Is It Possible to Reduce the Number of Exponential Terms in the Equations that Describe the Kinetic Behaviour of an Enzyme System? A General Solution

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Abstract

In many enzyme-catalysed reactions, the time-course equation for the concentration of the involved species consists in a polynomial part, generally up to a degree of two, and a multi-exponential part, which generally contains several exponential terms. The accurate fit of the experimental time-progress curves to these equations, done to evaluate the kinetic parameters involved, can become very difficult if the number of exponential terms in the equation exceeds two. To circumvent the difficulties that arise from multi-exponential kinetic behaviours, multi-exponential equations are often approached and reduced to uni- or bi-exponential expressions. This reduction is possible provided that one or two of the exponential terms is assumed to be much larger than the others from a short reaction time after the onset of the reaction. Uni- or bi-exponential equations are easy to fit, and therefore it is more suitable to suggest experimental designs and kinetic data analyses. We herein present a general procedure that can be applied to any enzyme system described by a multi-exponential equation, which allowed us to

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obtain the expressions for the amplitudes and arguments involved in the exponential terms of the resulting reduced equation. Finally, the conditions under which this approach can be applied, and given the relationships between the individual rate constants and the initial ligand species concentrations, are discussed. To support our analysis and to verify its goodness, we use the Sequential Ordered Ter-Ter-Theorell Chance mechanism by way of example.

1. Introduction

The starting point of this contribution is strict multi-exponential transient phase equations for enzyme systems which fit the model described in different contributions [1-9]. For this model, to whose fit most enzyme systems, general and systematic methods have been proposed, which allow the manual [1, 2, 10], or both manual and computerised [3, 6], derivation of the time course equations that correspond to the concentration of the species involved. In most systems, these equations are multi-exponential, and are uni-exponential or bi-exponential in very simple reaction mechanisms.

In early contributions [11, 12], strict transient phase equations have been derived for the enzyme reactions that fit a model of enzyme reactions, in which the interconversions between the involved enzyme forms are reversible and of a first- or pseudo-first order. Galvez and Varon [1] extended the studies of Darvey [11, 12] to a more general model by including irreversible steps, e.g., inactivations or irreversible inhibitions, and they derived the corresponding time-course equations for the extended model. These equations are considered in the present contribution. Later [3, 6], the equations derived by Galvez and Varon [1] were improved by expressing them according to the quantities that can be systematically obtained, and the software that allows a quick easy acquisition of time-course equations was also provided. These equations are multi-exponential and too complex to be of practical interest due to the large number of exponential terms involved.

The first step of the strategy followed to evaluate the kinetic parameters involved in a scheme of enzyme reactions from the resulting multi-exponential transient phase equation for some involved species, generally a product of the reaction, is the experimental monitoring of the time-course concentration of the species. Next experimental kinetic data are fitted to the corresponding kinetic equation and some, or all, of the kinetic parameters are evaluated. In some cases, a first- or second-order dynamics is not enough to describe all the required phenomena in compartmental or enzyme models. Nevertheless, fitting to complex models with too many parameters is generally a poor measure as it can exaggerate minor fluctuations in the data. Even when the fitted model does not have too many parameters, the fitted

relationship is not expected to perform as well in a new data set than in the data set used for fitting [13, 14]. Conversely, uni- or bi-exponential equations are easily fitted to experimental kinetic data and are, therefore, much more appropriate for suggesting experimental designs and kinetic data analyses. So in order to circumvent these difficulties, multi-exponential equations are often approached and reduced to uni- or bi-exponential expressions, as long as the contributions from one (or two) of the exponential terms are much larger than those from the other terms for a short reaction time after the onset of the reaction [11, 12, 15-17]. In these works, however, reducing equations to uni- or bi-exponential equations has been carried out on an individual and independent basis for specific mechanisms, which does not allow generalisation to other mechanisms. Moreover, the conditions that enable such a reduction possible have not been provided.

The literature available on the model simplification of linear kinetic systems is considerable. Some well-known solutions based on, e.g. reducing the number of species/reactions and/or on the separation of fast and slow dynamics, have been reported [18, 19]. The aim of this work is to present a general procedure to reduce multi-exponential enzyme kinetic equations that differ from common existing approaches that is applicable to any enzyme system, and to obtain the expressions for the amplitudes, arguments and coefficients involved in the resulting reduced equations. Two strategies can reduce the number of exponential terms so that the resulting equations are easier to handle: 1) obtaining the reduced equation according to the assumptions that (i) some of the first- or pseudofirst-order rate constants are nearly of the same order of magnitude, and (ii) they are also much larger than the others [3, 6]; and 2) choosing the number of exponential terms that must appear in the reduced equations, and determine the relationships between the rate constants in order to achieve it.

2. Materials and methods

To obtain the conditions under which reducing the number of exponential terms can be assumed, a general polynomial theory and the relationships between the zeros of a polynomial and its coefficients, i.e. the so-called Euler's relationships, are applied.

Simulated progress curves are obtained by numerical integration of the set of differential equations that describe the kinetics of the reaction that evolves according to the corresponding mechanism in Scheme 3, and by also using values of the rate constants and

initial concentrations chosen arbitrarily, but within a realistic range. This numerical solution can be obtained by using the classical fourth-order Runge–Kutta formula, but by applying an adaptive step-size control, originally invented by Fehlberg [20-22], using the WES software implemented in Visual C++ 6.0 and other computer programs developed by our group [23-26]. The WES programme runs on a Dell workstation with 16 GB of RAM. The plots of the data obtained from the numerical integration, the plots of the equations made in Figs. 2 and 3, and the fittings of the simulated progress curves to Eq. (99), are carried out by the Sigma Plot Scientific Graphing System programme, v. 8.02 (2002, SPSS Inc).

To obtain the strict time-course equation of product P involved in Schemes 1 and 3, we apply the TRAPHAER [6] or the SKEE-w2013 [9] software, available together with the WES software, on http://oretano.iele-ab.uclm.es/~BioChem-mg/software.php.

3. Theory

3.1. The model of enzyme systems

The general model of the enzyme reaction to which the reduction process is applied has been previously described [1-9]. It consists of n enzyme species denoted arbitrarily by X_i ($i = 1,2,...,n$) (with the only restriction that X_1 is the free enzyme), and g ligand species (products, substrates, inhibitors and activators) denoted arbitrarily as Y_1, Y_2,..., Y_g; e.g. the conversion of any enzyme species, X_i into another, X_j, may be reversible or not. The corresponding *individual* rate constants are denoted by $k_{i,j}$.

We assume that the only enzyme species present upon the onset of the reaction is the free enzyme, X_1, and its initial concentration is $[E]_0$. In addition, the concentration of a ligand species Y_s ($s = 1,2,...,g$), which reacts with any enzyme species, remains nearly constant throughout the reaction. Under these conditions, any reaction step of the model is either of a first- or pseudo-first order. $K_{i,j}$ denotes $k_{i,j}$ or $k_{i,j}[Y_s]_0$ depending on whether the conversion of enzyme species X_i into X_j is of first or pseudo-first order, respectively. In some mechanisms, two steps or more between a pair of enzyme forms, i.e. parallel steps, can occur [27]. In this case, the first- or pseudo-first order rate constant involved in the parallel steps is denoted by numbered (or primed) symbols: $K_{i,j} = K_{i,j}(1) + K_{i,j}(2) + ...$ In these cases, the corresponding $K_{i,j}$ does not mean a first- or pseudo-first rate constant, but a sum of two of these rate constants or

more, each denoted by a numbered $K_{i,j}$, e.g. $K_{i,j}$(number). Thus, $K_{i,j}$(number) denotes $k_{i,j}$(number) or $k_{i,j}$(number)$[Y_s]_0$ depending on whether the conversion of enzyme species X_i into X_j via the number-th parallel step is of first or pseudo-first order, respectively.

In this paper, the term *rate constant* is used for (i) a first- or a pseudo-first order rate constant and coincides with a constant $K_{i,j}$ if there are no parallel steps between X_i and X_j, but also for (ii) with a numbered $K_{i,j}$(number) if the corresponding rate constant is associated with a parallel step. Thus when referring to *rate constant $K_{i,j}$*, or merely to $K_{i,j}$, this constant can be a rate constant or a sum of rate constants (in the case of the parallel steps between X_i and X_j). For more details about the model, see references [1-9]. If a rate constant is individual, they are specifically expressed as such.

Most enzyme reactions can be described by this enzyme model, which takes into account the presence of reversible or irreversible steps, parallel steps, loops, irreversible modifications, activations, etc., provided that the interconversions between the different involved enzyme species are of first or pseudo-first order.

3.2. Additional notation

For a better understanding of the reduction procedures presented below, this work provides a reduced set of the symbols that appear. More details about the genesis and how these quantities are obtained are provided extensively in the literature about transient phase equations of enzyme systems.

$D(\lambda)$: a secular determinant of the set of n differential linear equations with constants coefficients [the constants $K_{i,j}$ $(i,j = 1,2,...,n)$] that describe the kinetic of the enzyme species in the reaction mechanism under study. The expansion of this determinant yields:

$$D(\lambda) = (-1)^n \lambda^c T(\lambda) \tag{1}$$

where:

$$T(\lambda) = \sum_{q=0}^{u} F_q \lambda^{u-q} \tag{2}$$

Thus c and u are the number of null and non-null roots of $D(\lambda)$, respectively, and their values depend on the specific reaction mechanism. Obviously, $n = c + u$. The non-null roots of $D(\lambda)$ are the roots of polynomial $T(\lambda)$, i.e. the roots of the equation:

$$\sum_{q=0}^{u} F_q \lambda^{u-q} = 0 \tag{3}$$

F_q ($q=0,1,2,...,u$): coefficient F_0 is always unity, i.e.:

$$F_0 = 1 \tag{4}$$

Coefficient F_1 is the sum of all the rate constants $K_{i,j}$ ($i \neq j$):

$$F_1 = \sum_{\substack{i,j=1 \\ i \neq j}}^{n} K_{i,j} \tag{5}$$

The other coefficients F_q ($q = 2,3,...,u$) consist of one term, or a sum of terms, and each one is a product of q different rate constants $K_{i,j}$'s, so they had a different first subindex without including the symmetric or ring combinations of $K_{i,j}$'s. The symmetric combinations of $K_{i,j}$'s are those of the $K_{a,b}K_{b,a}$-type while the ring combinations of $K_{i,j}$'s are of the $K_{a,b}K_{b,c}....K_{v,w}K_{w,a}$-type ($K_{a,b}$, $K_{b,c}$,..., $K_{v,w}$ and $K_{w,a}$ can be anywhere in the term). Note that the u-value coincides with the maximal number of $K_{i,j}$'s in a term of the above characteristics and, therefore, also with the value of the subindex of the last F_q.

$\lambda_1, \lambda_2,..., \lambda_u$: roots of polynomial $T(\lambda)$. These roots are real and negative, or complex with a negative real part [1, 21, 28]. Moreover, the following relationships from the polynomial theory are fulfilled:

$$\left.\begin{aligned}
&\lambda_1 + \lambda_2 + \cdots + \lambda_u = -F_1 \\
&\lambda_1\lambda_2 + \lambda_1\lambda_3 + \cdots + \lambda_{u-1}\lambda_u = F_2 \\
&\quad\vdots \\
&\lambda_1\lambda_2 \cdots \lambda_m + \lambda_1\lambda_2 \cdots \lambda_{m+1} + \cdots + \lambda_{u-m}\lambda_{u-m+1} \cdots \lambda_u = (-1)^m F_m \\
&\lambda_1\lambda_2 \cdots \lambda_{m+1} + \lambda_1\lambda_2 \cdots \lambda_{m+2} + \cdots + \lambda_{u-m-1}\lambda_{u-m} \cdots \lambda_u = (-1)^{m+1} F_{m+1} \\
&\lambda_1\lambda_2 \cdots \lambda_{m+2} + \lambda_1\lambda_2 \cdots \lambda_{m+3} + \cdots + \lambda_{u-m-2}\lambda_{u-m-1} \cdots \lambda_u = (-1)^{m+2} F_{m+2} \\
&\quad\vdots \\
&\lambda_1\lambda_2 \cdots \lambda_u = (-1)^u F_u
\end{aligned}\right\} \tag{6}$$

Coefficients $f_{i,q}$ $(i = 1,2,...,n; q = 0,1,2,...,u)$:

These coefficients can be zero or positive.

In particular, the $f_{i,0}$ -values $(i = 1,2,..., n)$ are:

$$f_{i,0} = \begin{cases} 1 & \text{if } i = 1 \\ 0 & \text{if } i > 1 \end{cases} \tag{7}$$

If coefficients $f_{i,q}$ $(i=1,2,...,n; q=1,2,...,u)$ are not zero, they consist in one term, or a sum of terms, with each one being the product of q $K_{i,j}$'s. The definition of these coefficients and the corresponding coefficient F_q has been well explained in previous contributions by our group [6, 9, 29]. It is possible to obtain coefficients $f_{i,q}$ easily from F_q, as explained in these works.

3.3. Time-course equations

3.3.1. Enzyme species

We employ the strict transient phase equations by Garcia Meseguer et al. [29] and those by Varon et al. [6]. These equations, where $[X_i]$ is used to denote the instantaneous concentration of any enzyme species are, after a minor adaptation of the existing notation:

$$[X_i] = A_{i,0} + \sum_{h=1}^{u} A_{i,h} e^{\lambda_h t} \qquad (i=1,2,...,n) \tag{8}$$

where:

$$A_{i,0} = \frac{f_{i,u}[\text{E}]_0}{F_u} \qquad (i=1,2,...,n) \tag{9}$$

$$A_{i,h} = \frac{(-1)^{u+1}[\text{E}]_0 \sum\limits_{q=0}^{u} f_{i,q} \lambda_h^{u-q}}{\lambda_h \prod\limits_{\substack{p=1 \\ p \neq h}}^{u} (\lambda_p - \lambda_h)} \qquad (i=1,2,...,n; h=1,2,...,u; \text{If } u=1, \text{ then denominator is } \lambda_1) \tag{10}$$

3.3.2. Ligand species

From the contribution made by Varon et al. [6], after making minor changes in notation, when using $[Y_s]$ to denote the instantaneous concentration of any ligand species, we obtain:

$$[Y_s] - [Y_s]_0 = \beta_s + \alpha_s t + \sum_{h=1}^{u} \gamma_{s,h} e^{\lambda_h t} \quad (s=1,2,...,g) \tag{11}$$

where:

$$\alpha_s = \frac{N_{s,u}[E]_0}{F_u} \qquad (s = 1,2,...,g) \tag{12}$$

$$\beta_s = \frac{N_{s,u-1}[E]_0}{F_u} - \frac{F_{u-1}}{F_u} \alpha_s \qquad (s = 1,2,...,g) \tag{13}$$

$$\gamma_{s,h} = (-1)^{u+1} [E]_0 \frac{\displaystyle\sum_{q=0}^{u} N_{s,q} \lambda_h^{u-q}}{\lambda_h^2 \displaystyle\prod_{\substack{p=1 \\ p \neq h}}^{u} (\lambda_p - \lambda_h)} \quad (s = 1,2,...,g;\ h=1,2,...,u) \ \ \text{(If } u=1, \text{ then denominator is } \lambda_1^2) \tag{14}$$

In Eqs. (12)-(14), coefficients $N_{s,q}$ $(s = 1,2,...,g;\ q = 0,1,2,...,u)$ are provided by:

$$N_{s,q} = \sum_{(i,j)} [K_{j,i} f_{j,q} - K_{i,j} f_{i,q}] \tag{15}$$

In Eq. (15), the summation limit extends to all pairs of values (i,j), where i and j are the subindices of enzyme species X_i and X_j that participate in one step or more, where X_i reacts with ligand species Y_s $(s = 1,2,...,g)$ in a reversible or irreversible way to yield X_j (by releasing another ligand species or not). Note that the brackets of Eq. (15) contain two terms separated by a minus sign, which are respectively related to the formation and consumption of ligand species Y_s in this step. For a given pair (i,j), neither of these two terms is null if the step is reversible. However, if the step is irreversible, one of these two terms is zero. In the pair (i,j), $K_{j,i}$ $(K_{i,j})$ is numbered if the corresponding step in which Y_s is formed (or consumed) belongs to a set of parallel steps by connecting X_j (X_i) with X_i (X_j).

In Eqs. (8) and (11), coefficients $A_{i,h}$ and γ_h $(h = 1,2,…,n)$ are the *amplitude* of the corresponding exponential term with *argument* λ_h $(h = 1,2,…,n)$. So the following sections refer to λ_h $(h = 1,2,…,n)$ as a root of Eq. (3) [or of polynomial $T(\lambda)$ in Eq. (2)], or as an argument in Eqs. (8) or (11).

3.4. Reducing the number of exponential terms in a multi-exponential equation

Two different procedures to reduce the number of exponential terms from a multi-exponential kinetic equation by providing the time course of the concentration of the species involved in an enzyme reaction are proposed. In many of the following equations, symbol $\simeq$ that separates both sides of the equations is more appropriate than symbol $=$, used for simplicity reasons.

3.4.1. Procedure A

Reduction is due to some first or pseudo-first rate constants being nearly of the same order of magnitude and much larger than the remaining constants. The statement that rate constants are of the same order of magnitude means that the quotient of any two of them goes neither to 0 nor to ∞; e.g. they are of the same infinite order. In this paper these rate constants, which are much larger than others and are not very different mutually, are denominated as a *large rate constant*. We assign the value 1 to the infinite order of the *large rate constants* and the value 0 to the remaining rate constants. For more details on the infinite order, see [30]. Thus any rapid equilibrium assumptions require these conditions being fulfilled:

$$\left.\begin{array}{l} \textit{large rate constants} \gg \text{ all the other constants} \\ \textit{large rate constants} \text{ are of the same infinite order} \end{array}\right\} \qquad (16)$$

The general conditions (16) are expressed in more suitably for the purpose of this contribution as:

$$\left.\begin{array}{l} \textit{large rate constants} \rightarrow \infty \\ \textit{large rate constants} \text{ are of the same infinite order} \end{array}\right\} \qquad (17)$$

Thus reduced transient phase equations derive from the strict transient phase equations by introducing the conditions (17) into them; e.g.:

reduced transient phase equation = $\lim\limits_{conditions(17)}$ strict transient phase equations $\qquad$ (18)

We denote the number of *large rate constants* involved in a reaction mechanism by m. The value $m = 0$ corresponds to strict equations. Any other m-value leads to a reduced transient phase equation.

3.4.1.1. Effect of conditions (17) on the different coefficients and parameters involved in strict transient-phase equations.

We now go on to study how the insertion of conditions (17) affects the strict transient-phase equations; that is, the effect on the different coefficients and parameters that appear in strict equations.

Effect on coefficients F_q ($q = 0,1,2,...,u$) and the roots of polynomial $T(\lambda)$

The insertion of conditions (17) into F_0, equal to the unity, has no effect. Nevertheless with the other coefficients F_q ($q=1,2,...,u$), there may be some terms of the coefficient that contain *smaller rate constants* than other terms of the same coefficient, which means that such terms may be neglected. Given the nature of these coefficients and their systematic formation law [3, 5, 6, 22, 24], we find:

1) m coefficients F_1, F_2, ..., F_m ($m \leq u$) have at least one term with 1, 2,...,m *large rate constants*, respectively.

2) Coefficients F_{m+1}, F_{m+1},..., F_u do not contain terms with more than m *large rate constants* because, otherwise, a symmetric or ring combinations of $K_{i,j}$'s would exist in that term.

3) After eliminating any negligible terms from a coefficient F_q ($q=1,2,...,u$), the resulting coefficient consists of one term or more that (all) contain(s) the q of the *large rate constants* if $q \leq m$, and a maximum of m of these constants if $q \geq m$. We denote these resulting coefficients as $F_q^{\cdot}$ ($q = 0,1,2,...,u$; $F_0^{\cdot} = F_0 = 1$):

$$F_q^{\cdot} = \lim\limits_{conditions(17)} F_q \qquad\qquad (q=0,1,2,...,u) \qquad (19)$$

As $F_0^{\cdot}=1$, we assign an infinite order 0 to $F_0^{\cdot}$. If we assign an infinite order 1 to a *large rate constant* and an infinite order of 0 to a finite rate constant, the infinite order of the resulting coefficients $F_q^{\cdot}$ $(q=1,2,\ldots,u)$ after applying conditions (17) are:

$$\text{infinite order of } F_q^{\cdot} = \begin{cases} q \text{ if } q \le m \\ \text{at the most } m \text{ if } q > m \end{cases} \qquad (q=1,2,\ldots,u) \qquad (20)$$

From the above results, we obtain:

$$\frac{F_q^{\cdot}}{F_m^{\cdot}} \to 0 \ \ \text{if } q < m \qquad\qquad (m \le u) \qquad (21)$$

and

$$\frac{F_q^{\cdot}}{F_m^{\cdot}} = \begin{cases} 1 \text{ if } q = m \\ \text{a finite quantity if } q > m \text{ and the infinite order of } F_q^{\cdot} \text{ is } m \\ \text{an infinitesimal quantity if } q > m \text{ and the infinite order of } F_q^{\cdot} \text{ is } < m \end{cases} \qquad (m \le u) \quad (22)$$

As explained below, polynomial $T(\lambda)$, which results after applying conditions (17), usually has *finite* and *infinite roots*; i.e. roots with a finite or infinite absolute value. *Infinite roots* have, if they exist, an infinite order of 1, which is the same as that of $F_1^{\cdot}$, e.g., the same as that of the *large rate constants*, and as deduced from the first of Eqs. (6). *Finite roots* have an infinite order of 0. In short:

$$\text{infinite order of the roots of } T(\lambda) = \begin{cases} 0 \text{ if the root is finite} \\ 1 \text{ if the root is infinite} \end{cases} \qquad (23)$$

An *infinite root* means that it is much larger, in absolute values, than *finite* roots.

If in Eq. (2) we take into account Eq. (19) and we divide both sides by $F_m^{\cdot}$, it can be rewritten as:

$$\sum_{q=0}^{u} \frac{F_q^{\cdot}}{F_m^{\cdot}} \lambda^{u-q} = 0 \qquad\qquad (24)$$

According to Eq. (21) the terms $\dfrac{F_q^\cdot}{F_m^\cdot}\lambda^{u-q}$ for $q < m$ can be neglected in Eq. (24) which becomes:

$$\sum_{q=m}^{u}\frac{F_q^\cdot}{F_m^\cdot}\lambda^{u-q}=0 \qquad \text{(when attempting to obtain \textit{finite roots})} \qquad (25)$$

Therefore, polynomial $T(\lambda)$ has $u-m$ *finite roots,* and consequently m *infinite roots*.

If $u-m = w,$ the w *finite* roots of $T(\lambda)$ are denoted as $\lambda_1, \lambda_2,...,\lambda_w$ and the remaining m *infinite roots* as $\lambda_{w+1},\lambda_{w+2},...,\lambda_u.$ The infinite order of roots $\lambda_1, \lambda_2,...,\lambda_w$ is 0 and that of roots $\lambda_{w+1},\lambda_{w+2},...,\lambda_u$ is 1 since the sum of u roots equals minus the sum of the rate constants, which are an order of the infinite of 0 or 1.

If λ_i is an *infinite root* ($i=w+1,\ w+2,...,u$) and λ_j a *finite* one ($j=1,2,...,w$), then:

$$\lambda_i + \lambda_j = \lambda_i \qquad (i=w+1,\ w+2,...,u;\ j=1,2,...,w) \qquad (26)$$

$$\lambda_i - \lambda_j = \lambda_i \qquad (i=w+1,\ w+2,...,u;\ j=1,2,...,w) \qquad (27)$$

$$\left|\lambda_i\right|\to\infty \quad (\lambda_i\to-\infty \text{ if } \lambda_i \text{ is real}) \qquad (i=w+1,\ w+2,...,u) \qquad (28)$$

If on both sides of all Eqs. (6) the terms with an infinite order less than that of other terms of the same side are neglected, after some rearrangements we obtain:

$$\lambda_{w+1}+\lambda_{w+2}+\cdots+\lambda_u = -F_1^\cdot \qquad (29)$$

$$\lambda_{w+1}\lambda_{w+2}+\lambda_{w+1}\lambda_{w+3}+\cdots+\lambda_{u-1}\lambda_u = F_2^\cdot \qquad (30)$$

$$\lambda_{w+1}\lambda_{w+2}\cdots\lambda_u = (-1)^m F_m^\cdot \qquad (31)$$

$$\left(\lambda_1 + \lambda_2 +\cdots+\lambda_w\right)\lambda_{w+1}\lambda_{w+2}\cdots\lambda_u = (-1)^{m+1} F_{m+1}^\cdot \qquad (32)$$

$$\left(\lambda_1\lambda_2 + \lambda_1\lambda_3 +\cdots+\lambda_{w-1}\lambda_w\right)\lambda_{w+1}\lambda_{w+2}\cdots\lambda_u = (-1)^{m+2} F_{m+2}^\cdot \qquad (33)$$

$$\lambda_1\lambda_2\cdots\lambda_w\lambda_{w+1}\lambda_{w+2}\cdots\lambda_u = (-1)^u F_u^\cdot \qquad (34)$$

In turn, from Eqs. (27) and (31) we acquire:

$$\prod_{\substack{p=1\\p\neq h}}^{u}\left(\lambda_p-\lambda_h\right)=\begin{cases}(-1)^m F_m^{\bullet}\prod_{\substack{p=1\\p\neq h}}^{w}\left(\lambda_p-\lambda_h\right) & \text{if } w>1\\[2ex](-1)^m F_m^{\bullet} & \text{if } w=1\end{cases}\qquad (h=1,2,...,w)\qquad (35)$$

Effect on coefficients $f_{i,q}$ (i=1,2,...,n; q=0,1,2,...u) involved in Eqs. (9), (10) and (15)

The insertion of conditions (17) into the expressions of coefficients $f_{i,q}$ ($i = 1,2,...,n$; $q = 0,1,2,...,u$), which correspond to strict equations, has no effect on the coefficients that are 0 or 1. Nevertheless in the remaining coefficients, some terms may include *smaller rate constants* than other terms of the same coefficient, therefore, the former are negligible compared to the latter. In the following we denote the coefficient that results from $f_{i,q}$ as $f_{i,q}^{\bullet}$ after inserting conditions (17), as so:

$$f_{i,q}^{\bullet} = \lim_{conditions\,(17)} f_{i,q} \qquad (36)$$

From the definition of coefficients $f_{i,q}$ ($i = 1,2,...,n$; $q = 0,1,2,...,u$), and from the procedure followed to acquire them from the corresponding coefficient F_q, the following relationships exist between the infinite orders of coefficients $f_{i,q}^{\bullet}$ ($i = 1,2,...,n$; $q = 0,1,2,...,u$) and $F_q^{\bullet}$ ($q=0,1,2,...,u$):

$$\text{infinite order of } f_{i,q}^{\bullet} \leq \text{infinite order of } F_q^{\bullet} \qquad (q = 0,1,2,...,u) \qquad (37)$$

$$\text{infinite order of } f_{i,0}^{\bullet}, f_{i,1}^{\bullet},..., f_{i,m-1}^{\bullet} < \text{infinite order of } F_m^{\bullet} \qquad (38)$$

Hence as the infinite order of *finite roots* λ_h ($h=1,2,...,w$) is 0, we obtain:

$$\text{If } q < m, \text{ then } \frac{f_{i,q}^{\bullet}\lambda_h^{u-q}}{F_m^{\bullet}} \to 0 \qquad (39)$$

Effect on $N_{s,q}$

The effect of conditions (17) on the expressions of $[K_{j,i}f_{j,q} - K_{i,j}f_{i,q}]$ ($q = 0,1,2,...,u$) involved in $N_{s,q}$ ($s = 1,2,...,g$; $q = 0,1,2,...,u$) [see Eq.(15)] is the removal of the terms in square brackets with a number of *large rate constants* below the maximum number of *large rate constants* in

any of their terms. This maximum number of *large rate constants* in a term coincides with the infinite order of $[K_{j,i}f_{j,q} - K_{i,j}f_{i,q}]$ which is, at the most, $q+1$ because the infinite order of $f_{j,q}$ and $f_{i,q}$ is, at the very most, q, and one of the two rate constants $K_{j,i}$ or $K_{i,j}$ can be a *large rate constant*. We denote the resulting expression for $\left[K_{j,i}f_{j,q} - K_{i,j}f_{i,q} \right]$ as $\left[K_{j,i}f_{j,q} - K_{i,j}f_{i,q} \right]^{\cdot}$ when conditions (17) are applied, and as $N_{s,q}^{\cdot}$ for the resulting expression of $N_{s,q}$, given by Eq. (15), when the expressions in this equation $\left[K_{j,i}f_{j,q} - K_{i,j}f_{i,q} \right]$ are replaced with $\left[K_{j,i}f_{j,q} - K_{i,j}f_{i,q} \right]^{\cdot}$, e.g.:

$$\left[K_{j,i}f_{j,q} - K_{i,j}f_{i,q} \right]^{\cdot} = \lim_{condition\ (17)} \left[K_{j,i}f_{j,q} - K_{i,j}f_{i,q} \right] \tag{40}$$

and

$$N_{s,q}^{\cdot} = \sum_{(i,j)} [K_{j,i}f_{j,q} - K_{i,j}f_{i,q}]^{\cdot} \tag{41}$$

Note that the maximum infinite order of $N_{s,q}^{\cdot}$ is $q+1$.

3.4.1.2. Effect on Eqs. (8) and (11)

By taking into account Eq. (28), the m exponential terms in Eqs. (8) and (11), which involve *m infinite roots* $\lambda_{w+1}, \lambda_{w+2}, ..., \lambda_u$, can be neglected (remember that roots $\lambda_1, \lambda_2, ..., \lambda_u$, irrespectively of whether they are *finite* or *infinite*, are real negative or complex with a negative real part; due to Eqs. (33) and (34), the amplitudes, arguments and coefficients in the reduced equation are also simplified). In this case, the transient phase equations are:

Enzyme species:

$$[X_i] = A_{i,0} + \sum_{h=1}^{w} A_{i,h} e^{\lambda_h t} \qquad (i=1,2,...,n) \tag{42}$$

where $\lambda_1, \lambda_2,...,\lambda_w$ are the roots of Eq. (25), and

$$A_{i,0} = \frac{f_{i,u}^{\cdot}[E]_0}{F_u^{\cdot}} \qquad (i=1,2,...,n) \tag{43}$$

$$A_{i,h} = \frac{(-1)^{w+1}[E]_0 \left(\sum\limits_{q=m}^{u} f_{i,q} \lambda_h^{u-q} \right)^{\bullet}}{\lambda_h F_m^{\bullet} \prod\limits_{\substack{p=1 \\ p \neq h}}^{w} (\lambda_p - \lambda_h)} \qquad (i=1,2,\ldots,n;\ h=1,2,\ldots,w) \qquad (44)$$

Since not all the terms in $\sum\limits_{q=m}^{u} f_{i,q} \lambda_h^{u-q}$ are of the same order of infinite, only those with

the highest order remain and the others can be neglected. This is expressed as $\left(\sum\limits_{q=m}^{u} f_{i,q} \lambda_h^{u-q} \right)^{\bullet}$

Ligand species:

$$[Y_s] - [Y_s]_0 = \beta_s + \alpha_s t + \sum\limits_{h=1}^{w} \gamma_s e^{\lambda_h t} \qquad (s=1,2,\ldots,g) \qquad (45)$$

where $\lambda_1, \lambda_2, \ldots, \lambda_w$ are the roots of Eq. (25) and

$$\alpha_s = \frac{N_{s,u}^{\bullet}[E]_0}{F_u^{\bullet}} \qquad (s=1,2,\ldots,g) \qquad (46)$$

$$\beta_s = \frac{N_{s,u-1}^{\bullet}[E]_0}{F_u^{\bullet}} - \frac{F_{u-1}^{\bullet}}{F_u^{\bullet}} \alpha_s \qquad (s=1,2,\ldots,g) \qquad (47)$$

$$\gamma_{s,h} = (-1)^{w+1}[E]_0 \frac{\left(\sum\limits_{q=m}^{u} N_{s,q}^{\bullet} \lambda_h^{u-q} \right)^{\bullet}}{\lambda_h^2 F_m^{\bullet} \prod\limits_{\substack{p=1 \\ p \neq h}}^{w} (\lambda_p - \lambda_h)} \quad (s=1,2,\ldots,g;\ h=1,2,\ldots,u) \quad \text{(If } u=1, \text{ then the denominator is } \lambda_1^2 \text{)} \qquad (48)$$

As previously shown, in $\sum\limits_{q=m}^{u} N_{s,q}^{\bullet} \lambda_h^{u-q}$ only the terms with the highest order remain and the

others can be neglected. This is expressed as $\left(\sum\limits_{q=m}^{u} N_{s,q}^{\bullet} \lambda_h^{u-q} \right)^{\bullet}$

Eqs. (42)-(48) are general and can be applied to any case. Nevertheless, they become considerably simplified when $w = 1$ and $w = 0$.

w = 1:

In this case, since $m = u-1$, index q can only take values $u-1$ and u. The time-course equations are:

Enzyme species:

$$[X_i] = A_{i,0} + A_{i,1} e^{\lambda_1 t} \qquad\qquad (i=1,2,...,n) \qquad\qquad (49)$$

where

$$\lambda_1 = -\frac{F_u^{\cdot}}{F_{u-1}^{\cdot}} \qquad\qquad\qquad (50)$$

$$A_{i,0} = \frac{f_{i,u}^{\cdot}[E]_0}{F_u^{\cdot}} \qquad\qquad (i=1,2,...,n) \qquad\qquad (51)$$

$$A_{i,1} = -\frac{[E]_0 \left(f_{i,u-1}^{\cdot} \lambda_1 + f_{i,u}^{\cdot} \right)^{\cdot}}{F_u^{\cdot}} \qquad\qquad (i=1,2,...,n) \qquad\qquad (52)$$

Ligand species:

$$[Y_s] - [Y_s]_0 = \alpha_s t + \beta_s \left(1 - e^{\lambda_1 t} \right) \qquad\qquad (s=1,2,...,g) \qquad\qquad (53)$$

where α_s, β_s and λ_1 are given by Eqs. (46), (47) and (50). Eq. (53) results from the fact that in this case we obtain:

$$\gamma_{s,1} = -\beta_s \qquad\qquad (s = 1,2,... ,g) \qquad\qquad (54)$$

w = 0:

In this case, index q only takes the value u because $m=u-w=u$; thus there are no *finite roots*. Therefore, u roots λ_1, λ_2, ... λ_u of polynomial $T(\lambda)$ are *infinite*. Hence all the exponential terms can be neglected in Eqs. (8) and (11). Since the infinite order of $F_{u-1}^{\cdot}$ (equal to $u-1$) is less than that of $F_u^{\cdot}$ (equal to u), the second term on the right-hand side of Eq. (47) vanishes. The infinite order of $N_{s,u-1}^{\cdot}$ is $\leq u$, which is the same as that of F_u, and, in principle,

the first term on the right-hand side of Eq. (47) does not necessarily vanish; so Eq. (47) becomes $\beta_s = N^{\cdot}_{s,u-1}[E]_0 / F^{\cdot}_u$ (obviously β_s can be zero). Thus Eqs. (8) and (11) for this case reduce to:

$$[X_i] = A_{i,0} \qquad\qquad (i = 1,2,...,n) \qquad\qquad (55)$$

$$[Y_s] - [Y_s]_0 = \frac{N^{\cdot}_{s,u-1}[E]_0}{F^{\cdot}_u} + \alpha_s t \qquad\qquad (s = 1,2,...,g) \qquad\qquad (56)$$

where $A_{i,0}$ and α_s are given by Eqs. (43) and (46). Note that in Eqs. (42)-(56), the coefficients with a lower value of subindex q than m do not appear.

3.4.2. Procedure B

Another way to reduce the number of exponential terms in a multi-exponential equation is to simply set the number of exponential terms (generally one or two) that we require in the reduced expressions. To make this reduction possible, certain relationships between rate constants are required. Let the number of exponential terms in the resulting reduced equation be w ($w < u$, $w = u-m$). This means that there are w roots of $T(\lambda)$ with small values (we denote them as $\lambda_1, \lambda_2, \cdots, \lambda_w$) and m roots with large values (we denote them as $\lambda_m, \lambda_{m+1}, \cdots, \lambda_u$). If we assign infinite orders of 0 and 1 respectively to the *finite* and *infinite rate constants,* we find from Eqs. (6) the following after neglecting the terms with low infinite orders on the left-hand side of these equations:

$$\lambda_{w+1} + \lambda_{w+2} + \cdots + \lambda_u = -F_1 \qquad\qquad (57)$$

$$\lambda_{w+1}\lambda_{w+2} + \lambda_{w+1}\lambda_{w+3} + \cdots + \lambda_{u-1}\lambda_u = F_2 \qquad\qquad (58)$$

$$\lambda_{w+1}\lambda_{w+2} \cdots \lambda_u = (-1)^m F_m \qquad\qquad (59)$$

$$\left(\lambda_1 + \lambda_2 + \cdots + \lambda_w\right)\lambda_{w+1}\lambda_{w+2} \cdots \lambda_u = (-1)^{m+1} F_{m+1} \qquad\qquad (60)$$

$$\left(\lambda_1\lambda_2 + \lambda_1\lambda_3 + \cdots + \lambda_{w-1}\lambda_w\right)\lambda_{w+1}\lambda_{w+2} \cdots \lambda_u = (-1)^{m+2} F_{m+2} \qquad\qquad (61)$$

$$\lambda_1\lambda_2 \cdots \lambda_w\lambda_{w+1}\lambda_{w+2} \cdots \lambda_u = (-1)^u F_u \qquad\qquad (62)$$

Therefore, the infinite orders of F_1, F_2, …, F_m equal 1, 2, …, m, while the infinite order of coefficients F_{m+1},…,F_u equal, at the most, m.

From Eqs. (57)-(62), we obtain:

$$\lambda_1 + \lambda_2 + \cdots + \lambda_w = -\frac{F_{m+1}}{F_m} \tag{63}$$

$$\lambda_1\lambda_2 + \lambda_1\lambda_3 + \cdots + \lambda_{w-1}\lambda_w = \frac{F_{m+2}}{F_m} \tag{64}$$

$$\lambda_1\lambda_2 \cdots \lambda_w = (-1)^{w+1}\frac{F_u}{F_m} \tag{65}$$

i.e., the w arguments with small values are the roots of equations.

$$\sum_{q=m}^{u}\frac{F_q}{F_m}\lambda^{u-q} = 0 \tag{66}$$

and so,

$$\prod_{\substack{p=1 \\ p\neq h}}^{u}\left(\lambda_p - \lambda_h\right) = \begin{cases} (-1)^m F_m \displaystyle\prod_{\substack{p=1 \\ p\neq h}}^{w}\left(\lambda_p - \lambda_h\right) & \text{if } w > 1 \\ (-1)^m F_m & \text{if } w = 1 \end{cases} \tag{67}$$

If we assign an infinite order of 1 to *large rate constants*, we obtain from Eqs. (57)-(62):

$$\frac{F_1}{F_2}, \frac{F_2}{F_3}, \cdots, \frac{F_{m-1}}{F_m} \to 0 \tag{68}$$

or alternatively:

$$F_1 \ll F_2 \ll F_3 \ll \cdots \ll F_m \tag{69}$$

Finally, by admitting that there are w *finite roots*, the exponential terms in which the $u-w$ arguments are involved can be neglected, and the equations become formally the same Eqs. (42)-(48), but by replacing coefficients $F_q^{\cdot}$ $(q = 0,1,2,\ldots,u)$, $f_{i,q}^{\cdot}$ $(i = 1,2,\ldots n,$

$q = 0,1,2,…u)$ and $N^*_{s,q}$ $(s = 1,2,…g; q = 0,1,2,…,u)$ by F_q $(q = 0,1,2,…,u),$ $f_{i,q}$ $(i = 1,2,…n,$

$q = 0,1,2,…u)$ and $N_{s,q}$ $(s = 1,2,…g; q = 0,1,2,…,u).$

4. Results and discussion

The evaluation of the individual rate constants in an enzyme reaction mechanism requires experimentally monitoring the time evolution of the concentration of one species or more involved in the transient phase. These experiments are normally performed under certain conditions; e.g. with a limiting enzyme and during a reaction time so that the initial concentration of the ligand species, which binds an enzyme species, remains approximately constant. Next experimental data must be fitted to the corresponding theoretical equations, which are generally multi-exponential. In principle, individual rate constants can be obtained from these fittings. In practice, and when the corresponding expression involves more than two exponential terms, equations are too complex to obtain estimates of rate constants by fitting them to experimental data. Hence the importance and usefulness of the reduction process (especially to one or two exponential term(s)) proposed in this paper.

Rrducing the number of exponential terms offers advantages as fitting is easier or possible, but there is one disadvantage; since the resulting expression is only an approached equation, fewer individual rate constants (or the kinetic parameters related to these constants) can be evaluated. Moreover, estimates of individual rate constants and kinetic parameters determined in this way can significantly differ in some cases from their actual values. Overall this contribution addresses, for the first time, the complex problem of the kinetic data analysis of any enzyme system whose kinetic behaviour is described by multi-exponential equations. To this end, two general and systematic procedures, A and B, are proposed.

4.1. About Procedure A

Procedure A is interpretative and based on the fact that some rate constants in numerous enzyme reactions are much smaller than others. As a result, the number of exponential terms, and the expressions of the amplitudes, arguments and the remaining coefficients involved in the original equation, are considerably reduced. Note, however, that reduced expressions depend strongly on the set of rate constants that are considered *large rate constants*. In other words, if we consider a different set of *large rate constants,* the

expressions of the amplitudes, arguments and coefficients also differ, even if the number of exponential terms does not vary.

The reduced equations obtained by procedure A are much simpler than those derived by procedure B, although they only are valid for the specific set of conditions (17) chosen.

The literature contains different examples of reducing the number of exponential terms in the kinetic equations that correspond to specific reaction mechanisms when assuming the partial or total rapid equilibrium approach [5, 6, 29, 31-37]. For this reduction to be applied, the values of rate constants (of first or pseudo-first orders) must allow reversible steps to be in equilibrium from practically the onset of the reaction. In any case, we emphasise that these procedures to reduce the terms of exponential terms according to the assumptions of partial or total rapid equilibrium are a particular case of the procedure herein suggested because the rate constants that are much larger than others are not limited to those involved in reversible steps.

In turn, the practical consequence of applying conditions (17) to a reaction mechanism is that the mechanism reduces to a much simpler process with fewer enzyme species and/or reversible steps in rapid equilibrium (see the example in Subsection 4.3 below).

To explain this procedure, we frequently use the term *infinite order* and the fact that different infinite orders exist. The following mathematical example can help to better understand this concept. Let's consider the following limit:

$$\lim_{x \to \infty} x^n \to \infty \quad (x > 1, n = 1,2,\ldots) \tag{70}$$

The infinite order of the above limit equals n for x>1 and n = 1,2,3,…., but if $n=0$, the infinite order of x^n is 0 because $x^0 = 1$. Another example is geometric: for a single point, its infinite order can be assigned a 0, while the infinite orders of the points contained in a segment, a square and a cube are 1, 2 and 3.
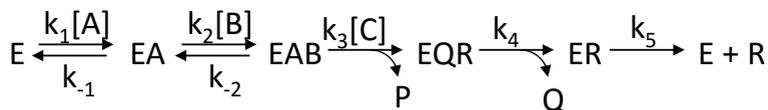
4.2. About Procedure B

Conversely, procedure B is descriptive and establishes a single relationship between coefficients F_q $(q=1,2,\ldots,u)$ in the non-reduced equation and the desired number of exponential terms $(=w < u)$ in the reduced equation. This relationship is defined by Eqs. (68) and (69), and is independent of rate constants, which must be much larger than the others.

Generally different sets of *large rate constants* exist for which the relations in Eqs. (68) and (70) are fulfilled. For them all, the same reducing equation with w exponential terms is valid. This is the main advantage of Procedure B compared to Procedure A; that is, it provides a single reduced equation with the desired number of exponential terms in which the expressions for amplitudes, arguments and coefficients are the same regardless of the set of rate constants. This enables Eqs. (68) and (69) to be fulfilled. The expressions that derive by using procedure B include, as particular cases, any possible reduced equation obtained by applying procedure A with the same number of exponential terms. However, one disadvantage of procedure B is that it is descriptive; i.e. macroscopic, and does not justify the facts that lead to reducing the equation. Nevertheless, we believe that Procedures A and B are necessary and complementary.

4.3. An analytical example

To support our analysis and to verify its goodness, we resort to the example of the well-known Sequential Ordered Ter-Ter Theorell Chance mechanism [17, 38] shown in Scheme 1. In this scheme, $[A]_0$, $[B]_0$ and $[C]_0$ are the initial concentrations of the substrates (which remain approximately constant if the free enzyme is limiting), and P, Q and R are the products. To avoid making this paper too long, we stuck to the time course of P and assumed that the only species present at the onset of the reaction are the free enzyme, E, and the three substrates. The strict transient phase equations under these conditions are shown in Appendix A and the kinetic equations that correspond to any other species can be analogously dealt with. Note, however, that the procedures shown here are applicable to all types of enzyme systems, irrespectively of their complexity, and whose kinetic equations consist in a polynomial part and a multi-exponential part.

$$E \underset{k_{-1}}{\overset{k_1[A]}{\rightleftarrows}} EA \underset{k_{-2}}{\overset{k_2[B]}{\rightleftarrows}} EAB \overset{k_3[C]}{\underset{P}{\searrow}} EQR \overset{k_4}{\underset{Q}{\searrow}} ER \overset{k_5}{\longrightarrow} E + R$$

Scheme 1

If we denote enzyme species E, EA, EAB, EQR and ER as X_1, X_2, X_3, X_4 and X_5, then non-null constants $K_{i,j}$ ($i,j=1,2,3,4,5$; $i \neq j$) involved in Scheme 1 are:

$$\left.\begin{array}{l} K_{1,2} = k_1[A]_0 \\ K_{2,1} = k_{-1} \\ K_{2,3} = k_2[B]_0 \\ K_{3,2} = k_{-2} \\ K_{3,4} = k_3[C]_0 \\ K_{4,5} = k_4 \\ K_{5,1} = k_5 \end{array}\right\} \tag{71}$$

All the rate constants are of first order, except $K_{1,2}$, $K_{2,3}$, and $K_{3,4}$, which are of pseudo-first order.

4.3.1. Procedure A

We obtain the corresponding reduced transient phase equation from Eq. (A.1) when the following assumptions are considered in Scheme 1:

$$\left.\begin{array}{l} K_{1,2}, K_{2,1}, K_{2,3}, K_{3,2} \text{ and } K_{4,5} \to \infty \\ K_{1,2}, K_{2,1}, K_{2,3}, K_{3,2} \text{ and } K_{4,5} \text{ are of the same infinite order} \end{array}\right\} \tag{72}$$

If conditions (72) are inserted into Eq. (A.1) and those related, we obtain:

$$F_1^{\cdot} = k_1[A]_0 + k_{-1} + k_2[B]_0 + k_{-2} + k_4 \tag{73}$$

$$F_2^{\cdot} = k_1[A]_0k_2[B]_0 + k_1[A]_0k_{-2} + k_1[A]_0k_4 + k_{-1}k_{-2} + k_2[B]_0k_4 + k_{-2}k_4 \tag{74}$$

$$F_3^{\cdot} = k_1[A]_0k_2[B]_0k_4 + k_1[A]_0k_{-2}k_4 + k_{-1}k_{-2}k_4 \tag{75}$$

$$F_4^{\cdot} = k_1[A]_0k_2[B]_0k_3[C]_0k_4 + k_1[A]_0k_2[B]_0k_4k_5 + k_1[A]_0k_{-2}k_4k_5 + k_{-1}k_{-2}k_4k_5 \tag{76}$$

Note that the infinite order of $F_1^{\cdot}$, $F_2^{\cdot}$, $F_3^{\cdot}$ and $F_4^{\cdot}$ are 1, 2, 3 and 3, respectively. Therefore $m = 3$ and $w = 1$; that is, there are only one finite argument and three infinite ones. Since $w = 1$, Eqs. (53) and (54) apply and we obtain:

$$[P] = \alpha t + \beta\left(1 - e^{\lambda_1 t}\right) \tag{77}$$

with:

$$\lambda_1 = -\frac{F_4^{\cdot\cdot}}{F_3^{\cdot\cdot}} \tag{78}$$

$$\alpha = \frac{N_4^{\cdot}[E]_0}{F_4^{\cdot\cdot}} \tag{79}$$

$$\beta_s = \frac{N_3^{\cdot}[E]_0}{F_4^{\cdot\cdot}} - \frac{F_3^{\cdot\cdot}}{F_4^{\cdot\cdot}}\alpha \tag{80}$$

In turn, the insertion of conditions (72) into Eqs. (A.11) and (A.12) leads to:

$$N_3^{\cdot} = k_3[C]_0 k_1[A]_0 k_2[B]_0 k_4 \tag{81}$$

$$N_4^{\cdot} = k_3[C]_0 k_1[A]_0 k_2[B]_0 k_4 k_5 \tag{82}$$

If in Eqs. (78)-(80) quantities $F_3^{\cdot\cdot}$, $F_4^{\cdot\cdot}$, $N_3^{\cdot}$ and $N_4^{\cdot}$ are replaced with the corresponding Eqs. (73)-(76), (81) and (82), considerable simplifications and cancelations result, which have been omitted.

As previously mentioned, the application of conditions (17) to a reaction mechanism has the additional effect that the mechanism can be considered to take place with fewer kinetic steps. Thus applying conditions (72) transforms the reaction mechanism in Scheme 1 into Scheme 2.
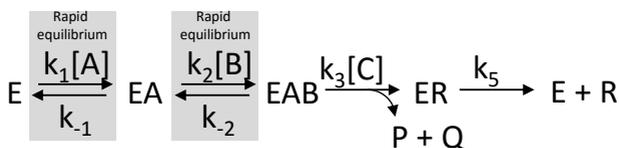
Scheme 2

4.3.2. Procedure B

If we wish to transform Eq. (A.1) into a reduced equation with only one exponential term, we set $w = 1$ in Eq. (72). Therefore, because $u = 4$, then $m = u-w = 3$. Accordingly, the necessary and sufficient condition for $w = 1$ is that:

$$F_1 \ll F_2 \ll F_3 \tag{83}$$

Note that the conditions (72) imposed in Procedure A agree with conditions (83), but other relationships between the rate constants that differ from conditions (72) can also comply with conditions (83).

As previously indicated, the reduced equation obtained by procedure B is:

$$[P] = \alpha t + \beta \left(1 - e^{\lambda_1 t}\right) \tag{84}$$

where λ_1, α and β are provided by:

$$\lambda_1 = -\frac{F_4}{F_3} \tag{85}$$

$$\alpha = \frac{N_4 [E]_0}{F_4} \tag{86}$$

$$\beta = \frac{N_3 [E]_0}{F_4} - \frac{F_3}{F_4} \alpha \tag{87}$$

The expressions for F_3, F_4, N_3 and N_4 coincide with Eqs. (A.5), (A.6), (A.11) and (A.12) for the tetra-exponential equation. Note that expressions for λ_1, γ_1, α and β are more complex that those obtained by procedure A because they include all the rate constants involved in the reaction mechanism.

As mentioned above, Eqs. (84)-(87) are valid for all the sets of rate constants that fulfil conditions (83); for example, the following sets given in (88), where the *large rate constants* with the same *infinite order* are marked in bold:

1. $\boldsymbol{k_1 [A]_0}, \boldsymbol{k_{-1}}, \boldsymbol{k_2 [B]_0}, \boldsymbol{k_{-2}}, k_3 [C]_0, \boldsymbol{k_4}, k_5$ [these are the conditions (72) used in the example for Procedure A]
2. $\boldsymbol{k_1 [A]_0}, \boldsymbol{k_{-1}}, \boldsymbol{k_2 [B]_0}, \boldsymbol{k_{-2}}, k_3 [C]_0, \boldsymbol{k_4}, \boldsymbol{k_5}$
3. $\boldsymbol{k_1 [A]_0}, k_{-1}, \boldsymbol{k_2 [B]_0}, k_{-2}, k_3 [C]_0, \boldsymbol{k_4}, k_5$
4. $\boldsymbol{k_1 [A]_0}, k_{-1}, \boldsymbol{k_2 [B]_0}, k_{-2}, k_3 [C]_0, \boldsymbol{k_4}, \boldsymbol{k_5}$
5. $\boldsymbol{k_1 [A]_0}, \boldsymbol{k_{-1}}, k_2 [B]_0, k_{-2}, \boldsymbol{k_3 [C]_0}, k_4, \boldsymbol{k_5}$
6. $k_1 [A]_0, k_{-1}, \boldsymbol{k_2 [B]_0}, \boldsymbol{k_{-2}}, k_3 [C]_0, \boldsymbol{k_4}, \boldsymbol{k_5}$

and many more possibilities.

$$\left.\right\} \tag{88}$$

4.4. A numerical example

This section provides a numerical example that illustrates the reduction process. This example reveals that although the reduced equations imply loss of accuracy, this is compensated by the easy (and sometimes the only possible) processing of experimental data. The chosen reaction mechanism is shown in Scheme 3, which corresponds to the reaction mechanism for the action of an enzyme, E, on a suicide substrate [39-44].

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} X \xrightarrow{k_3} E + P$$

$$\downarrow k_4$$

$$E_i$$
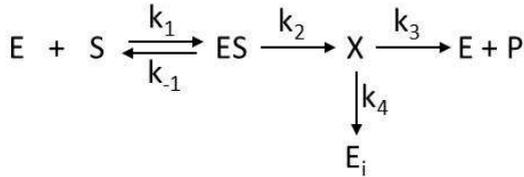
Scheme 3

Under the conditions of (i) limiting enzyme, (ii) considering a reaction time for which the concentration of the accumulated product is much lower than the initial substrate concentration, and (iii) assuming that there is no product upon the onset of the reaction, the strict analytical solution for product accumulation is given by:

$$[P] = \beta + \gamma_1 e^{\lambda_1 t} + \gamma_2 e^{\lambda_2 t} + \gamma_3 e^{\lambda_3 t} \tag{89}$$

where λ_1, λ_2 and λ_3 are the roots of Eq. (3), where $u = 3$ and F_1, F_2 and F_3 are:

$$F_1 = k_1[S]_0 + k_{-1} + k_2 + k_3 + k_4 \tag{90}$$

$$F_2 = k_1(k_2 + k_3 + k_4)[S]_0 + (k_{-1} + k_2)(k_3 + k_4) \tag{91}$$

$$F_3 = k_1 k_2 k_4 [S]_0 \tag{92}$$

In turn:

$$\beta = r[E]_0 \tag{93}$$

and

$$\gamma_h = \frac{k_1 k_2 k_3 [S]_0 [E]_0}{\lambda_h \prod_{\substack{p=1 \\ p \neq h}}^{3} (\lambda_p - \lambda_h)} \qquad (h=1,2,3) \tag{94}$$

The kinetic parameter r in Eq. (93) is the so-called partitio ratio and is given by:

$$r = k_3 / k_4 \tag{95}$$

4.4.1. Procedure A

Let rate constants $k_1[S]_0$, k_{-1} and k_3 be much larger than k_2 and k_4. Then procedure A leads to:

$$F_1^{\cdot} = k_1[S]_0 + k_{-1} + k_3 \tag{96}$$

$$F_2^{\cdot} = k_3 \left(k_{-1} + k_1[S]_0 \right) \tag{97}$$

$$F_3^{\cdot} = k_1 k_2 k_4 [S]_0 \tag{98}$$

Note that the infinite orders of $F_1^{\cdot}$, $F_2^{\cdot}$ and $F_3^{\cdot}$ respectively are 1,2 and 1; e.g., $m=2$ so that $w = u-m = 1$. Thus according to procedure A, the time accumulation of P by assuming that $[P]_0=0$ is:

$$[P] = \beta \left(1 - e^{\lambda_1 t} \right) \tag{99}$$

where β is given by Eq. (93) and

$$\lambda_1 = -\frac{F_3^{\cdot}}{F_2^{\cdot}} = -\frac{k_2[S]_0}{r\left(K_1 + [S]_0 \right)} \tag{100}$$

where equilibrium constant K_1 is:

$$K_1 = \frac{k_{-1}}{k_1} \tag{101}$$

In Fig. 1 we compare the simulated progress curves of [P] (acting as experimental progress curves), computed by numerical integration of the set of differential equations that

describe the kinetic behaviour of the enzyme system into Scheme 3, with the corresponding plots obtained from Eqs. (89) and (99). Comparisons were made for three different cases of the values of the rate constants, and at a fixed value of $[E]_0$ and $[S]_0$. The values of these rate constants, together with the values of F_1, F_2, y F_3 [Eqs. (90)-(92)], are shown in Table 1. In turn, the values of β [Eq. (93)], the values of λ_1, λ_2 and λ_3 (obtained from Eq. (3) with $u=3$, and the values of γ_1, γ_2 and γ_3 [Eq. (94)], which are necessary to plot Eq. (89), are given in Table 2. Finally, the values of β, K_1 and λ_1 [obtained from Eqs. (93), (101) and (100)], which are needed to plot Eq. (99), are listed in Table 3.
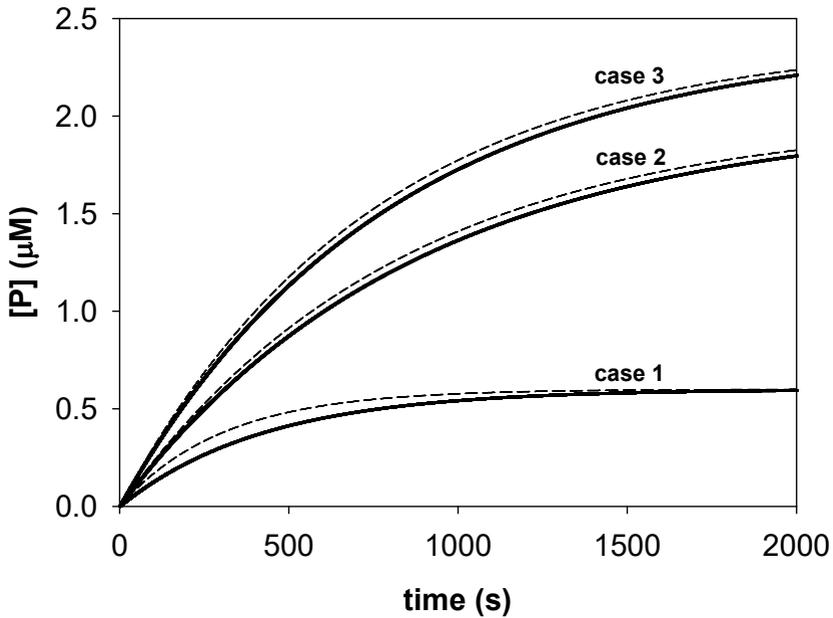

Figure 1. Simulated progress curves of [P] (——) and those obtained from Eq. (89) (which in the three cases overlap the simulated progress curves) and from Eq. (99) (------) for cases 1-3 in Table 1.

Table 1

Values of the rate constants for which the curves that correspond to cases 1-3 in Fig. 1 were obtained. In all cases, values $[E]_0$ and $[S]_0$ are 10^{-7} M and 10^{-4} M, respectively. The last three columns show the corresponding values of F_1, F_2 and F_3.

Set	k_1 ($M^{-1}s^{-1}$)	k_{-1} (s^{-1})	k_2 (s^{-1})	k_3 (s^{-1})	k_4 (s^{-1})	F_1 (s^{-1})	F_2 (s^{-2})	F_3 (s^{-3})
1	10^3	5	1	30	5	41.1	213.6	0.5
2	5×10^4	200	1	100	5	311	21635	25
3	10^5	300	1	120	5	436	38885	50

Table 2

The kinetic parameter values needed to plot Eq. (89) for cases 1, 2 and 3. These parameters were obtained from Eq. (3) with $u=3$ and from Eqs. (90)-(94).

	case 1	case 2	case 3
β (μM)	0.6	2	2.4
λ_1 (s^{-1})	- 2,34188 x 10^{-3}	-1.15556 x 10^{-3}	- 1.28586 x 10^{-3}
λ_2 (s^{-1})	- 34.9970	- 205.952	- 310.947
λ_3 (s^{-1})	- 6.10062	- 105.047	- 125.052
γ_1 (μM)	- 6.00271 x 10^{-1}	- 2.00002	- 2.40003 x 10^{-1}
γ_2 (μM)	- 8.47706 x 10^{-6}	- 1.16823 x 10^{-5}	- 6.67640 x 10^{-7}
γ_3 (μM)	2.79059 x 10^{-4}	4.49050 x 10^{-5}	4.12797 x 10^{-6}

Table 3

The kinetic parameter values needed to plot Eq. (99) for cases 1-3. These parameters were obtained from Eqs. (93), (101) and (100).

case	β (μM)	K_1 (mM)	λ_1 (s^{-1})
1	0.6	5	1/306
2	2	4	1/820
3	2.4	3	1/744

Note that as rate constants k_1, k_{-1} and k_3 increase, the deviations between the simulated progress curves and those obtained with simplified equations become smaller. Note also that the simulated progress curves and the corresponding plots from the strict solution [Eq. (89)] practically overlap, so they are indistinguishable in Fig. 1.

Evaluation of kinetic parameters

To evaluate from experimental time-course curves kinetic parameters r, k_2 and equilibrium constant K_1 (assuming that Eq. (99) holds), the time progress curves of [P] at different values of $[E]_0$ and $[S]_0$ are obtained. According to Eq. (93), a fit of the β-values obtained per curve vs. $[E]_0$ allows the r-value to be obtained. Also from Eq. (100). a plot of $-1/\lambda_1$ vs. $1/[S]_0$ gives a straight line with slope rK_1/k_2 and ordinate intercept r/k_2. From these parameters, and because r is already known, the k_2 and K_1-values are obtained. Note that as we use a reduced equation, not all the kinetic parameters in Scheme 3 can be evaluated. Thus, k_{-1} and k_1, and k_3 and k_4, cannot be evaluated separately, but only their quotients K_1 and r.

In Fig. 2 we show the simulated progress curves of [P] for the set of rate constants that correspond to case 2 in Table 1. These curves are computed at different values of $[E]_0$ and $[S]_0$, which are shown in Table 4. By fitting each progress curve in Fig. 2 to Eq. (99), we obtain the corresponding values of β and λ_1 which are also offered in Table 4. Next, and by proceeding as previously mentioned, e.g., using plots of β vs. $[E]_0$ and of $-1/\lambda_1$ vs. $1/[S]_0$, the following values of r, r/k_2 and rK_1/k_2 are obtained: $r = 19.93 \pm 0.07$, $r/k_2 = 21.3 \pm 0.3$ s and $rK_1/k_2 = (8.447 \pm 0.005) \times 10^{-2}$ M·s. Hence $k_2 = 0.93 \pm 0.02$ s^{-1} and $K_1 = 3.96 \pm 0.08$ mM. If we proceed in the same way for cases 1 and 3, the corresponding values of r, k_2 and K_1 are also obtained. The estimates of these parameters for cases 1-3, together with their actual values, are summarised in Table 5. As expected, the deviations for k_2 and K_1 become smaller as rate constants k_1, k_{-1} and k_3 increase.
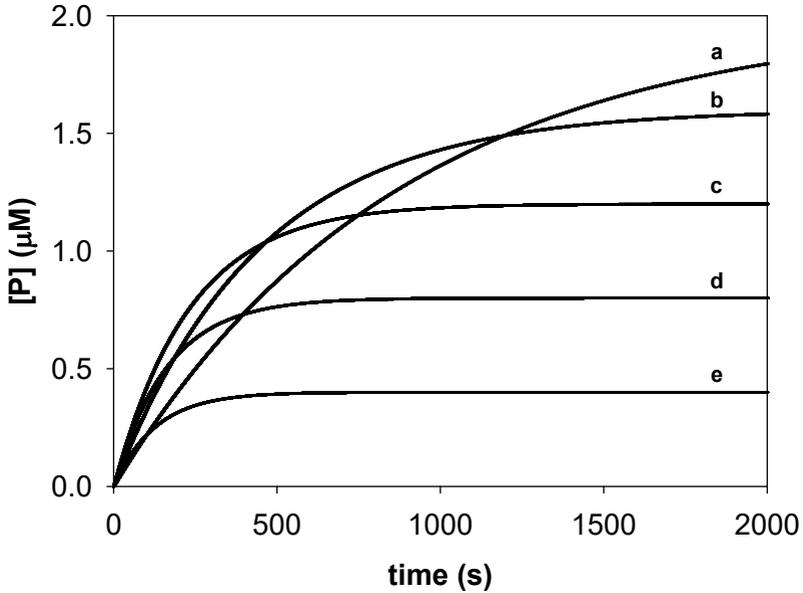
Figure 2. Simulated progress curves of [P] for the set of rate constants corresponding to case 2 in Table 1 computed at the five different sets of values of $[E]_0$ and $[S]_0$ shown in Table 4.

Table 4

The $[E]_0$ and $[S]_0$ values used to compute the five simulated progress curves in Fig. 2 correspond to case 2 in Table 1. The last two columns show the values of both β and λ_1 obtained from the fits of these curves to Eq. (99).

set	$[E]_0$ (nM)	$[S]_0$ (mM)	β (μM)		$-\lambda_1$ (s^{-1}) x 10^3	
			Value	StdErr	Value	StdErr
a	100	0.1	1.993	5.628 x 10^{-5}	1.155	7.130 x 10^{-5}
b	80	0.2	1.599	1.231 x 10^{-5}	2.251	1.018 x 10^{-7}
c	60	0.4	1.200	2.159 x 10^{-6}	4.301	4.123 x 10^{-5}
d	40	0.6	0.800	3.773 x 10^{-7}	6.173	1.918 x 10^{-5}
e	20	0.8	0.400	1.018 x 10^{-7}	7.890	1.518 x 10^{-5}

Table 5

The values of r, k_2 and K_1 for cases 1, 2 and 3, obtained from the time progress curves by applying the kinetic data analysis, are explained in the main text. If we compare the k_2- and K_1-values with their actual values, we find that the corresponding deviations for k_2 and K_1 increase from case 1 to case 3. In turn, the r-values coincide practically with the actual values in the three cases, although deviations increase slightly from cases 1 to 3. This is because the curves in Fig. 1 have been simulated in all cases for a reaction time of 2,000 s. Under these conditions, the curves for case 1 show more values of [P] near β than for cases 2 and 3. Hence in case 1, the fit uses more points in this region than in the other cases.

case	r			k_2 (s^{-1})			K_1 (mM)		
	Obtained	Actual	Error	Obtained	Actual	Error	Obtained	Actual	Error
1	5.998 ± 0.002	6	0.04%	0.8523 ± 0.0004	1	14.77%	5.987 ± 0.003	5	19.74%
2	19.93 ± 0.06	20	0.35%	0.9340 ± 0.0150	1	6.60%	3.9586 ± 0.0796	4	1.05%
3	23.91 ± 0.08	24	0.38%	0.9413 ± 0.0022	1	5.87%	3.0130 ± 0.0174	3	0.43%

4.4.2. Procedure B

If we wish to reduce the three exponential terms in Eq. (89) to only one term ($w=1$), then $m = u\text{-}1 = 2$ and, according to the results obtained for procedure B in Section 3.4.2, we find that the time equation for [P] coincides with Eq. (99) where β is given by Eq. (93), but λ_1 is now defined by:

$$\lambda_1 = -\frac{F_3}{F_2} = -\frac{k_1 k_2 k_4 [S]_0}{k_1 (k_2 + k_3 + k_4)[S]_0 + (k_{-1} + k_2)(k_3 + k_4)} \tag{102}$$

Eq. (102) can be rewritten in a more compact way by dividing both the numerator and denominator by k_1:

$$\lambda_1 = -\frac{k_2 k_4 [S]_0}{\{k_2 + k_4 (r+1)\}[S]_0 + K_m k_4 (r+1)} \tag{103}$$

where, K_m is the Michaelis constant $= (k_{-1}+k_2)/k_1$. Parameter r can be obtained as in procedure A; that is, by fitting the β-values for different $[E]_0$-values to Eq. (93).

To evaluate λ_1 from the simulated time progress curves obtained in different sets of values of $[E]_0$ and $[S]_0$, we proceed as in procedure A, but now according to Eq. (103), a plot of $-1/\lambda$ vs. $1/[S]_0$ gives a straight line with slope $K_m(r+1)/k_2$ and ordinate intercept

$\{k_2+k_4(r+1)\}/(k_2k_4)$. As r is known (its value is obtained as in procedure A and coincides with those listed in Table 5), the values of global kinetic parameters K_m/k_2 and $\{k_2+k_4(r+1)\}/(k_2k_4)$ can be also estimated. Note that if the conditions to assume $F_1 \ll F_2$ are the same as for procedure A, e.g., k_1, k_{-1} and $k_3 \gg k_2$ and k_4, then the above slope and intercept ordinate coincide with those obtained for procedure A; e.g. K_1/k_2 and r/k_2.

Appendix A

Strict transient-phase equations for Scheme 1

If we assume that no product P exists upon the onset of the reaction, its instantaneous concentration, denoted as [P], is given by:

$$[P] = \beta + \alpha t + \gamma_1 e^{\lambda_1 t} + \gamma_2 e^{\lambda_2 t} + \gamma_3 e^{\lambda_3 t} + \gamma_4 e^{\lambda_4 t} \tag{A.1}$$

where arguments λ_1, λ_2, λ_3 and λ_4 are the roots of the equation

$$\lambda^4 + F_1\lambda^3 + F_2\lambda^2 + F_3\lambda + F_4 = 0 \tag{A.2}$$

where:

$$F_1 = k_1[A]_0 + k_{-1} + k_2[B]_0 + k_{-2} + k_3[C]_0 + k_4 + k_5 \tag{A.3}$$

$$F_2 = k_1[A]_0k_2[B]_0 + k_1[A]_0k_{-2} + k_1[A]_0k_3[C]_0 + k_1[A]_0k_4 + k_1[A]_0k_5 + k_{-1}k_{-2} + k_{-1}k_3[C]_0 + \\ + k_2[B]_0k_4 + k_2[B]_0k_5 + k_{-2}k_4 + k_{-2}k_5 + k_3[C]_0k_4 + k_3[C]_0k_5 + k_4k_5 \tag{A.4}$$

$$F_3 = k_1[A]_0k_2[B]_0k_3[C]_0 + k_1[A]_0k_2[B]_0k_4 + k_1[A]_0k_2[B]_0k_5 + k_1[A]_0k_{-2}k_4 + k_1[A]_0k_{-2}k_5 + \\ + k_1[A]_0k_3[C]_0k_4 + k_1[A]_0k_3[C]_0k_5 + k_1[A]_0k_4k_5 + k_{-1}k_{-2}k_4 + k_{-1}k_{-2}k_5 + k_{-1}k_3[C]_0k_4 + \\ + k_{-1}k_3[C]_0k_5 + k_{-1}k_4k_5 + k_2[B]_0k_3[C]_0k_4 + k_2[B]_0k_3[C]_0k_5 + k_2[B]_0k_4k_5 + k_{-2}k_4k_5 + \\ + k_3[C]_0k_4k_5 \tag{A.5}$$

$$F_4 = k_1[A]_0k_2[B]_0k_3[C]_0k_4 + k_1[A]_0k_2[B]_0k_3[C]_0k_5 + k_1[A]_0k_2[B]_0k_4k_5 + k_1[A]_0k_{-2}k_4k_5 + \\ + k_1[A]_0k_3[C]_0k_4k_5 + k_{-1}k_{-2}k_4k_5 + k_{-1}k3[C]_0k_4k_5 + k_2[B]_0k_3[C]_0k_4k_5 \tag{A.6}$$

Hence:

$$\gamma_h = -[E]_0 \frac{N_2\lambda_h^2 + N_3\lambda_h + N_4}{\lambda_h^2 \prod\limits_{\substack{p=1 \\ p \neq h}}^{4} \left(\lambda_p - \lambda_h \right)} \qquad (h=1,2,3,4) \qquad (A.7)$$

$$\alpha = \frac{N_4[E]_0}{F_4} \qquad (A.8)$$

$$\beta = \frac{N_3[E]_0}{N_4} - \frac{F_3}{F_4}\alpha \qquad (A.9)$$

N_2, N_3 and N_4 are:

$$N_2 = k_3[C]_0 k_1[A]_0 k_2[B]_0 \qquad (A.10)$$

$$N_3 = k_3[C]_0 k_1[A]_0 k_2[B]_0 k_4 + k_3[C]_0 k_1[A]_0 k_2[B]_0 k_5 \qquad (A.11)$$

$$N_4 = k_3[C]_0 k_1[A]_0 k_2[B]_0 k_4 k_5 \qquad (A.12)$$

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