

# An alternative analysis of enzyme systems based on the whole reaction time: evaluation of the kinetic parameters and initial enzyme concentration

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This work presents an alternative analysis of the integrated rate equations corresponding to the simple Michaelis-Menten mechanism without product inhibition. The suggested new results are reached under a minimal set of assumptions and include, as a particular case, the classical integrated Michaelis-Menten equation. Experimental designs and a kinetic data analysis are suggested to the estimation of the maximum steady-state rate,  $V_{\max}$ , the Michaelis-Menten constant,  $K_m$ , the initial enzyme

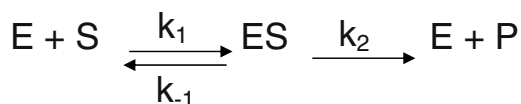
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concentration,  $[E]_0$ , and the catalytic constant,  $k_2$ . The goodness of the analysis is tested with simulated time progress curves obtained by numerical integration.

**KEY WORDS:** Enzyme kinetics, Michaelis–Menten, integrated equation, product rate, substrate rate, numerical integration

## 1. Introduction

The simple Michaelis–Menten reaction mechanism shown in scheme 1 (see below) is the most widely used in enzyme kinetics analysis, in spite of only few enzyme reactions evolve according to this mechanism. In scheme 1,  $E$  denotes the free enzyme,  $S$  the substrate,  $ES$  the complex enzyme–substrate, and  $P$  is the product of the reaction and  $k_1$ ,  $k_{-1}$  and  $k_2$  the rate constants corresponding to the elementary reaction steps. The steady-state parameters describing scheme 1 are the maximum initial rate,  $V_{\max}$  (i.e.  $k_2[E]_0$ ,  $[E]_0$  being the initial enzyme concentration), the catalytic constant,  $k_{\text{cat}}$  (i.e.  $k_2$ ) and the Michaelis–Menten constant  $K_m$  [i.e.  $(k_{-1} + k_2)/k_1$ ]. The reason for the wide use of scheme 1 is that most of the enzyme systems can be apparently described by means of the same equations corresponding this scheme, but using apparent maximum initial, apparent catalytic constant and apparent Michaelis–Menten constant, which are composed of algebraic combinations of the individual rate constants.



Scheme 1.

The most frequently used equation related with reaction mechanisms fitting scheme 1 is the called Michaelis–Menten equation which gives the initial steady-state rate,  $v$ , of product formation,  $P$ , at the steady-state of the reaction as a function of the initial enzyme and substrate concentrations, the rate constant  $k_2$  and the global kinetic parameter  $K_m$  [1,2]. By deriving this equation, it is assumed that the initial substrate concentration remains approximately constant during the reaction time assayed. To reach experimentally this condition it is necessary and sufficient that the initial substrate concentration,  $[S]_0$ , is much higher than that one of the free enzyme,  $[E]_0$ , and that the reaction time assayed is such that the product concentration at this time,  $[P]$  is much less than the initial substrate concentration, e.g.  $[P] = 0.05[S]_0$ .

But under some experimental situations can be not advisable to restrict the reaction extension to a small reaction progress. Some examples of these situations are: (1)  $K_m$  is very small. In this case, the initial concentration of the substrate used must be also very small in order to the product rate is  $[S]_0$ -dependent and thus can use the steady-state rate equation. But if  $[S]_0$  is very small, then, the concentration of the formed product is also very small and difficult to measure and, moreover, the substrate concentration diminishes rapidly and

the assumption of its constancy cannot more be made; (2) If the sensibility of the method to measure the product concentration is small, then it is difficult to detect small concentrations of product, i.e. small variations in the substrate concentration and, therefore, is necessary to let the reaction to progress until a considerable amount of substrate has been converted into product; (3) Sometimes the product concentration cannot be directly measured and indeed the loss of substrate concentration must be measured, what might require a considerable diminution of its concentration [3].

The above situations can be handled by means of the actual so called classical Michaelis–Menten integrated rate equation which is generally used from the steady-state up, assumed to be reached from  $t = 0$ , to the end of the reaction, i.e. in the final phase of the reaction and also assuming that the initial enzyme concentration is much less than the initial substrate concentration [4–12]. Kellershon and Laurent [13] gave methods for analysing the time course of an enzyme system evolving according to scheme 1 when the concentration of the enzyme is high and the derived the corresponding integrated equation for this situation and which become the classical one of Michaelis–Menten in the case of very diluted enzymes.

The different existent contributions about the Michaelis–Menten integrated rate equation are focused to suggest experimental design and kinetic data analysis to evaluate the kinetic parameters  $K_m$  and  $V_m$  and sometimes also the individual rate constants [6]. But, as far as we know, no contributions exist carrying out neither procedures to evaluate from the time course progress of  $S$  and/or  $P$  the enzyme concentration (apart from  $K_m$  and  $V_m$ ) nor a complete analysis about the limits of applicability of the classical integrated rate equations used.

In our opinion, the Michaelis–Menten integrated equation for scheme 1 has some limitations which can be partially avoided if it would be possible to obtain an equivalent equation using less simplifying assumptions than those used by deriving the actual integrated equation. Therefore, the main purposes of this contribution are as follows:

- (1) To analyse the degree of approach of the actual Michaelis–Menten integrated rate equation corresponding to scheme 1. For this purpose we compare the predicted results from the actual integrated equation with those obtained from numerical integration of the system of differential equations describing the behaviour of the enzyme system.
- (2) To derive new equations for scheme 1, in an integrated or differential form, that fits better to the simulated results and, therefore, that improves the actual equation analysis.
- (3) To suggest experimental designs and kinetic data analyses based in the improved equations which allow us to estimate the kinetic  $K_m$ ,  $V_{max}$ ,  $[E]_0$  and  $k_{cat}(k_2)$ .

## 2. Materials and methods

Simulated progress curves were obtained by numerical solution of the set of differential equations (1)–(4), using arbitrary sets of rate constants and initial concentration values. This numerical solution was found by the Runge–Kutta–Fehlberg algorithm [14–16] using a computer program implemented in Visual C++ 6.0 [17]. The above program was run on a PC-compatible computer based on a Pentium III/450 MHz processor with 128 MB of RAM. Data obtained in this way and the corresponding analytical solutions were plotted using the SigmaPlot Scientific Graphing System for Windows Version 8.02 (SPSS Inc.).

## 3. Kinetic analysis

The kinetic behaviour of enzyme systems evolving according to the reaction mechanism in scheme 1 is given by the set of differential equations:

$$\frac{d[E]}{dt} = -k_1[E][S] + (k_{-1} + k_2)[ES], \quad (1)$$

$$\frac{d[ES]}{dt} = k_1[E][S] - (k_{-1} + k_2)[ES], \quad (2)$$

$$\frac{d[S]}{dt} = -k_1[E][S] + k_{-1}[ES], \quad (3)$$

$$\frac{d[P]}{dt} = k_2[ES]. \quad (4)$$

Now we assume that the only species presents at the onset of the reaction are  $E$  and  $S$ . Hence, the mass balances equations are:

$$[S]_0 = [S] + [ES] + [P], \quad (5)$$

$$[E]_0 = [E] + [ES], \quad (6)$$

where  $[E]$ ,  $[ES]$ ,  $[S]$  and  $[P]$  mean the instantaneous concentrations of  $E$ ,  $ES$ ,  $S$  and  $P$ , respectively, and  $[E]_0$  and  $[S]_0$  the initial concentrations of  $E$  and  $S$ , respectively.

From the time at which can be assumed that the system has reached the steady-state, during any elementary time interval,  $dt$ , between the reaction times  $t$  and  $t + dt$ , the concentrations of  $S$ ,  $E$  and  $ES$  remain approximately constant and equal to their instantaneous values at reaction time  $t$ ,  $[S]$ ,  $[E]$  and  $[ES]$ .

We will call the time from which the system can be considered in a (variable) steady-state as  $t_\theta$ . The condition of each steady-state during the elementary time interval  $dt$  is that  $ES$  is formed, approximately (non-equal), at the same rate it disappears, i.e.:

$$k_1[E][S] \approx (k_{-1} + k_2)[ES] \quad (t \geq t_\theta). \quad (7)$$

Obviously  $t_\theta \geq 0$  because at  $t = 0$  is  $[ES] = 0$  and equation (7) is not fulfilled. Note that relationship (7) could not be observed at the end of the reaction when  $[S] \rightarrow 0$  before  $[ES] \rightarrow 0$ . From equations (6) and (7) one obtains:

$$[E] \approx \frac{K_m[E]_0}{K_m + [S]} \quad (t \geq t_\theta), \quad (8)$$

$$[ES] \approx \frac{[E]_0[S]}{K_m + [S]} \quad (t \geq t_\theta), \quad (9)$$

where  $K_m$  is the well-known Michaelis–Menten constant:

$$K_m = \frac{k_{-1} + k_2}{k_1}. \quad (10)$$

Equations (7) and (8) allows us to assume that for any elementary time interval,  $dt$ , between  $t$  and  $t + dt$  the system remains in at instantaneous steady-state which infinitesimally changes from an interval  $dt$  to the next one between  $t + dt$  and  $t + 2dt$ , and so on.

This previous assumption allows us to carry out the following kinetic analysis furnishing both the substrate and product concentrations.

### 3.1. Relationship between $[S]$ and $[P]$ at any reaction time

If equation (9) is inserted into equation (5) we obtain, after some simple algebraic steps, the following result:

$$[S] \approx \frac{-(K_m + [E]_0 + [P] - [S]_0) + \sqrt{(K_m + [E]_0 + [P] - [S]_0)^2 + 4K_m([S]_0 - [P])}}{2} \quad (t \geq t_\theta), \quad (11)$$

which explicitly relates  $[S]$  with  $[P]$  or:

$$[P] \approx [S]_0 - [S] \left( 1 + \frac{[E]_0}{K_m + [S]} \right) \quad (t \geq t_\theta), \quad (12)$$

which explicitly relates  $[P]$  with  $[S]$ .

### 3.2. Relationship between $d[S]/dt$ and $d[P]/dt$ at any reaction time

If we derivate both sides of equation (5) with respect to time,  $t$ , we have:

$$0 = \frac{d[S]}{dt} + \frac{d[ES]}{dt} + \frac{d[P]}{dt}. \quad (13)$$

Hence, deriving both sides of equation (9) with respect to time we obtain:

$$\frac{d[ES]}{dt} \approx \frac{K_m[E]_0}{(K_m + [S])^2} \frac{d[S]}{dt} \quad (t \geq t_\theta). \quad (14)$$

If now equation (14) is inserted into equation (13), we get the following equation (15) relating to the instantaneous rates, at a same reaction time, of the substrate and the product:

$$\frac{d[S]}{dt} \approx -\frac{1}{1 + \frac{K_m[E]_0}{(K_m + [S])^2}} \frac{d[P]}{dt} \quad (t \geq t_\theta) \quad (15)$$

or also:

$$\frac{d[P]}{dt} \approx -\left(1 + \frac{K_m[E]_0}{(K_m + [S])^2}\right) \frac{d[S]}{dt} \quad (t \geq t_\theta). \quad (16)$$

### 3.3. Relationship between $d[P]/dt$ and $[S]$ at any reaction time

If in equation (4) we replace  $[ES]$  by its expression given in equation (9), we have:

$$\frac{d[P]}{dt} \approx \frac{V_{\max}[S]}{K_m + [S]} \quad (t \geq t_\theta) \quad (17)$$

from where:

$$[S] = \frac{K_m \frac{d[P]}{dt}}{V_{\max} - \frac{d[P]}{dt}} \quad (t \geq t_\theta). \quad (18)$$

### 3.4. Integrated rate equation

If we now replace in equation (17)  $d[P]/dt$  by its expression given in equation (16), the result is:

$$\frac{d[S]}{dt} \approx -\frac{V_{\max}[S](K_m + [S])}{(K_m + [S])^2 + [E]_0 K_m} \quad (t \geq t_\theta). \quad (19)$$

By integrating equation (19) one obtains the following relationship between  $[S]$  and  $t$ :

$$V_{\max}(t - t_\theta) \approx [S]_\theta - [S] + K_m \ln \frac{[S]_\theta}{[S]} + [E]_0 \ln \frac{[S]_\theta (K_m + [S])}{[S] (K_m + [S]_\theta)} \quad (t \geq t_\theta), \quad (20)$$

where  $[S]_\theta$  is the substrate concentration at any arbitrarily chosen reaction time  $t_\theta$  at which the attainment of the steady-state is considered to have been reached. Equation (20) implicitly relates  $[S]$  with  $t$ . If in equation (20) we insert equation (11) we would have an implicit relationship between  $[P]$  and  $t$ .

## 4. Results and discussion

In the previous section, we suggest a new, improved, integrated rate equation [equation (20)] for scheme 1 alternative to that used actually for the same reaction scheme. In this section, we analyse the applicability limits of both of the actual integrated rate equations. Next we will carry out a comparative study of the results obtained and we compare these results with those ones obtained by numerical integration of the set of differential equations (1)–(4) describing the kinetic behaviour of systems evolving according to scheme 1. Finally, we suggest experimental designs and kinetic data analysis allowing the evaluation of  $K_m$ ,  $V_{\max}$ ,  $[E]_0$  and  $k_2$ . For a better comprehension of this section, we begin it remembering the major items in the derivation of the actual integrated rate equation of Michaelis–Menten for scheme 1.

### 4.1. The classical integrated Michaelis–Menten equation

The derivation of the actual integrated rate equation for enzyme reactions evolving according to mechanisms as that in scheme 1 is based on the following three implicit assumptions (a)–(c), which next we summarise.

**Assumption (a).** The steady-state of the reaction is reached from practically  $t = 0$ .

Using our notation this assumption means that  $t_\theta \approx 0$ ,  $[S]_\theta = [S]_0$  and  $[P]_\theta = 0$ .

**Assumption (b).** The rate of substrate disappearance is, at each reaction time, equal to the formation rate of the substrate at the same time and this rate is given by an equation formally analogous to that of Michaelis–Menten corresponding to the steady-state but in which the initial substrate concentration,  $[S]_0$ , is replaced by the instantaneous one,  $[S]$ , i.e.:

$$\frac{d[S]}{dt} = -\frac{d[P]}{dt} \quad (t \geq 0), \quad (21)$$

$$\frac{d[S]}{dt} = -\frac{V_{\max}[S]}{K_m + [S]} \quad (t \geq 0). \quad (22)$$

From equation (22) it results, after integration and some rearrangements:

$$V_{\max}t = [S]_0 - [S] + K_m \ln \frac{[S]_0}{[S]} \quad (t \geq 0). \quad (23)$$

**Assumption (c).** The amount of substrate consumed during the reaction time  $t$  is equal to the amount of product formed during the same time  $t$ , i.e. the enzyme-substrate complex concentration  $[ES]$ , at any reaction time is ignored so that the following relationship is assumed:

$$[S]_0 - [S] = [P] \quad (t \geq 0). \quad (24)$$

Having equation (24) into account in equation (23), the later can be rewritten as:

$$V_{\max}t = P + K_m \ln \frac{[S]_0}{[S]_0 - [P]} \quad (t \geq 0), \quad (25)$$

which yet admits other arrangement forms, all of them equivalent. Equations (23) and (25) are the two most frequently used forms of the integrated rate equation of Michaelis–Menten for scheme 1.

#### 4.2. Limitations and applicability of the integrated rate equation (20) suggested here

The kinetic analysis suggested by us in section 3 is based on the fulfillment of infinite and successive instantaneous steady states each of them during an infinitesimal time interval  $dt$  time which each of them binds to the following elementary steady state during the reaction progress. In each of these elementary, virtual steady-state equation (7) showing that the formation rate of  $ES$  is approximately equal to its transformation in  $E$  must be observed. Nevertheless, this approach will not be observed until a certain reaction time is elapsed and that was above denoted as  $t_\theta$ . For example, at  $t = 0$  the formation rate of  $ES$



is  $k_1[E]_0[S]_0$  whereas the rate of  $ES$  transformation is zero and a certain time must be elapsed before both rates,  $k_1[E][S]$  and  $(k_{-1}+k_2)[ES]$ , are approximately equal and they remain approximately equal (although being different the values of these rates at each reaction time because the changes in the  $[E]$ -,  $[S]$ - and  $[ES]$ -values) during the whole course of the reaction.

Once defined conceptually the time  $t_\theta$  as the time at which the steady-state is reached, an operational definition of it is needed. An operational definition we suggest, and that we will call definition A, consists on taking as  $t_\theta$  the  $t$ -value at which the system reaches the steady-state the first time from the onset of the reaction. This time can be quantified as the time for which the quotient,  $r$ , defined in equation (26), reaches the first time, from the onset of the reaction, the unity value or the nearest to the unity value.

$$r = \frac{\frac{[E][S]}{[ES]}}{K_m} = \frac{[E][S]}{K_m[ES]}. \quad (26)$$

To support this section, in table 1 we show, as examples, four cases of enzyme systems evolving according to the reaction mechanism in scheme 1 which only differ in the  $[E]_0$ - and/or  $[S]_0$ -values. In table 1 are also shown the time the reaction elapsed to  $[P] = 0.9999[S]_0$ , which we will take as the time the reaction run, i.e. the time from  $t = 0$  at which the reaction can be considered finished. In the following we will refer this time as  $t_\infty$ . From the simulated time progress curves of each of these cases it is easy to determine the corresponding  $t_\theta$ -values which are summarised in table 2. Note that this value is generally small. Thus, with this operational definition  $t_\theta$  is the minimal value of the reaction time from which the system remains (approximately) in a steady-state. The values of  $[S]$  and  $[P]$  corresponding to this  $t_\theta$ -value are those of  $[S]_\theta$  and  $[P]_\theta$ . The value of  $r$  corresponding to this  $t_\theta$ -value will be denoted as  $r_\theta$ . In table 2 we show  $t_\theta$ -,  $r_\theta$ -,  $[S]_\theta$ - and  $[P]_\theta$ -values for each one of the cases in table 1 obtained from the simulated time progress curves, i.e. from the numerical integration of the set of differential equations (1)–(4). This definition has the advantage that  $t_\theta$  can be unequivocally determined, but it has the inconvenient that it can only be determined from the simulated progress curves and has, therefore, a fundamentally meaning theoretical. Thus, in the practice, other operational definitions are needed.

A second operational definition, given arbitrarily and that we will call operational definition B, consists on taking as  $t_\theta$  any reaction time we assume it is higher than the  $t_\theta$ -value corresponding to definition A. Below we show how to determine if the reaction time chosen as  $t_\theta$  in definition B is higher (and therefore, adequate) than the  $t_\theta$ -value corresponding to definition A. The decision about what reaction time to choose is obviously arbitrary. For example, it can be chosen, from the time progress curve of  $S$  or  $P$ , as the reaction time corresponding to a determined concentration of the substrate or product concentration.

Table 1

Cases differing in the  $[E]_0$ - and  $[S]_0$ - values used as examples. In all cases the values of the rate constants, chosen arbitrarily but in the usual rank in enzyme reactions, are  $k_1 = 1 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ ,  $k_{-1} = 115 \text{ s}^{-1}$  y  $k_2 = 10 \text{ s}^{-1}$ . Therefore, the Michaelis–Menten constant,  $K_m$ , in all of the cases is  $1.25 \times 10^{-6} \text{ M}$ . On the fourth–sixth columns the values of  $[E]_0/K_m$ ,  $[E]_0/[S]_0$  and  $V_{\max}$  corresponding to each case are indicated. On the last column is indicated the time the reaction elapses to  $[P] = 0.9999[S]_0$  and that can be taken as the time the reaction is ended, denoted as  $t_\infty$ .

Case	$[E]_0(\mu\text{M})$	$[S]_0(\text{mM})$	$[E]_0/K_m$	$[E]_0/[S]_0$	$V_{\max}(\mu\text{M s}^{-1})$	$t_\infty(\text{s})$
1	0.1	0.01	0.08	0.01	1	22.150
3	0.1	1	0.08	0.0001	1	1086.5
4	1	0.01	0.8	0.1	10	2.8366
7	10	0.01	8	1	100	1.0463

Table 2

Values of  $t_\theta$ ,  $r_\theta$ ,  $[S]_\theta$  and  $[P]_\theta$  for each of cases 1–7 in table 1 according to the definition A explained in the main text.

Case	$t_\theta(\text{ms})$	$r_\theta$	$[S]_\theta(\mu\text{M})$	$[P]_\theta(\text{nM})$
1	10.3	1.000	9.9028	8.3920
3	0.8036	1.000	999.56	337.56
4	8.5763	1.000	9.0540	67.349
7	6.2993	1.000	2.7515	372.45

In table 3 we shown the  $t_\theta$  for cases 1–7 in table 1 as the time the system elapses to  $[S] = 0.95[S]_0$ . Likewise, the  $t_\theta$ -value in definition B could be merely chosen as any reaction time less than  $t_\infty$  at which we consider that the system already is in the steady-state. Obviously, the less the chosen value for  $t_\theta$  the more experimental data are available for the kinetic data analysis. In table 4 we have arbitrarily chosen  $t_\theta$  as the 10% approximately of the total reaction time,  $t_\infty$ .

In figure 1, we show as example, the time course of the species involved in scheme 1 for case 4 in table 1 and in figures 2(a)–4(a), we show the corresponding time course of  $r$  for cases 4, 1 and 7 in table 1 obtained from the time progress curves of  $E$ ,  $S$  and  $ES$  and equation (26).

#### 4.3. Comparison of the validity of equations (20) and (23)

In figures 2(b)–4(b), we show the instantaneous values of the quotient,  $q$ , given by equation (27)

$$q = \frac{V_{\max}(t - t_\theta)}{[S]_\theta - [S] + K_m \ln \frac{[S]_\theta}{[S]} + [E]_0 \ln \frac{[S]_\theta(K_m + [S])}{[S](K_m + [S]_\theta)}} \quad (27)$$

Table 3

Values of  $t_\theta$ ,  $r_\theta$ ,  $[S]_\theta$  and  $[P]_\theta$  for each of cases 1–7 in table 1 according to the definition B explained in the main text taking as  $t_\theta$  the time the system elapses for  $[S] = 0.95[S]_0$  (therefore  $[S]_\theta = 0.95[S]_0$ ). On the third and fourth columns the corresponding values of  $r_\theta$  and  $[P]_\theta$  are shown. Note that the  $t_\theta$ -value taken for cases 4 and 7 are not adequate because  $r_\theta$  is far from the unity.

Case	$t_\theta$ (s)	$r_\theta$	$[S]_\theta$ ( $\mu\text{M}$ )	$[P]_\theta$ ( $\mu\text{M}$ )
1	0.4614	0.9999	9.5	0.40797
3	49.961	1.000	950	49.897
4	$0.7504 \times 10^{-3}$	7.6655	9.5	0.0215
7	$5.2795 \times 10^{-5}$	144.46	9.5	$1.3436 \times 10^{-4}$

Table 4

Values of  $t_\theta$ ,  $r_\theta$ ,  $[S]_\theta$  and  $[P]_\theta$  for each of cases in table 1 according to the definition B explained in the main text taking as  $t_\theta$  a value of the reaction time approximately equal to a 10% of  $t_\infty$ .

Case	$t_\theta$ (s)	$r_\theta$	$[S]_\theta$ ( $\mu\text{M}$ )	$[P]_\theta$ ( $\mu\text{M}$ )
1	1	0.9999	9.0296	0.8826
3	50	1.0000	949.96	49.935
4	0.15	0.9988	0.9711	1.2999
7	0.1	0.9391	73.637	5.4089

during the whole course from  $t = t_\theta$  for cases 1, 4 and 7 in table 1. The  $t$ -values and the corresponding  $[S]$ -values, were obtained from numerical integration of the set of differential equations (1)–(4). The  $t_\theta$ - and  $[S]_\theta$ -values were those in table 2. According to equation (20) this quotient should be ever equal to the

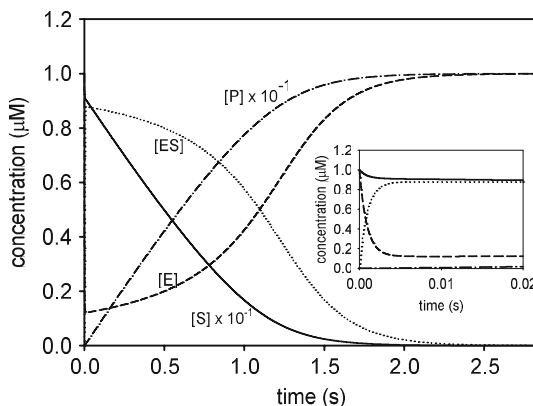


Figure 1. Time progress curves of  $E$ ,  $ES$ ,  $S$  and  $P$  corresponding to case 4 in table 1.

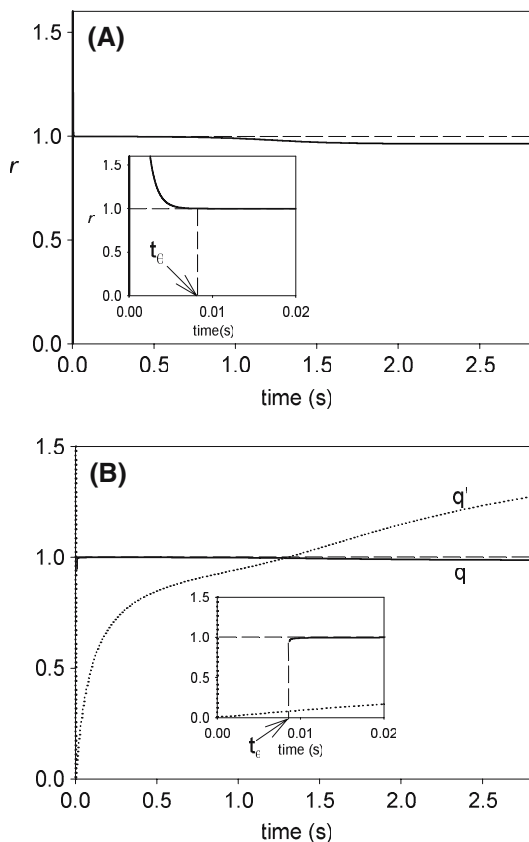


Figure 2. (a) Time course of  $r$  corresponding to case 4 in table 1. We also indicate (---) the ideal  $r$  value equal to the unity. Insert: Details at the onset of the reaction where the  $t_{\theta}$ -value arisen from operational definition A is indicated. (b) Time course of  $q$  (—) and  $q'$  (.....) corresponding to case 4 in table 1. We also indicate (---) the ideal  $q$ - and  $q'$ -values equal to the unity. Insert. Details at the onset of the reaction where it is indicated the time (the  $t_{\theta}$ ) from which our analysis is valid.

unity if equation (20) would be observed. Thus, the nearer is  $q$  of the unity the higher is the accuracy of equation (20).

To quantify the validity of equation (23) we define  $q'$  as the quotient:

$$q' = \frac{V_{\max} t}{[S]_0 - [S] + K_m \ln \frac{[S]_0}{[S]}} \quad (t \geq 0). \quad (28)$$

In figures 2(b)–4(b), we show the instantaneous values of the quotient,  $q'$ , given by equation (28) during the whole course of the reaction for cases 1, 4 and 7 in table 1. The  $t$  values and the corresponding  $[S]$  values to be inserted into above quotient were obtained from numerical integration of the set of differential equations (1)–(4). According to equation (23), this quotient should be ever equal to the unity. Thus, the nearest is  $q'$  of the unity the higher is the accuracy of equation (23).

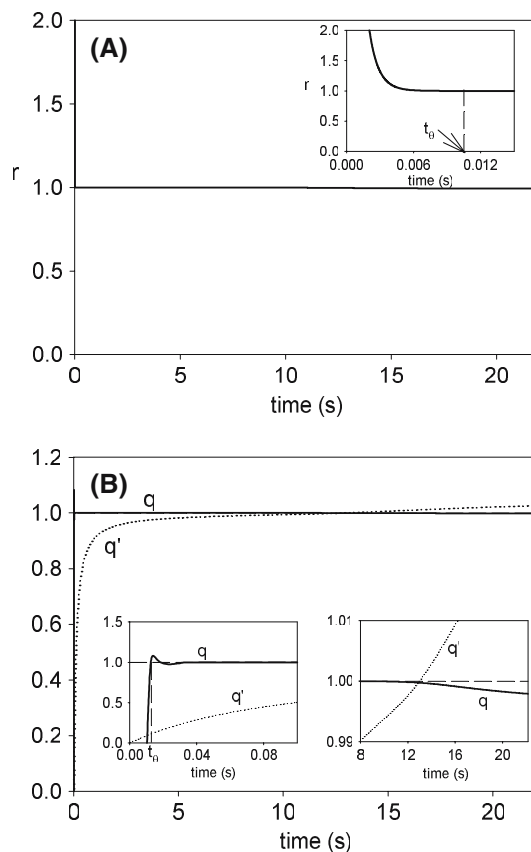


Figure 3. (a) Time course of  $r$  corresponding to case 1 in table 1. We also indicate (---) the ideal  $r$  value equal to the unity. Insert. Details at the onset of the reaction where the  $t_{\theta}$ -value arisen from operational definition A is indicated. (b) Time course of  $q$  (—) and  $q'$  (.....) corresponding to case 4 in table 1. We also indicate (---) the ideal  $q$ - and  $q'$ -values equal to the unity. Left hand insert: Details at the onset of the reaction where it is indicated the time (the  $t_{\theta}$ ) from which our analysis is valid. Right hand insert: Details at the end of the reaction.

Briefly, from figures 2(b)–4(b) it is evident that the application of integrated rate equation (20) is much more adequate, in all cases, that the actual integrated rate equation (23). This result will be also confirmed from the kinetic data analysis suggested below.

#### 4.4. Actual classical integrated Michaelis–Menten equations are particular cases of that suggested here

Our integrated rate equation (20) contains, as a particular case, the actual integrated rate equations (24) and (25). Effectively, due that it is ever observed in equation (20) that  $[S] \leq [S]_{\theta}$  (equal at  $t = t_{\theta}$  and less at any time  $t > t_{\theta}$ ), the

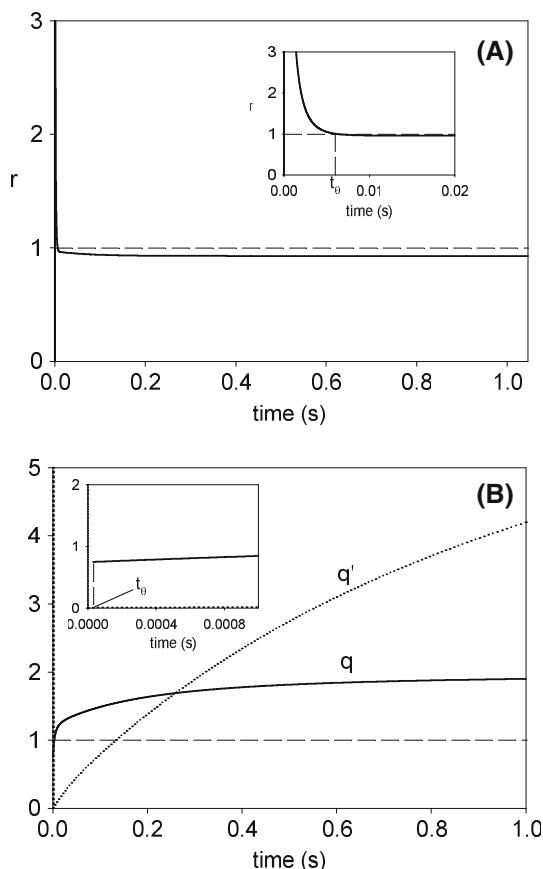


Figure 4. (a) Time course of  $r$  corresponding to case 7 in table 1. We also indicate (---) the ideal  $r$  value equal to the unity. Insert. Details at the onset of the reaction where the  $t_\theta$ -value arisen from operational definition A is indicated. (B) Time course of  $q$  (—) and  $q'$ (.....) corresponding to case 7 in table 1. We also indicate (---) the ideal  $q$ - and  $q'$ -values equal to the unity. Insert. Details at the onset of the reaction where it is indicated the time ( $t_\theta$ ) from which our analysis is valid.

following relationship will be ever observed:

$$\frac{[S]_\theta (K_m + [S])}{[S] (K_m + [S]_\theta)} \leq \frac{[S]_\theta}{[S]} \quad (t \geq t_\theta). \quad (29)$$

So that in those cases in which it is fulfilled that:

$$[E]_0/K_m \ll 1 \quad (30)$$

the following approach can be made:

$$K_m \ln \frac{[S]_\theta}{[S]} + [E]_0 \ln \frac{[S]_\theta (K_m + [S])}{[S] (K_m + [S]_\theta)} \approx K_m \ln \frac{[S]_\theta}{[S]} \quad (t \geq t_\theta) \quad (31)$$

and equation (20) becomes:

$$V_{\max}(t - t_{\theta}) \approx [S]_{\theta} - [S] + K_m \ln \frac{[S]_{\theta}}{[S]} \quad (t \geq t_{\theta}). \quad (32)$$

Moreover it is to be note that if in equation (12) condition (30) is inserted, then equation (24) is approximately fulfilled. If now we insert equation (24) into equation (32), it results:

$$V_{\max}(t - t_{\theta}) = [P] - [P]_{\theta} + K_m \ln \frac{[S]_0 - [P]_{\theta}}{[S]_0 - [P]} \quad (t \geq t_{\theta}). \quad (33)$$

Note that if in equations (32) and (33) we set  $t_{\theta} = 0$  and, therefore  $[S]_{\theta} = [S]_0$  and  $[P]_{\theta} = 0$ , these equations become equations (23) and (25), respectively, i.e., the actual integrated rate equations for scheme 1 are particular cases of equation (20).

#### 4.5. Kinetic data analysis: evaluation of $V_{\max}$ , $K_m$ , $[E]_0$ and $k_2$

##### 4.5.1. Revision of the methods based on the actual integrated rate equations (23) and (25)

From actual integrated equation (23) a kinetic data analysis is suggested to evaluate the kinetic parameters  $V_{\max}$  and  $K_m$  [3]. Effectively, equation (24) can be rearranged as:

$$\frac{1}{t} \ln \frac{[S]_0}{[S]} = -\frac{1}{K_m} \frac{[S]_0 - [S]}{t} + \frac{V_{\max}}{K_m} \quad (t \geq 0) \quad (34)$$

and, therefore,  $V_{\max}$  and  $K_m$  could be obtained from the time progress curve of S by plotting  $(1/t) \ln([S]_0/[S])$  versus  $([S]_0 - [S])/t$ , which results in a straight line with the ordinate intercept  $V_{\max}/K_m$  and with the slope  $-1/K_m$  so that the determination of  $V_{\max}$  and  $K_m$  is immediate. But, due to the fact that very approached character of equation (23) and, therefore, of equation (34), the mentioned plot exhibits a peculiar shape and the values of  $K_m$  and  $V_{\max}$  obtained considerably can differ from those ones which would have been obtained, i.e. the real ones, as we shown using data obtained from numerical integration. In figure 5(a), we show the plots of  $(1/t) \ln([S]_0/[S])$  versus  $([S]_0 - [S])/t$  for case 4 in table 1. Note that the plot does not correspond to a straight line with a negative slope until a certain reaction time  $t_a$ , is elapsed. In table 5, we summarise the values of  $K_m$  and  $V_{\max}$  obtained from the portion of curve between points  $a$  and  $b$  in the plot [the only which can be fitted to an equation like equation (34)]. In the following we will refer to this method as IM(S).

Likewise, from actual integrated rate equation (25) a kinetic data analysis is suggested to evaluate the kinetic parameters  $V_{\max}$  and  $K_m$  [3]. Effectively, from equation (25) we have that a plot of  $(1/t) \ln([S]_0/([S]_0 - [P]))$  versus  $[P]/t$

Table 5

Values of the parameters  $K_m$ ,  $V_{\max}$ ,  $[E]_0$  and  $k_2$  obtained for cases 1–4 according to some of the methods 1–3 described in the main text. The values obtained from the actual integrated rate equation [either from both IM(P) and IM(S) methods or from one of them] are also indicated. The true values of these parameters are summarised in table 1 and marked in this one in bold on the first row of each case.

Case	Method	$K_m$ ( $\mu\text{M}$ )	$V_{\max}$ ( $\mu\text{M s}^{-1}$ )	$[E]_0$ ( $\mu\text{M}$ )	$k_2$ ( $\text{s}^{-1}$ )
1		<b>1.25</b>	<b>1</b>	<b>0.1</b>	<b>10</b>
1	1	1.2405 $\pm$ 0.0001	0.9979 $\pm$ 0.0008	0.0992 $\pm$ 0.0001	10.059 $\pm$ 0.019
1	2	1.3314 $\pm$ 0.0001	1.0317 $\pm$ 0.0001	0.0537 $\pm$ 0.0001	19.213 $\pm$ 0.038
1	3	1.2525 $\pm$ 0.0001	1.0001 $\pm$ 0.0001	0.0917 $\pm$ 0.0001	10.906 $\pm$ 0.013
1	IM(S)	1.4624 $\pm$ 0.0052	1.0471 $\pm$ 0.0067	–	–
1	IM(P)	1.32254 $\pm$ 0.0006	1.0066 $\pm$ 0.0010	–	–
3		<b>1.25</b>	<b>1</b>	<b>0.1</b>	<b>10</b>
3	1	1.2563 $\pm$ 0.0024	1.0000 $\pm$ 0.0001	0.1005 $\pm$ 0.0002	9.9502 $\pm$ 0.0208
3	2	1.2521 $\pm$ 0.0001	1.0007 $\pm$ 0.0001	0.0965 $\pm$ 0.0001	10.435 $\pm$ 0.012
3	3	1.2502 $\pm$ 0.0001	1.0000 $\pm$ 0.0001	0.0998 $\pm$ 0.0001	10.020 $\pm$ 0.011
3	IM(S)	4.7506 $\pm$ 0.1437	1.0052 $\pm$ 0.0610	–	–
3	IM(P)	1.2509 $\pm$ 0.0093	1.0000 $\pm$ 0.0149	–	–
4		<b>1.25</b>	<b>10</b>	<b>1</b>	<b>10</b>
4	1	1.1895 $\pm$ 0.0050	10.092 $\pm$ 0.0002	0.9849 $\pm$ 0.0050	10.247 $\pm$ 0.053
4	2	1.1909 $\pm$ 0.0002	9.6968 $\pm$ 0.0002	0.9445 $\pm$ 0.0003	10.267 $\pm$ 0.004
4	3	1.2627 $\pm$ 0.0001	10.007 $\pm$ 0.001	0.9438 $\pm$ 0.0008	10.603 $\pm$ 0.001
4	IM(S)	3.1289 $\pm$ 0.0084	14.143 $\pm$ 0.060	–	–
4	IM(P)	2.1368 $\pm$ 0.0040	10.832 $\pm$ 0.035	–	–
7		<b>1.25</b>	<b>100</b>	<b>10</b>	<b>10</b>
7	1	1.1390 $\pm$ 0.0057	118.15 $\pm$ 5.80	9.8170 $\pm$ 0.4859	12.035 $\pm$ 1.187
7	2	0.8886 $\pm$ 0.0344	39.679 $\pm$ 2.179	3.5135 $\pm$ 0.6212	11.293 $\pm$ 2.617
7	3	1.1116 $\pm$ 0.0004	97.466 $\pm$ 0.034	9.7656 $\pm$ 0.0012	9.9805 $\pm$ 0.0047
7	IM(S)	(impossible to apply)	–	–	–
7	IM(P)	34.471 $\pm$ 0.1719	319.44 $\pm$ 0.08	–	–

should be a straight line with the ordinate intercept  $V_{\max}/K_m$  and the slope  $-1/K_m$  so that the evaluation of  $V_{\max}$  and  $K_m$  is immediate. But, due to the very approached character of equation (25), the mentioned plot exhibits a peculiar shape [see figure 5(b)] and the values of  $K_m$  and  $V_{\max}$  obtained considerably can differ from those ones which would have been obtained. In figure 5(b), we show the plot of  $(1/t) \ln([S]_0/([S]_0 - [P]))$  versus  $[P]/t$  for case 4 in tables 1 and 5, we summarise the values of  $K_m$  and  $V_{\max}$  obtained from the portion of curve between points *a* and *b* in the plot (the only which can be fitted to a straight line with a negative slope). In the following, we will refer to this method as IM(P).

Note that only suitable results by applying methods IM(S) and IM(P) are to be expected when equations (23) and (25) are justified, i.e. when condition



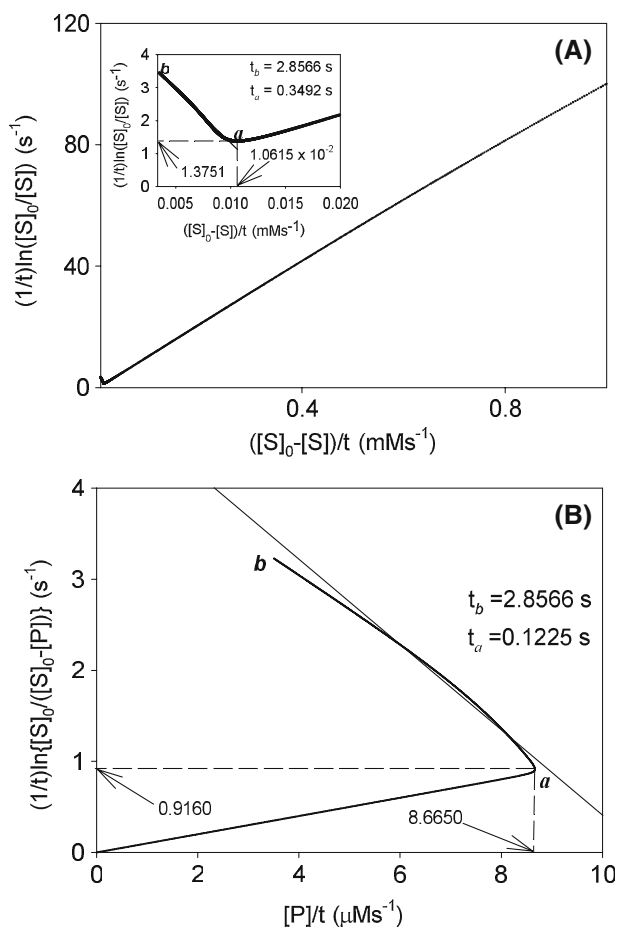


Figure 5. (a) Plot of  $(1/t) \ln([S]_0/[S])$  versus  $([S]_0-[S])/t$  from  $t = 0$  for case 4 in table 1. Note that only from  $t = t_a = 0.3492$  the plot approaches a straight line with positive ordinate intercept and negative slope (Insert). This plot is needed to apply method IM(S). (b) Plot of  $(1/t) \ln\{[S]_0/([S]_0-[P])\}$  versus  $[P]/t$  from  $t = 0$  for case 4 in table 1. Note that only from  $t = t_a = 0.3492$  s the plot approaches a straight line with positive ordinate intercept and negative slope. This plot is needed to apply method IM(P).

(30) is observed. Thus, in case 4 where  $[E]_0/K_m = 0.8$  the results are bad, whereas in cases 1 and 3, where  $[E]_0/K_m = 0.08$ , the results are much better, as indicated in table 5. In case 7, where  $[E]_0/K_m = 8.0$  method IM(S) is inapplicable and method IM(P) gives an enormous error. Therefore, a strategy to use methods IM(S) and/or IM(P) is to decrease the initial enzyme concentration.

#### 4.5.2. Methods based on our results

Next, we suggest experimental designs and kinetic data analysis based on other of our results in this paper to evaluate not only  $K_m$  and  $V_{max}$ , but also  $[E]_0$

and, therefore,  $k_2$ . We will distinguish three different situations: (a) One disposes of the time course of  $S$  and  $P$ ; (b) One disposes of the time course of  $S$ , but not that of  $P$ ; (c) One disposes of the time course of  $P$ , but not of that of  $S$ .

(a) *One disposes of the time course progress curves of both  $S$  and  $P$  (method 1)*

In this case, the procedure we suggest consists in the following steps (1)–(3).

(1) Equation (12) relating  $[P]$  with  $[S]$  can also be rewritten in the following forms more adequate for our purpose:

$$\frac{[S]_0 - [P]}{[S]} \approx \frac{K_m + [E]_0 + [S]}{K_m + [S]} \quad (t \geq t_\theta) \quad (35)$$

or

$$\frac{[S]_0 - [P]}{[S]} \approx \frac{a + b[S]}{1 + b[S]} \quad (t \geq t_\theta). \quad (36)$$

A fit by non-linear regression to the two parameters rational equation (36) of the experimental data of  $[P]$  and  $[S]$  allows us to evaluate the parameters  $a$  [which, according to equation (35) is equal to  $1 + ([E]_0/K_m)$ ] and  $b$  [equal to  $1/K_m$  according to equation (35)]. Therefore, evaluation of  $K_m$  and  $[E]_0$  is immediate. In figure 6(a) we plotted the dependence of  $([S]_0 - [P])/[S]$  on  $[S]$  for case 4. From the values for  $a$  and  $b$  from the fitting to equation (36) we obtained the values  $K_m$  and  $[E]_0$ .

(2) Hence, from the  $[E]_0$ - and  $K_m$ -values obtained above, the  $[S]$ -values at the different reaction times,  $t$ , the  $t_\theta$ - and  $[S]_\theta$ -values, one plot of

$$[S]_\theta - [S] + K_m \ln \frac{[S]_\theta}{[S]} + [E]_0 \ln \left\{ \frac{[S]_\theta (K_m + [S])}{[S] (K_m + [S]_\theta)} \right\} \text{ versus } (t - t_\theta) \text{ gives,}$$

according to equation (20) a straight line through the origin with the slope  $V_{\max}$ . In figure 6(b) we plotted this dependence for case 4.

(3) From the  $V_{\max}$ - and  $[E]_0$ -values above obtained the  $k_2$ -value is immediately obtained as the quotient  $V_{\max}/[E]_0$ .

In table 5, we summarise the  $K_m$ -,  $[E]_0$ -,  $V_{\max}$ - and  $k_2$ -values obtained using this method for case 4.

(b) *One disposes of the time course of  $S$ , but not of that of  $P$  (method 2)*

From the time progress curve of  $S$  we construct the time progress curve of  $(1/[S])(d[S]/dt)$ . From equation (19) we have:

$$-\frac{1}{[S]} \frac{d[S]}{dt} \approx \frac{a + b[S]}{1 + c[S] + d[S]^2} \quad (t \geq t_\theta), \quad (37)$$

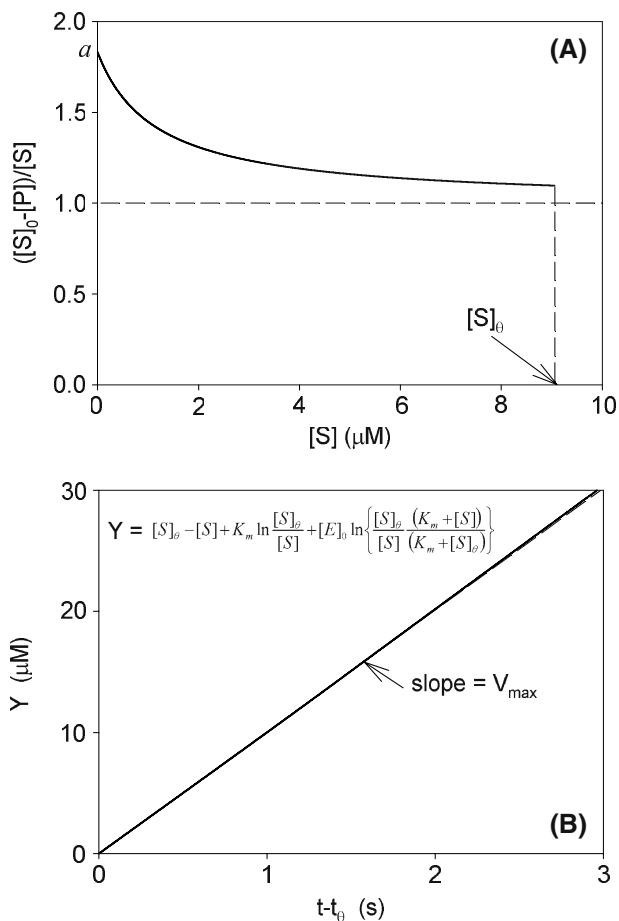


Figure 6. Plots needed to apply method 1 suggested in this paper. (a) Plot of  $([S]_0 - [P])/[S]$  versus  $[S]$  from  $[S] = [S]_\theta$  for case 4 in table 1. A fit of this curve to equation (36) allows to determine the parameters  $a$  and  $b$  involved. (b) Plot of  $[S]_\theta - [S] + K_m \ln \frac{[S]_\theta}{[S]} + [E]_0 \ln \left\{ \frac{[S]_\theta (K_m + [S])}{[S] (K_m + [S]_\theta)} \right\}$  versus  $t - t_\theta$  from  $t = t_\theta$ . for case 4 in table 1 (—). A fit of this plot to a straight line through the origin (---) allows to determine  $V_{\max}$  equal to its slope.

where:

$$a = \frac{V_{\max}}{K_m + [E]_0}, \tag{38}$$

$$b = \frac{V_{\max}}{K_m(K_m + [E]_0)}, \tag{39}$$

$$c = \frac{2}{K_m + [E]_0}, \tag{40}$$

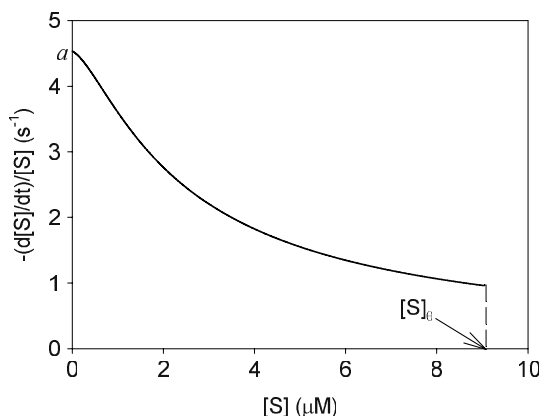


Figure 7. Plot of  $-(d[S]/dt)/[S]$  versus  $[S]$  from  $[S] = [S]_{\theta}$  for case 4 in table 1 needed to apply method 2 suggested in this paper. A fit of this curve to equation (37) allows determining the parameters  $a$ ,  $b$ ,  $c$  and  $d$  involved.

$$d = \frac{1}{K_m(K_m + [E]_0)}. \quad (41)$$

Then a fit of the data  $(1/[S])(d[S]/dt)$  to the four parameters rational equation (37) allows to evaluate  $a$ ,  $b$  and  $c$ . By combining these values those of  $K_m$ ,  $V_{\max}$ , and  $[E]_0$  are obtained [ $K_m = a/b$ ,  $V_{\max} = 2a/c$ ,  $[E]_0 = (2b-ac)/(bc)$ ]. Hence, from the  $V_{\max}$ - and  $[E]_0$ -values,  $k_2$  is obtained because  $k_2 = V_{\max}/[E]_0$ .

In figure 7, we plotted the dependence of  $(1/[S])(d[S]/dt)$  on  $[S]$  for case 4. From the values for  $a$ ,  $b$  and  $c$  obtained from the fitting to equation (36) and from equations (38)–(40), the values of the kinetic parameters  $K_m$ ,  $V_{\max}$ ,  $[E]_0$ , and  $k_2$  can be easily obtained. In table 5, we show these values for cases 3, 4 and 7. Obviously, this method 2 can also be applied when method 1 is possible.

(c) *One disposes only of the time course of  $P$  (and, therefore, of its first derivative  $d[P]/dt$ ), but not of that of  $S$ . (method 3).*

If in equation (12) we insert equation (18) we have, after some algebraic rearrangement:

$$\frac{[S]_0 - [P]}{\frac{d[P]}{dt}} = \frac{a + b \frac{d[P]}{dt}}{1 + c \frac{d[P]}{dt}}, \quad (42)$$

where:

$$a = \frac{K_m + [E]_0}{V_{\max}}, \quad (43)$$

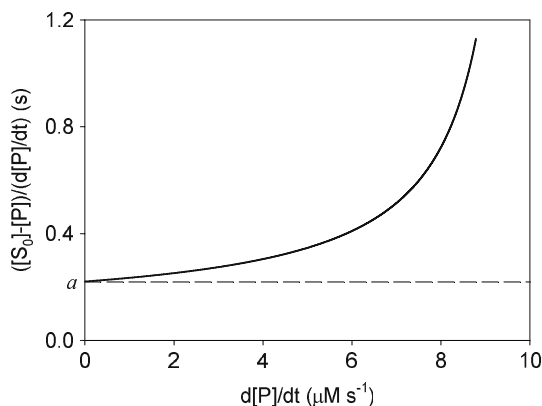


Figure 8. Plot of  $([S]_0 - [P]) / (d[P]/dt)$  versus  $d[P]/dt$  from  $t = t_\theta$  for case 4 in table 1 needed to apply method 3 suggested in this paper. A fit of this curve to equation (42) allows to determine the parameters  $a$  and  $b$  involved.

$$b = -\frac{[E]_0}{V_{\max}^2}, \quad (44)$$

$$c = -\frac{1}{V_{\max}}. \quad (45)$$

Thus, if we fit to equation (42) the experimental dependence of  $([S]_0 - [P]) / (d[P]/dt)$  on  $d[P]/dt$  we obtain the values of  $a$ ,  $b$  and  $c$  and, from them, those ones of  $K_m$ ,  $V_{\max}$  and  $[E]_0$ .

In figure 8, we plotted the dependence of  $([S]_0 - [P]) / (d[P]/dt)$  on  $d[P]/dt$  for case 4. From the values for  $a$ ,  $b$  and  $c$  obtained from the fitting to equation (42) and from equations (43)–(45), the values of the kinetic parameters  $K_m$ ,  $V_{\max}$ ,  $[E]_0$ , and  $k_2$  can be easily obtained. In table 5, we show these values for cases 1–7. Obviously, this method 3 can also be applied when method 1 is possible.

#### 4.6. Choose of an adequate $t_\theta$ -value

Methods 1–3 are based on the knowledge of the  $t_\theta$ -value. In the results summarised in tables 6 and 7 we have used in each case the  $t_\theta$ -value obtained from the operational definition A (see table 2). Nevertheless, the results would have been similar if we had taken, in each case, any other  $t_\theta$ -value higher than the corresponding one from the operational definition A. But, as commented above, the value experimentally chosen for  $t_\theta$  is always submitted to the doubt whether it is correct or not, i.e. if at this time value the steady-state has already been reached or it is not more observed. The kinetic data analysis here suggested

Table 6

Values of  $K_m$ ,  $V_{\max}$ ,  $[E]_0$  and  $k_2$  for case 3 obtained from the time progress curve of  $P$ , i.e. by using method 3, but starting at the different  $t$ -values on the first column. The true values of  $K_m$ ,  $V_{\max}$ ,  $[E]_0$  and  $k_2$  are 1.25, 1, 0.1  $\mu\text{M}$  and 10  $\text{s}^{-1}$ , respectively.

$t_\theta$ (s)	$K_m$ ( $\mu\text{M}$ )	$V_{\max}$ ( $\mu\text{M s}^{-1}$ )	$[E]_0$ ( $\mu\text{M}$ )	$k_2$ ( $\text{s}^{-1}$ )
$0.8036 \times 10^{-3}$	1.2502 $\pm$ 0.0001	1.0000 $\pm$ 0.0001	0.0998 $\pm$ 0.0001	10.020 $\pm$ 0.012
10.220	1.2502 $\pm$ 0.0001	1.0000 $\pm$ 0.0001	0.0983 $\pm$ 0.0001	10.173 $\pm$ 0.012
50.343	1.2502 $\pm$ 0.0001	1.0000 $\pm$ 0.0001	0.0982 $\pm$ 0.0001	10.183 $\pm$ 0.012

Table 7

Values of  $K_m$ ,  $V_{\max}$ ,  $[E]_0$  and  $k_2$  for case 1 obtained from the time progress curve of  $P$ , i.e. by using method 3, but starting at the different  $t$ -values on the first column. The true values of  $K_m$ ,  $V_{\max}$ ,  $[E]_0$  and  $k_2$  are 1.25  $\mu\text{M}$ , 1  $\mu\text{M}$ , 0.1  $\mu\text{M}$  and 10  $\text{s}^{-1}$ , respectively.

$t_\theta$ (s)	$K_m$ ( $\mu\text{M}$ )	$V_{\max}$ ( $\mu\text{M s}^{-1}$ )	$[E]_0$ ( $\mu\text{M}$ )	$k_2$ ( $\text{s}^{-1}$ )
0.0103	1.2525 $\pm$ 0.0001	1.0001 $\pm$ 0.0001	0.0917 $\pm$ 0.0001	10.906 $\pm$ 0.013
2.0115	1.2523 $\pm$ 0.0001	1.0002 $\pm$ 0.0001	0.0910 $\pm$ 0.0001	10.991 $\pm$ 0.013
5.0078	1.2536 $\pm$ 0.0001	1.0003 $\pm$ 0.0001	0.0897 $\pm$ 0.0001	11.152 $\pm$ 0.014

can help us to solve this dilemma. It would be sufficient to take as  $t_\theta$  some different reaction times arbitrarily chosen and distributed on the total reaction time,  $t_\infty$ . Any of the  $t_\theta$ -value belonging to a set of consecutive  $t_\theta$ -values giving all of them similar results for  $K_m$  and  $V_{\max}$  (by using the same method) than any other  $t_\theta$ -value of the set might be taken as the  $t_\theta$ -value for the analysis (see tables 6–9). In tables 6–8 we summarise the values of  $K_m$ ,  $V_{\max}$ ,  $[E]_0$  and  $k_2$  obtained by using method 3 for cases 3, 1 and 4, respectively, starting at different  $t_\theta$ -values and in table 9 we indicate the values of  $K_m$ ,  $V_{\max}$ ,  $[E]_0$  and  $k_2$  obtained by using method 2 for case 1 for different  $t_\theta$ -values. Note the good correspondence between these values and the true ones

Because working with experimental progress curves we do not know the values of the kinetic parameters, we will not be never sure which among the different suitable  $t_\theta$ -values furnishes the more accurate results. Thus, to take the average value of the kinetic parameters obtained for some different  $t_\theta$ -values will furnish, generally, the most reliable results. Thus, in examples in tables 8 and 9, the average values of  $K_m$ ,  $V_{\max}$  and  $[E]_0$  taking as  $t_\theta$ -values all of them giving similar results (those ones after dotted line in table 8 and up to dotted line in table 9) and the corresponding  $k_2$  value obtained from the average values of  $V_{\max}$  and  $[E]_0$  are indicated in the table caption. This same procedure could be used for any case and any method.

Table 8

Values of  $K_m$ ,  $V_{max}$  and  $[E]_0$  for case 7 obtained from the time progress curve of S, i.e. by using method 3, but starting at the different  $t_\theta$ -values on the first column. Note that as  $t_\theta$  can be taken any time after 6.2993 ms. The true values of  $K_m$ ,  $V_{max}$ ,  $[E]_0$  and  $k_2$  are 1.25, 100,  $10\mu\text{M}$  and  $10\text{s}^{-1}$ , respectively. Before the third  $t_\theta$ -value no reaction time should be taken as  $t_\theta$ -value due that the corresponding values obtained for the kinetic parameters greatly differ from those on the third row. That is marked by a dot line ( $\dots\dots$ ). The average values of each of  $K_m$ ,  $V_{max}$  and  $[E]_0$  from the third row are  $K_m = 1.1317 \pm 0.0001\mu\text{M}$ ,  $V_{max} = 97.968 \pm 0.010\mu\text{M}$  and  $[E]_0 = 9.8016 \pm 0.0009\mu\text{M}$  and, therefore, we obtain for  $k_2$  the value  $9.9951 \pm 0.0052\text{s}^{-1}$ .

$t_\theta(\text{s})$	$K_m (\mu\text{M})$	$V_{max}(\mu\text{M s}^{-1})$	$[E]_0(\mu\text{M})$	$k_2 (\text{s}^{-1})$
0.0104	$(2.7285 \pm 0.1061) \times 10^{-6}$	$9.4607 \pm 0.0372$	$1.4938 \pm 0.0641$	$6.3333 \pm 0.2967$
2.8816	$(4.0563 \pm 0.0111) \times 10^{-4}$	$58.140 \pm 0.002$	$6.6624 \pm 0.0184$	$8.7266 \pm 0.0244$
6.2993	$1.1116 \pm 0.0004$	$97.466 \pm 0.034$	$9.7656 \pm 0.0012$	$9.9805 \pm 0.0047$
10.000	$1.1503 \pm 0.0001$	$98.328 \pm 0.003$	$9.8232 \pm 0.0011$	$10.010 \pm 0.002$
40.000	$1.1287 \pm 0.0001$	$98.135 \pm 0.002$	$9.8232 \pm 0.0006$	$9.9901 \pm 0.0009$
60.000	$1.1361 \pm 0.0001$	$97.943 \pm 0.001$	$9.7943 \pm 0.0004$	$10.000 \pm 0.001$

Table 9

Values of  $K_m$ ,  $V_{max}$ ,  $[E]_0$  and  $k_2$  for case 1 obtained from the time progress curve of S, i.e. by using method 2, but starting at the different  $t_\theta$ -values on the first column. On the second column the corresponding  $[S]_\theta$ -values are shown. The true values of  $K_m$ ,  $V_{max}$ ,  $[E]_0$  and  $k_2$  are 1.25, 1,  $0.1\mu\text{M}$  and  $1\text{s}^{-1}$ , respectively. After the penultimate  $t_\theta$ -value no reaction time should be taken as  $t_\theta$ -value due that the corresponding values obtained for the kinetic parameters greatly differ from the those on penultimate row. That is marked by a dotted line ( $\dots\dots\dots$ ). The average values of  $K_m$ ,  $V_{max}$  and  $[E]_0$ , excluding those on the last row are:  $K_m = 1.2817 \pm 0.0001\mu\text{M}$ ,  $V_{max} = 1.0109 \pm 0.0001\mu\text{M}$  and  $[E]_0 = 0.0748 \pm 0.0001\mu\text{M}$  and, therefore, we obtain for  $k_2$  the value  $13.515 \pm 0.020\text{s}^{-1}$ .

$t_\theta(\text{s})$	$[S]_\theta(\mu\text{M})$	$K_m (\mu\text{M})$	$V_m(\mu\text{M s}^{-1})$	$[E]_0(\mu\text{M})$	$k_2 (\text{s}^{-1})$
0.0103	9.9028	$1.3314 \pm 0.0001$	$1.0317 \pm 0.0001$	$0.0537 \pm 0.0001$	$19.212 \pm 0.038$
2.0060	8.1573	$1.3283 \pm 0.0001$	$1.0303 \pm 0.0001$	$0.0548 \pm 0.0001$	$18.8011 \pm 0.037$
5.0029	5.6253	$1.3205 \pm 0.0001$	$1.0275 \pm 0.0001$	$0.0588 \pm 0.0001$	$13.923 \pm 0.021$
10.0031	1.9669	$1.2859 \pm 0.0001$	$1.0128 \pm 0.0001$	$0.0738 \pm 0.0001$	$10.506 \pm 0.012$
13.0015	0.5732	$1.2351 \pm 0.0001$	$0.9919 \pm 0.0001$	$0.0964 \pm 0.0001$	$8.6553 \pm 0.0085$
15.0116	0.17034	$1.1892 \pm 0.0001$	$0.9712 \pm 0.0001$	$0.1146 \pm 0.0001$	$8.4747 \pm 0.0083$
17.0085	0.0421	$0.6022 \pm 0.0003$	$0.6331 \pm 0.0002$	$0.2478 \pm 0.0002$	$2.5549 \pm 0.0029$

4.7. Final remarks

In this contribution, we have carried out an analysis of the enzyme systems evolving according to reaction mechanism in scheme 1 and we have obtained, among other results, integrated rate equations [equation (20) and that resulting inserting it in to equation (11)] that include, as particular cases (when

$[E]_0/K_m \ll 1$ ) the actual integrated rate equations [equations (23) and (25)] for the same type of enzyme systems. Therefore, Eqs. (23) and (25) will furnish suitable results when applied to an enzyme system when  $[E]_0/K_m \ll 1$  (see table 5). Moreover, in all studied cases, the actual method based in the time progress curve of  $P$  [we call as method IM( $P$ )] give better results than the actual one based on the time progress curve of  $S$  [we call as method IM( $S$ )]; both methods described in Segel [3] and summarised above.

Besides to establish the applicability limits of the actual integrated rate Michaelis–Menten equation, our analysis allows us to suggest three different methods (methods 1–3) which give suitable results irrespective of the relative values of the initial enzyme and substrate concentrations and the Michaelis–Menten constant, although the accuracy of the results increases when  $[E]_0/K_m$  decreases and, for a given value of  $[E]_0/K_m$ , it increases when the ratio  $[E]_0/[S]_0$  decreases (see table 5). Methods 1–3 allow to determine not only  $K_m$  and  $V_{\max}$ , but also  $[E]_0$  and, therefore, from  $V_{\max}$  and  $[E]_0$ , of  $k_2$ . Our analysis requires to fit the corresponding experimental (simulated in this paper) time progress curves from a certain reaction time,  $t_\theta$ , generally low, that the worker can chose arbitrarily and then confirm if it is or not adequate, as explained above. Generally, the decreasing order in the accuracy of the results by using methods 1–3 is: method 1  $\approx$  method 3 > method 2, and by using any of these methods, the decreasing order in the accuracy of the results is generally:  $V_{\max} > K_m > [E]_0 \approx k_2$ .

In those cases in which methods IM( $S$ ) and IM( $P$ ) furnishes acceptable results (i.e., when  $[E]_0/K_m \ll 1$ , e.g. cases 1 and 3) the goodness of the results obtained for  $K_m$  and  $V_{\max}$  by using methods IM( $P$ ) and IM( $S$ ) are similar or worse, respectively, than those ones obtained with any of methods 1–3. In those cases in which  $[E]_0/K_m$  is not much less than the unity (e.g. cases 4 and 7) the use (not recommendable) of methods IM( $S$ ) and IM( $P$ ) is only viable if it is possible to have a curve portion in the corresponding plot approximately linear with negative slope and they yield enormously erroneous values for  $K_m$  and  $V_{\max}$ . Nevertheless, even in these cases, the use of any of our methods 1–3 renders suitable estimates of the real values of  $V_{\max}$  and  $K_m$  (e.g. cases 4 and 7 in table 5, for which  $[E]_0/K_m = 0.8$  and  $[E]_0/K_m = 8$ , respectively).

The analysis carried out here to obtain alternative integrated rate equations and methods to obtain the values of the kinetic parameters and initial enzyme concentration for enzyme systems evolving according to the reaction mechanism in scheme 1 could be extrapolated to those enzyme systems fitting to more complex mechanisms, e.g. those involving product inhibition [3,4,6,11,12], unstable enzymes [7], coupled reactions [18], etc. although the more complex is the enzyme system, the more laborious will result the analysis.



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