined into one and the individual error associated with v_0 fluctuates. Moreover, the equation defined is a double-reciprocal one like the Lineweaver–Burk equation, and the increase in scatter also generates bias in the fitting [2]. Another data normalization procedure, namely combination plots, was given by Chan [8]. Here, V_{max} replaces the term v_0 in fractional activities, thus avoiding the need to determine v_0 at each substrate concentration. Combination plots are therefore subject to less scattering, because V_{max} is an estimate that is deduced from a number of v_0 values [8]. Nevertheless, combination plots have also been developed for studying enzyme inhibition, and the normalization we propose is likely to be used both in the presence and absence of inhibitors and is useful in the characterization of complex systems. Additionally, all the data are normalized by dividing by the same experimental value, which furthermore is chosen in the design of the experiment so that the associated error is minimal. This procedure circumvents the need of prior knowledge of V_{max} and introduces minimal additional scattering.

Presence of effectors: an inhibitor that binds E as an example

In the absence of products, the presence of an inhibitor that specifically binds to the free form of the enzyme in a Bi Bi ordered system modifies the steady-state equation as follows:

$$v = \frac{V_{max} AB}{K_{iA}K_{mB}\left(1 + \frac{I}{K_i}\right) + K_{mB}A + K_{mA}B\left(1 + \frac{I}{K_i}\right) + AB} \quad (5)$$

The normalized velocity in the presence of a times A and b times B is:

$$\bar{V}_{\substack{a,b \\ I \to E}} = \frac{K_{iA}K_{mB}\left(1 + \frac{I}{K_i}\right) + K_{mB}aA + K_{mA}bB\left(1 + \frac{I}{K_i}\right) + aAbB}{ab \cdot den1} \quad (6)$$

Where $I \to E$ represents the presence of the inhibitor that binds the free form of the enzyme.

We isolate the effect of the presence of I by defining the increment in normalized velocity as:

$$\Delta\bar{V}_{\substack{a,b \\ I \to E}} = \bar{V}_{\substack{a,b \\ I \to E}} - \bar{V}_{a,b} \quad (7)$$

Hence, according to eqns (4) and (6), the increment caused in the normalized velocity by the presence of I will be:

$$\Delta\bar{V}_{\substack{a,b \\ I \to E}} = \frac{\frac{I}{K_i}K_{iA}K_{mB} + \frac{I}{K_i}K_{mA}bB}{ab \cdot den1} \quad (8)$$

RESULTS AND DISCUSSION

Design of the experiment in the absence of products

Three series of experiments are prepared: (1) varying substrate concentrations, keeping $a = b$; (2) varying substrate B concentration, keeping $a = 1$; (3) varying substrate A concentration, keeping $b = 1$.

Thus, when one wishes to assay six concentrations of each substrate, only $(3 \times 6) - 2 = 16$ data need to be collected instead of the 36 required by other methods. The generic number of data for a Bi Bi reaction and n concentrations tested for each substrate will be $3n - 2$ instead of n^2. This is one of the strong points of the normalized plot: the lessened demand on data acquisition. In the classical approach, when the model is known, maximal precision in the determination of the p parameter values is obtained if measurements are made only in p experimental points, but when the model is unknown, it is advisable to make measurements that cover all independent variables at low, intermediate and high values [14]. The advantage of using fewer points in this normalized approach versus fewer points in the classical approach is that all the range of concentrations of substrates or effectors

Table 1 Parameters that describe the normalized velocity equation in different Bi Bi systems, in the absence of products

In (a), $den1 = K_{iA}K_{mB} + K_{mB}A + K_{mA}B + AB$; in (b), $den2 = K_{mB}A + K_{mA}B + AB$.

Term	$a = b$ Series	$a = 1$ Series	$b = 1$ Series
(a) Bi-Bi ordered systems and Bi Bi Theorell–Chance systems			
Second-order	$\frac{K_{iA}K_{mB}}{den1}$		
First-order	$\frac{K_{mB}A + K_{mA}B}{den1}$	$\frac{K_{iA}K_{mB} + K_{mB}A}{den1}$	$\frac{K_{iA}K_{mB} + K_{mA}B}{den1}$
Independent	$\frac{AB}{den1}$	$\frac{K_{mA}B + AB}{den1}$	$\frac{K_{mB}A + AB}{den1}$
(b) Bi Bi Ping Pong systems			
First-order	$\frac{K_{mB}A + K_{mA}B}{den2}$	$\frac{K_{mB}A}{den2}$	$\frac{K_{mA}B}{den2}$
Independent	$\frac{AB}{den2}$	$\frac{K_{mA}B + AB}{den2}$	$\frac{K_{mB}A + AB}{den2}$

is actually covered in the normalized plot design while allowing a proper distance between experimental points. Fewer points in the classical approach would in contrast compromise the quality of the constants obtained and/or would not fully explore the response of the system.

Graphic solutions in the absence of products

When graphic approaches are used, the data are linearized and plotted into families, which correspond to the rows or columns of the data matrices. The tendency of the plotted lines to convergence is studied. It is often necessary to resort to the replotting of slopes, x-intersects or y-intersects in order to characterize the system. Data-normalization procedures have been proposed to ease interpretation, but these have been developed for the presence of inhibitors in a Uni Uni system.

Absence of products in Bi Bi systems

Bi Bi ordered systems

The particular cases of eqn (4) for the three experimental series give rise to the expressions given in Table 1.

$$\bar{V}_{\substack{a,b\\a=b}} = \frac{K_{iA}K_{mB}}{den1}\frac{1}{a^2} + \frac{K_{mB}A + K_{mA}B}{den1}\frac{1}{a} + \frac{AB}{den1}$$

$$\bar{V}_{\substack{a,b\\a=1}} = \frac{K_{iA}K_{mB} + K_{mB}A}{den1}\frac{1}{b} + \frac{K_{mA}B + AB}{den1} \qquad (9)$$

$$\bar{V}_{\substack{a,b\\b=1}} = \frac{K_{iA}K_{mB} + K_{mA}B}{den1}\frac{1}{a} + \frac{K_{mB}A + AB}{den1}$$

Simultaneous plotting of these equations gives the characteristic representation for Bi Bi ordered systems: a parabola for the $a = b$ series, and two straight lines for the $a = 1$ and $b = 1$ series, with a common intersection point at (1, 1). The plots of the $a = b$ and $a = 1$ series also intersect at $1/a = K_{mA}B/K_{iA}K_{mB}$. The plots of the $a = b$ and $b = 1$ series also intersect at $1/a = -(A/K_{iA})$. The parabolic representation identifies the Bi Bi ordered mechanism. This characteristic plot is depicted in Figure 1.

Bi Bi Ping Pong systems

The velocity equation in the steady-state is:

$$v = \frac{V_{max}AB}{K_{mB}A + K_{mA}B + AB} \qquad (10)$$

and the normalized velocity in the absence of products will be:

$$\bar{V}_{a,b} = \frac{K_{mB}aA + K_{mA}bB + aAbB}{ab \cdot den2} \qquad (11)$$

where the parameter $den2$ is defined as:

$$den2 = K_{mB}A + K_{mA}B + AB$$

From the three series of experimental data, enough information will be obtained to discern Ping Pong systems from ordered systems:

$$\bar{V}_{\substack{a,b\\a=b}} = \frac{K_{mB}A + K_{mA}B}{den2}\frac{1}{a} + \frac{AB}{den2}$$

$$\bar{V}_{\substack{a,b\\a=1}} = \frac{K_{mB}A}{den2}\frac{1}{a} + \frac{K_{mA}B + AB}{den2} \qquad (12)$$

$$\bar{V}_{\substack{a,b\\b=1}} = \frac{K_{mA}B}{den2}\frac{1}{a} + \frac{K_{mB}A + AB}{den2}$$

The simultaneous plot of these equations yields the characteristic representation for Bi Bi Ping Pong systems: three straight lines with a common intersection point at (1, 1). This representation is depicted in Figure 2. The parameters that define the plots are given in Table 1.

Choice of the mechanism and acquisition of parameter values from plots

The usual choice between rival models, Bi Bi ordered and Ping Pong, depends on the tendency to convergence or on the parallelism between families of lines. In a Bi Bi ordered system, the plot $1/v$ versus $1/A$ at different fixed B intersect at $1/A = -(1/K_{iA})$. In a Bi Bi Ping Pong system, this double-reciprocal plot yields parallel lines at different B. The family of parallel lines

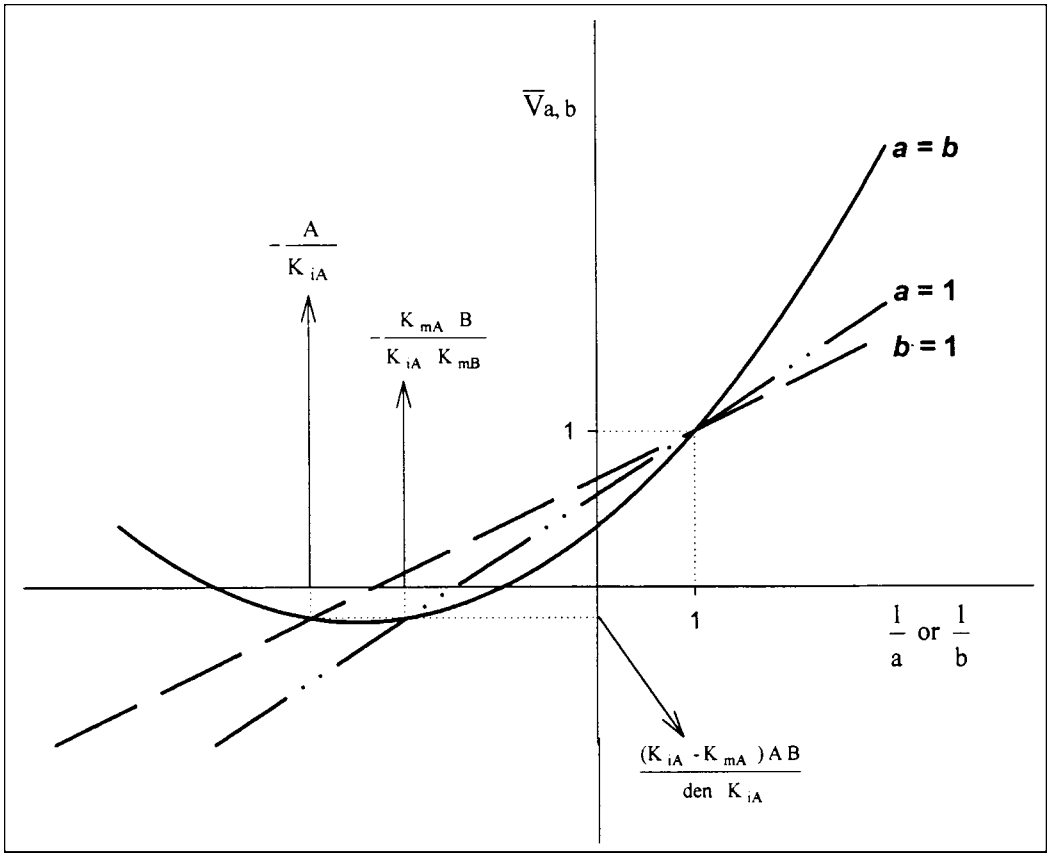

Figure 1 Ideal plot of $\overline{V}_{a,b}$ versus 1/*a* for a Bi Bi ordered system in the absence of products

The three experimental series are indicated. The parabolic representation for the $a = b$ series characterizes the Bi Bi ordered mechanism. The relative position of the $a = 1$ and $b = 1$ series depends on the actual data. The values for the slopes and intersection points are indicated in Table 1.

and the linear reciprocal plot when A and B are varied together identify a Ping Pong system. However, a Bi Bi ordered system with a very small K_{iA} as compared with the K_{mA} would yield the same results. Hence, it is quite possible that the best fit will be parallel lines, and product-inhibition studies will be necessary to distinguish between the two models [15].

When using normalized velocities and normalized substrate concentrations, the difference between Bi Bi Ping Pong and ordered systems is the inclusion of the additional addend $(K_{iA}K_{mB})/den$, responsible for the parabolic representation in a Bi Bi ordered system plot:

$$\overline{V}_{\substack{a,b \\ a=b}} \text{ versus } \frac{1}{a} \tag{13}$$

Thus discriminating between both models becomes a question of deciding whether the difference in squared differences in the fittings, if it exists, is large enough to justify choice of the second-order equation, i.e. the ordered model, instead of the first-order equation, i.e. the Ping Pong model. The option can be tested by using the F-statistic at the desired level of probability [14]. The advantage of this normalized approach versus the situation found when comparing parallel-like lines with the classic initial rate equations arises in the presence of a 'scientific constraint', following the nomenclature from Beechem [5], namely, the secondary parameter *den*. The value obtained for *den* must be checked against the one obtained by means of its definition. The agreement between both values will reflect the 'scientific goodness' of the mathematical fit. This self-consistency criterion would either support or call into question the biological significance of the best fit, making it necessary, if required, to reject the meaningless solution and to search for a mathematically poorer, but enzymically consistent fit.

With the currently available fitting software, we propose the equations for the $a = b$, $a = 1$ and $b = 1$ series to generate an over-determined linear equation system, as follows for a Bi Bi ordered system: α = second-order term in the $a = b$ series; $\beta+\gamma$ = first-order term in the $a = b$ series; δ = zero ordinate in the $a = b$ series; $\alpha+\beta$ = slope in the $a = 1$ series; $\gamma+\delta$ = zero ordinate in the $a = 1$ series; $\alpha+\gamma$ = slope in the $b = 1$ series; $\beta+\delta$ = zero ordinate in the $b = 1$ series.

We have used the following nomenclature for the sake of conciseness: $\alpha = (K_{iA}K_{mB})/den$, $\beta = (K_{mB}A)/den$, $\gamma = K_{mA}B/den$ and $\delta = AB/den$. However, experimental errors will usually generate an incompatible equation system. For this reason, here we propose an improvement in the fitting software; namely simultaneous fitting of the three series of experimental data $a = b$, $a = 1$ and $b = 1$. In this way, the least-squares algorithm would be performed sequentially on the equation for each series, varying the values of α, β, γ and δ at each step to generate a better approximation. The process would then be repeated, applying these better answers as initial estimates until the approximations converge to a stable set of answers [6]. Thus all the experimental data are fitted simultaneously, and the weighted

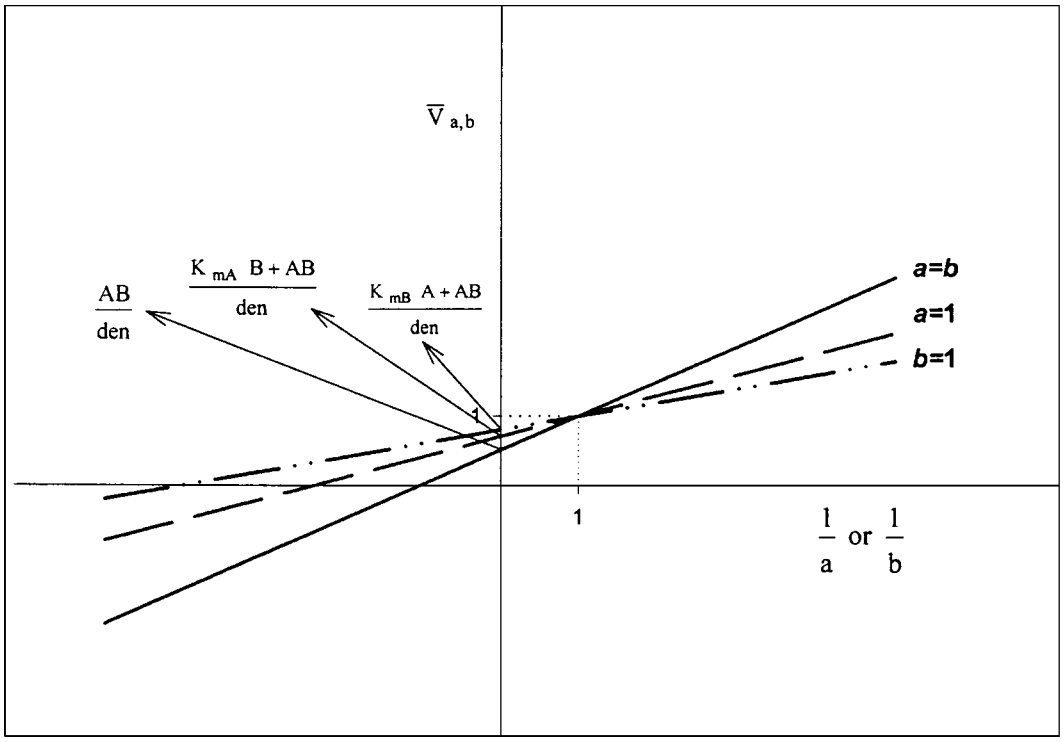

Figure 2 Ideal plot of $\bar{V}_{a,b}$ versus 1/*a* for a Bi Bi Ping Pong system in the absence of products

The three experimental series are indicated. The straight line for $a = b$ series characterizes the Bi Bi Ping Pong mechanism. The relative position of the $a = b$, $a = 1$ and $b = 1$ series depends on the actual data. The values for the slopes and intersection points are indicated in Table 1.

sum of the squared differences is minimized outright. The continuous monitoring of the value of *den* for each loop of the algorithm differentiates this fitting from that used in Cleland's original fitting routines, in which all datasets are fitted simultaneously. This procedure is comparable with the global data analysis proposed by Beechem [5], which performs a series of non-linear analyses, systematically altering each parameter, while adjusting all other fitting parameters so as to obtain the minimum possible χ^2 value. The values of the four parameters that we wish to fix, K_{iA}, K_{mA}, K_{mB} and *den1*, will be obtained from the overall interpretation of the results.

The secondary parameter, *den*, depends on the primary parameters, K_{iA}, K_{mA} and K_{mB}, and will therefore show covariance with all of them. This does not reduce the validity of the fittings, since there is covariance between the primary parameters, unless they are broken down into independent elementary rate constants, because they share these rate constants in their definitions [14]. In addition, the correlation between parameters can be modulated through an intelligent choice of the points on the *x*-axis and appropriate data managing. Moreover, it has been reported that some data transformations improve the acquisition of parameters when a correlation exists between them [16].

Presence of products in Bi Bi systems

Presence of products in a Bi Bi ordered system

In the presence of P and absence of Q, three terms appear in the steady-state velocity equation. When we normalize the concentrations of the substrates and the product, the velocity in the presence of *a* times *A*, *b* times *B* and *p* times *P* ($V_{a,b,p}$) is expressed as:

$$V_{a,b,p} = \frac{V_{max} aAbB}{K_{iA}K_{mB} + K_{mB}aA + K_{mA}bB + aAbB + \frac{K_{mQ}K_{mB}K_{iA}}{K_{iQ}K_{mP}}pP + \frac{K_{mQ}K_{mB}}{K_{iQ}K_{mP}}aApP + \frac{1}{K_{iP}}aAbBpP} \quad (14)$$

In addition, the normalized velocity will be:

$$\bar{V}_{a,b,p} = \frac{K_{iA}K_{mB} + K_{mB}aA + K_{mA}bB + aAbB + \frac{K_{mQ}K_{mB}K_{iA}}{K_{iQ}K_{mP}}pP + \frac{K_{mQ}K_{mB}}{K_{iQ}K_{mP}}aApP + \frac{1}{K_{iP}}aAbBpP}{abp \cdot den3} \quad (15)$$

In this case, the secondary parameter, *den3*, is:

$$den3 = K_{iA}K_{mB} + K_{mB}A + K_{mA}B + AB + \frac{K_{mQ}K_{mB}K_{iA}}{K_{iQ}K_{mP}}P + \frac{K_{mQ}K_{mB}}{K_{iQ}K_{mP}}AP + \frac{1}{K_{iP}}ABP \quad (16)$$

Four experimental series are prepared: (1) varying A, B and P concentrations, keeping $a = b = p$; (2) varying P concentrations,

Table 2 Parameters that describe the increments in the normalized velocity equation in different Bi Bi systems, in the presence of the product P

Term	$a = b$ Series	$a = 1$ Series	$b = 1$ Series
(a) Bi Bi Ordered systems			
Second-order	$\dfrac{\dfrac{K_{mQ}K_{mB}K_{iA}}{K_{iQ}K_{mP}}P}{den1}$		
First-order	$\dfrac{\dfrac{K_{mQ}K_{mB}}{K_{iQ}K_{mP}}AP}{den1}$	$\dfrac{\dfrac{K_{mQ}K_{mB}K_{iA}}{K_{iQ}K_{mP}}P+\dfrac{K_{mQ}K_{mB}}{K_{iQ}K_{mP}}AP}{den1}$	$\dfrac{\dfrac{K_{mQ}K_{mB}K_{iA}}{K_{iQ}K_{mP}}P}{den1}$
Independent	$\dfrac{\dfrac{1}{K_{iP}}ABP}{den1}$	$\dfrac{\dfrac{1}{K_{iP}}ABP}{den1}$	$\dfrac{\dfrac{1}{K_{iP}}ABP+\dfrac{K_{mQ}K_{mB}}{K_{iQ}K_{mP}}AP}{den1}$
(b) Bi Bi Ping Pong systems			
Second-order	$\dfrac{\dfrac{K_{iA}K_{mB}}{K_{iP}}P}{den2}$		
First-order	$\dfrac{\dfrac{K_{mB}}{K_{iP}}AP}{den2}$	$\dfrac{\dfrac{K_{iA}K_{mB}}{K_{iP}}P+\dfrac{K_{mB}}{K_{iP}}AP}{den2}$	$\dfrac{\dfrac{K_{iA}K_{mB}}{K_{iP}}P}{den2}$
Independent			$\dfrac{\dfrac{K_{mB}}{K_{iP}}AP}{den2}$
(c) Bi Bi Theorell–Chance systems			
Second-order	$\dfrac{K_{iB}P}{den1}$		
First-order	$\dfrac{\dfrac{K_{mB}}{K_{iP}}AP}{den1}$	$\dfrac{K_{iB}P+\dfrac{K_{mB}}{K_{iP}}AP}{den1}$	$\dfrac{K_{iB}P}{den1}$
Independent			$\dfrac{\dfrac{K_{mB}}{K_{iP}}AP}{den1}$

keeping $a = b = 1$; (3) varying A concentrations, keeping $b = p = 1$; and (4) varying B concentrations, keeping $a = p = 1$.

Thus, if six concentrations of A, B and P are tested, only $(4 \times 6) - 3 = 21$ data need to be collected, instead of the $6^3 = 216$ necessary in other methods. The generic number of data to be obtained when introducing one product and when testing n concentrations for A, B and P will be $4n - 3$ instead of n^3.

(i) Bi Bi ordered system: presence of P. We shall break down eqn (15) into its addends, as follows:

$$\alpha = \frac{K_{iA}K_{mB}}{den3} \quad \beta = \frac{K_{mB}A}{den3} \quad \gamma = \frac{K_{mA}B}{den3}$$

$$\delta = \frac{AB}{den3} \quad \epsilon = \frac{\dfrac{K_{mQ}K_{mB}K_{iA}}{K_{iQ}K_{mP}}P}{den3} \quad \zeta = \frac{\dfrac{K_{mQ}K_{mB}}{K_{iQ}K_{mP}}AP}{den3} \tag{17}$$

$$\eta = \frac{\dfrac{1}{K_{ip}}ABP}{den3}$$

Each set of experiments gives rise to a different mathematical expression:

$$\bar{V}_{\substack{a,b,p \\ a=b=p}} = \alpha\frac{1}{a^3} + (\beta+\gamma+\epsilon)\frac{1}{a^2} + (\delta+\zeta)\frac{1}{a} + \eta \tag{18}$$

where all the variables, a, b and p, have been renamed as a.

$$\bar{V}_{\substack{a,b,p \\ a=b=1}} = (\alpha+\beta+\gamma+\delta)\frac{1}{p} + (\epsilon+\zeta+\eta)$$

$$\bar{V}_{\substack{a,b,p \\ a=p=1}} = (\alpha+\beta+\epsilon+\zeta)\frac{1}{b} + (\gamma+\delta+\eta) \tag{19}$$

$$\bar{V}_{\substack{a,b,p \\ b=p=1}} (\alpha+\gamma+\epsilon)\frac{1}{a} + (\beta+\delta+\zeta+\eta)$$

Simultaneous plotting of these equations yields a third-order polynomial, $a = b = p$, and three straight lines for the other

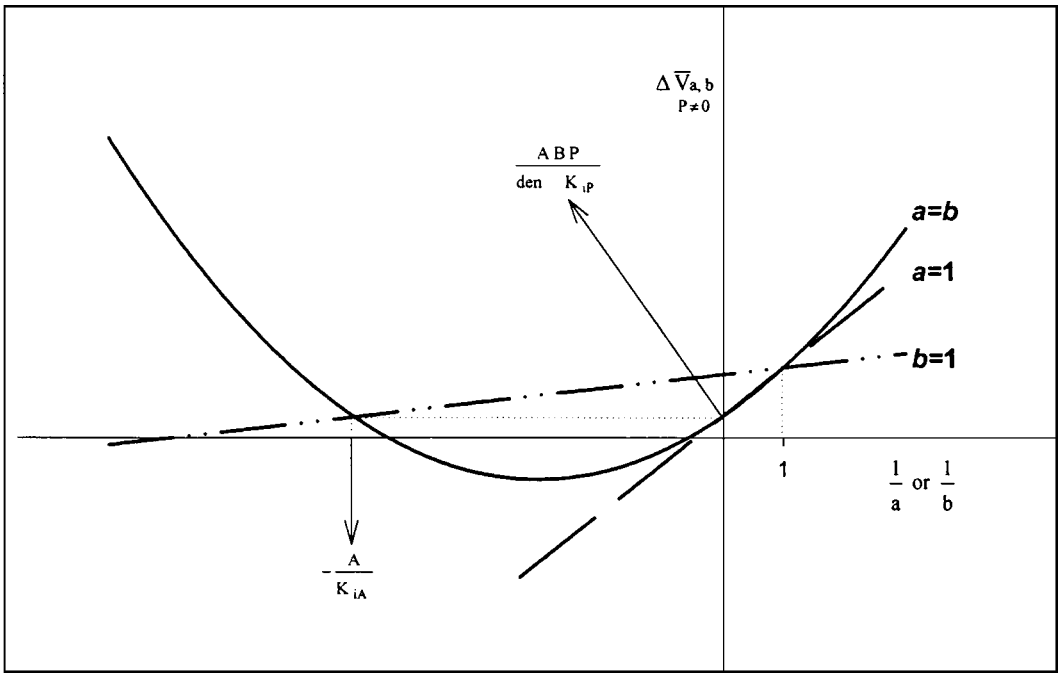

Figure 3 Ideal plot of $\Delta \bar{V}_{\substack{a,b \\ P\neq 0}}$ versus 1/*a* for a Bi Bi ordered system in the presence of the product P

The three experimental series are indicated. The intersection on the *y*-axis for $a = b$ and $a = 1$ series characterizes the inhibition by the product P in an ordered Bi Bi mechanism. The values for the slopes and intersection points are indicated in Table 2.

cases. All of them intersect at (1, 1). From the fitting of the four sets of experiments, we obtain ten linear equations built by the seven parameters to be found; i.e. an over-determined linear equation system that can be resolved. If the proposed simultaneous fitting is performed, the self-consistency achieved will guarantee that the equation system is compatible and determined, and the unknown parameter K_{iP} and the quotient $K_{mQ}/(K_{iQ}K_{mP})$ will be worked out directly from the fitting. Again, the parameter *den3* will give us a clue about the biological significance of the output.

(ii) Bi Bi ordered system: P as an inhibitor. The presence of product P diminishes the velocity and can therefore be studied as an inhibitor. In this case, more experiments are necessary. Three experimental series make up each data family: (1) varying A and B concentrations, keeping $a = b$; (2) varying A concentrations, keeping $b = 1$; and (3) varying B concentrations, keeping $a = 1$.

Each data family is determined at a different concentration of P and is compared with the data in the absence of any product. If six concentrations of A and B are tested, each data family will contain $(3 \times 6) - 2 = 16$ data points. If six concentrations of P are tested, $16 \times 6 = 96$ data points will be collected. This is still far from the $6^3 = 216$ data points necessary in the ordinary approach.

The normalized velocity equation in the presence of a times A and b times B and in presence of P is given by:

$$\bar{V}_{\substack{a,b \\ P\neq 0}} = \frac{K_{iA}K_{mB} + K_{mB}aA + K_{mA}bB + aAbB + \dfrac{K_{mQ}K_{mB}K_{iA}}{K_{iQ}K_{mP}}P + \dfrac{K_{mQ}K_{mB}}{K_{iQ}K_{mP}}aAP + \dfrac{1}{K_{iP}}aAbBP}{ab \cdot den1} \tag{20}$$

The increment in the normalized velocity due to the presence of P will be:

$$\Delta \bar{V}_{\substack{a,b \\ P\neq 0}} = \frac{\dfrac{K_{mQ}K_{mB}K_{iA}}{K_{iQ}K_{mP}}P + \dfrac{K_{mQ}K_{mB}}{K_{iQ}K_{mP}}aAP + \dfrac{1}{K_{iP}}aAbBP}{ab \cdot den1} \tag{21}$$

For the experimental series $a = b$, $a = 1$ and $b = 1$, we obtain three equations whose parameters are given in Table 2. Simultaneous plotting of these increments generates a parabola, the $a = b$ series, and two straight lines, the $a = 1$ and $b = 1$ series, with a common intersection point at:

$$\left(1, \frac{\dfrac{K_{mQ}K_{mB}K_{iA}K_{iP} + K_{mQ}K_{mB}K_{iP}A + K_{iQ}K_{mP}AB}{K_{iQ}K_{mP}K_{iP}}}{den1}P \right) \tag{22}$$

The second intersection point for the plots of the $a = b$ and $a = 1$ series is at $\{0, [(1/K_{iP})ABP]/den1\}$. The second intersection point for the plots of the $a = b$ and $b = 1$ series is at $\{-(A/K_{iA}), [(1/K_{iP})ABP]/\text{den1}\}$. This representation is depicted in Figure 3. From these results, we obtain values for K_{iP} and for the quotient $K_{mQ}/K_{iQ}K_{mP}$.

The parameters that describe the normalized velocity in a Bi Bi ordered system in the presence of Q are given in Table 3.

Table 3 Parameters that describe the increments in the normalized velocity equation in different Bi Bi systems, in the presence of the product Q

Term	$a = b$ Series	$a = 1$ Series	$b = 1$ Series
(a) Bi Bi ordered systems			
Second-order	$\frac{\frac{K_{mB}K_{iA}}{K_{iQ}}Q}{den1}$		
First-order	$\frac{\frac{K_{mA}}{K_{iQ}}BQ}{den1}$	$\frac{\frac{K_{mB}K_{iA}}{K_{iQ}}Q}{den1}$	$\frac{\frac{K_{mB}K_{iA}}{K_{iQ}}Q+\frac{K_{mA}}{K_{iQ}}BQ}{den1}$
Independent		$\frac{\frac{K_{mA}}{K_{iQ}}BQ}{den1}$	
(b) Bi Bi Ping Pong systems			
Second-order	$\frac{\frac{K_{iA}K_{mB}K_{mP}}{K_{mQ}K_{iP}}Q}{den2}$		
First-order	$\frac{\frac{K_{mA}}{K_{iQ}}BQ}{den2}$	$\frac{\frac{K_{iA}K_{mB}K_{mP}}{K_{mQ}K_{iP}}Q}{den2}$	$\frac{\frac{K_{iA}K_{mB}K_{mP}}{K_{mQ}K_{iP}}Q+\frac{K_{mA}}{K_{iQ}}BQ}{den2}$
Independent		$\frac{\frac{K_{mA}}{K_{iQ}}BQ}{den2}$	
(c) Bi Bi Theorell–Chance systems			
Second-order	$\frac{\frac{K_{mA}K_{iB}}{K_{iQ}}Q}{den1}$		
First-order	$\frac{\frac{K_{mA}}{K_{iQ}}BQ}{den1}$	$\frac{\frac{K_{mA}K_{iB}}{K_{iQ}}Q}{den1}$	$\frac{\frac{K_{mA}K_{iB}}{K_{iQ}}Q+\frac{K_{mA}}{K_{iQ}}BQ}{den1}$
Independent		$\frac{\frac{K_{mA}}{K_{iQ}}BQ}{den1}$	

Presence of products in a Bi Bi Ping Pong System

(i) Bi Bi Ping Pong system: presence of P. In the presence of P, two terms appear in the definition of the normalized velocity in a Bi Bi Ping Pong system:

$$\bar{V}_{a,b,p} = \frac{K_{mB}aA + K_{mA}bB + aAbB + \frac{K_{iA}K_{mB}}{K_{iP}}pP + \frac{K_{mB}}{K_{iP}}aApP}{abp \cdot den4} \quad (23)$$

We break down this equation to yield simple addends, as follows:

$$\alpha = \frac{K_{mB}A}{den4} \quad \beta = \frac{K_{mA}B}{den4} \quad \delta = \frac{AB}{den4}$$

$$\delta = \frac{\frac{K_{iA}K_{mB}}{K_{iP}}P}{den4} \quad \epsilon = \frac{\frac{K_{mB}}{K_{iP}}AP}{den4} \quad (24)$$

where the parameter *den4* is defined as:

$$den4 = K_{mB}A + K_{mA}B + AB + \frac{K_{mB}}{K_{iP}}AP + \frac{K_{iA}K_{mB}}{K_{iP}}$$

Thus the four experimental data series generate the following expressions.

$$\bar{V}_{\substack{a,b,p \\ a=b=p}} = (\alpha+\beta+\delta)\frac{1}{a^2} + (\gamma+\epsilon)\frac{1}{a}$$

$$\bar{V}_{\substack{a,b,p \\ a=b=1}} = (\alpha+\beta+\gamma)\frac{1}{p} + (\delta+\epsilon)$$

$$\bar{V}_{\substack{a,b,p \\ b=p=1}} = (\beta+\delta)\frac{1}{a} + (\alpha+\gamma+\epsilon) \quad (25)$$

$$\bar{V}_{\substack{a,b,p \\ a=p=1}} = (\alpha+\delta+\epsilon)\frac{1}{b} + (\beta+\gamma)$$

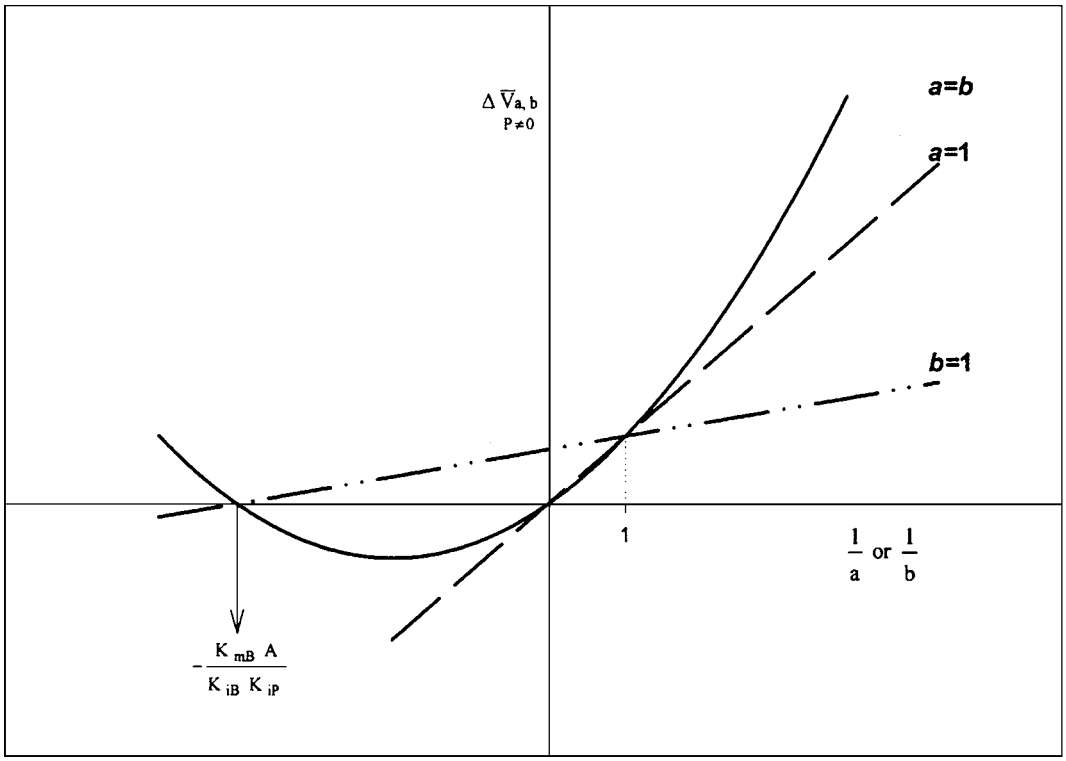

Figure 4 Ideal plot of $\Delta \bar{V}_{\substack{a,b \\ P\neq 0}}$ versus 1/*a* for a Bi Bi Ping Pong system in the presence of the product P

The three experimental series are indicated. The intercept at the origin for the $a = b$ and $a = 1$ series characterizes the inhibition by the product P in a Bi Bi Ping Pong mechanism. The values for the slopes and intersection points are indicated in Table 2.

As in Bi Bi ordered systems, simultaneous plotting of eqn (23) for the four experimental series provides enough information to characterize the system. The plot of the $a = b = p$ series yields a parabola instead of the third-order polynomial obtained from the same series in the Bi Bi ordered system. An over-determined equation system can be defined, and the values for K_{mA}, K_{mB}, K_{iP} and K_{iA} can be obtained from its resolution. The compatibility of the system and the uniqueness of the solution are achieved through the proposed simultaneous fitting. Again, checking of the value for *den4*, found in the solution, and the one obtained by applying its definition, reflects the biological goodness of the output.

(ii) Bi Bi Ping Pong system: P as an inhibitor. The effect of the presence of P on the velocity of reaction is isolated as the increment:

$$\Delta \bar{V}_{\substack{a,b \\ P\neq 0}} = \frac{\frac{K_{mB}}{K_{iP}} aAP + \frac{K_{iA}K_{mB}}{K_{iP}} P}{ab \cdot den2} \tag{26}$$

The same experimental design as in Bi Bi ordered systems is implemented, resulting in three data series. The equations that describe these series are given in Table 2. Simultaneous plotting of eqn (26) in the three experimental series generates a parabola and two straight lines with a common intersection point at $\{1, [(K_{iA} + A)K_{mB}/K_{iP}]/den2 \cdot P\}$. The absence of a zero ordinate in the parabola from the plot of the $a = b$ series differentiates between this Bi Bi Ping Pong system and the parabola in a Bi Bi ordered system, which bears a zero ordinate. The second intersection point for equations in $a = b$ and $b = 1$ is at $(-A/K_{iA}, 0)$. Simultaneous fitting will yield the value for K_{iP}. The overall representation is depicted in Figure 4. The description of the Bi Bi Ping Pong system in the presence of Q is depicted in Table 3.

Presence of products in a Theorell–Chance system

(i) Theorell–Chance system: presence of P. A Theorell–Chance mechanism defines a Bi Bi ordered hit-and-run reaction without a ternary complex. The equation in the absence of products is the same as for an Bi Bi ordered system. It is product inhibition that differentiates between these rival mechanisms. In the presence of P, two terms appear in the definition of the normalized velocity, as follows:

$$\bar{V}_{a,b,p} = \frac{K_{iB}K_{mA} + K_{mA}bB + K_{mB}aA + aAbB + K_{iB}pP + \frac{K_{mB}}{K_{iP}} aApP}{abp \cdot den5} \tag{27}$$

where the term *den5* is defined as:

$$den5 = K_{iB}K_{mA} + K_{mA}B + K_{mB}A + AB + K_{iB}P + \frac{K_{mB}}{K_{iP}} AP \tag{28}$$

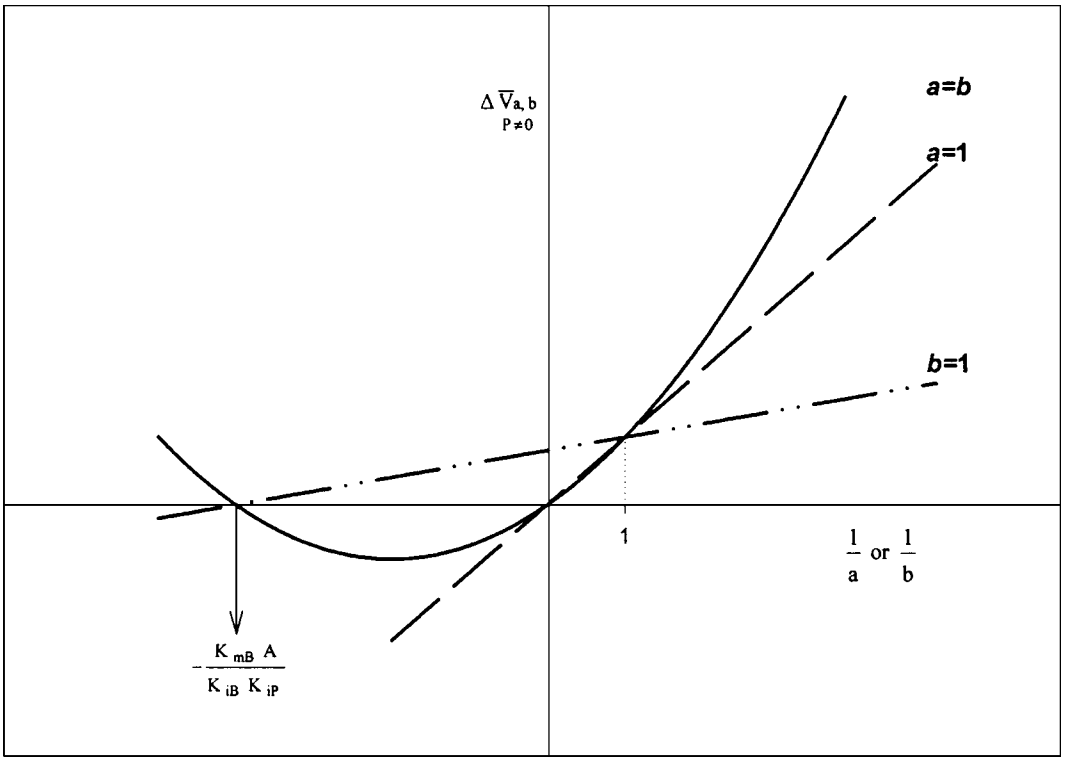

Figure 5 Ideal plot of $\Delta \bar{V}_{\substack{a,b \\ P\neq 0}}$ versus 1/*a* for a Theorell–Chance Bi Bi system in the presence of the product P

The three experimental series are indicated. The intercept at the origin for $a = b$ and $a = 1$ series characterizes the inhibition by the product P in a Theorell–Chance Bi Bi mechanism. The values for the slopes and intersections are indicated in Table 2. This representation is identical with that of a Ping Pong Bi Bi system in the presence of P.

The four experimental series of data give rise to the following equation system:

$$\bar{V}_{\substack{a,b,p \\ a=b=p}} = \alpha\frac{1}{a^3}+(\beta+\gamma+\delta)\frac{1}{a^2}+(\epsilon+\zeta)\frac{1}{a}$$

$$\bar{V}_{\substack{a,b,p \\ a=b=1}} = (\alpha+\beta+\gamma+\delta)\frac{1}{p}+(\epsilon+\zeta) \qquad (29)$$

$$\bar{V}_{\substack{a,b,p \\ a=p=1}} = (\alpha+\gamma+\epsilon+\zeta)\frac{1}{p}+(\beta+\delta)$$

$$\bar{V}_{\substack{a,b,p \\ b=p=1}} = (\alpha+\beta+\epsilon)\frac{1}{p}+(\gamma+\delta+\zeta)$$

where the greek symbols refer to:

$$\alpha = \frac{K_{iB}K_{mA}}{den5} \quad \beta = \frac{K_{mA}B}{den5} \quad \gamma = \frac{K_{mB}A}{den5}$$

$$\delta = \frac{AB}{den5} \quad \epsilon = \frac{K_{iB}P}{den5} \quad \zeta = \frac{\frac{K_{mB}}{K_{iP}}AP}{den5} \qquad (30)$$

Simultaneous plotting will yield a third-order polynomial with no zero ordinate for the $a = b = p$ series, and three straight lines, all of them intersecting at (1, 1). Simultaneous fitting will give the values for K_{mA}, K_{mB}, K_{iB} and K_{iP}, and will, moreover, differentiate between products P and Q, while at the same time ensuring biological consistence through the parameter *den*. Furthermore, it will discern between ordered and Theorell–Chance mechanisms, since the *F*-statistic test can be performed for choosing eqn (15), ordered, or eqn (27), Theorell–Chance, as the best fit.

(ii) Theorell–Chance system: P as an inhibitor. The increment in normalized velocity due to the presence of P is:

$$\Delta \bar{V}_{\substack{a,b \\ P\neq 0}} = \frac{K_{iB}P+\frac{K_{mB}}{K_{iP}}aAP}{ab\cdot den1} \qquad (31)$$

The experimental data series generate the expressions described in Table 2. The results from the fitting will yield the value for K_{iP} and will differentiate between P and Q. Also, the simultaneous plotting of the three equations generates a pattern that is different from a Bi Bi ordered system in the presence of P, thereby allowing one to discern between both mechanisms. The common intersection point for the three plots is at $\{1, [(K_{iB}\cdot K_{iP}+K_{mB}A)/K_{iP}]P/den1\}$. The second intersection point for the plots of the $a = b$ and $b = 1$ series is at $[-(K_{mB}A)/(K_{iB}K_{iP}), 0]$. The overall representation is depicted in Figure 5.

From the overall results, we can establish that the Bi Bi Theorell–Chance systems behave as Bi Bi ordered systems in the absence of products, and as Bi Bi Ping Pong systems in the presence of products, as observed in Tables 2 and 3.

Ter Ter systems

There exist four basic kinetic schemes for a Ter Ter system: one Ter Ter ordered system, and three Ter Ter Ping Pong systems. They can be easily discerned from each other by using normalized

velocity in the kinetic studies, while considerably reducing the number of experimental points to be determined. The studies of product inhibition with only one product will sharply differentiate between rival models. Solely the Hexa Uni Uni Ping Pong system, because of its inherent symmetry, needs further studies with analogues in order to determine the actual reaction pattern.

In the case of a Ter Ter ordered system in the absence of products, the experimental approach in the absence of products require the acquisition of four series of data: $a = b = c$, $a = b = 1$, $a = c = 1$ and $b = c = 1$. If six concentrations of each substrate will be tested, then $(6 \times 4) - 3 = 21$ data points will be enough for a proper description of the system, instead of the $6^3 = 216$ necessary when ordinary kinetic methods are used.

When the product P is introduced in the experimental design, five experimental series are needed to completely characterize the system: $a = b = c = p$, $b = c = p = 1$, $a = c = p = 1$, $a = b = p = 1$, and $a = b = c = 1$. This means that $(6 \times 5) - 4 = 26$ data will give a proper description of the system, instead of the $6^4 = 1296$ necessary when six concentrations of each substrate and product will be tested. It has been claimed that it is not strictly necessary to sweep on the whole experimental design space to achieve reliable results [17], but the economy in the practical approach of this normalized plot is anyway obvious.

This dramatic reduction in the amount of experimental data necessary for an appropriate description of a Ter Ter system illustrates the utility of the normalized plot, and opens a way for accelerating the characterization of complex enzyme systems.

Substrate inhibition

Substrate inhibition appears when any substrate binds the wrong form of the enzyme. This binding can generate a dead-end complex which sequesters the active enzyme, and is usually only apparent at high substrate concentrations and/or when the reaction is studied in the non-physiological sense [15]. Substrate inhibition is often avoided in the design of the experiments because of the additional non-linearity it introduces in the behaviour of the enzyme. Its study frequently needs interpolation and/or extrapolation from secondary plots, which in turn introduce uncertainty in the obtention of the parameters. Furthermore, the customary approach requires using separately low and high concentrations of the inhibitory substrate, as if the system showed sharp biphasic behaviour. Reality is usually very different, and estimation of asymptotes from non-linear double inverse plots is associated with high error levels [2].

The use of the normalized plot and simultaneous fitting opens a way for obtaining useful information from substrate-inhibited enzymes. When this approach is used, the inhibited enzyme form is easily identified, which could reflect changes in the enzyme conformation depending on the intermediate enzyme forms. The relationship between conformational changes occurring during the course of the reaction and changes in the enzyme activity can therefore be predicted for further studies. Moreover, the presence of substrate inhibition breaks down the inner symmetry inherent to Ping Pong systems. This facilitates the classification of the reaction sequence, since not only can the pairs AP and BQ be identified but, also, the A and B substrates can be discerned. Thus product inhibition should not be regarded as a situation to be avoided, but rather as a source of extra information that should be exploited. The accompanying paper [18] is an example of the information that can be achieved by using the normalized approach in the description of a substrate-inhibited enzyme.

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Conclusions

A new data-managing method based on velocity and substrate concentration normalization has been described. We have shown that this dramatically reduces the amount of experimental data necessary for a proper description of the system and provides a global statistical fitting algorithm to select kinetic models and extract constants. This reduction is greater for larger mechanisms. Thus, in the study of a Ter Ter reactant system in the presence of one of the products, 26 data points will give a correct description of the system, versus the 1296 data points required with the conventional approach, using 6×6^2 substrate matrices. In addition, a numeric constraint is introduced so that 'scientific goodness' of the best fit can be tested, therefore allowing one to reject a good but inconsistent fit. Visual inspection of the results yields easy discrimination between complex rival kinetic models, so that the intuitive aspects of the classical approaches are not lost in the normalized method. Also, if suitable software can be developed, the analytical treatment will help in the acquisition of reliable kinetic data. The proposed normalized plot is therefore likely to be useful when dealing with complex systems, inhibition and binding studies, and other possible applications not discussed here, such as integrated rate equations. Moreover, the normalized output is amenable to analysis by means of more robust methods, such as artificial neural networks.

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