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Time behaviour of the modifier involved in the general mechanism of Botts and Morales assuming rapid equilibrium in the modifier bindings

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To date, the classification as activator or inhibitor of a modifier involved in an enzyme catalysed reaction is established according to its kinetic behaviour at the steady state. Inhibitors and activators are defined as modifiers which decrease or increase, respectively, the steady state rate of an enzyme-catalysed reaction. At this state, in some cases, a modifier always acts as an activator or as an inhibitor for all its possible concentration values. In other cases the action of a modifier as activator or inhibitor depends on its concentration. In this paper we extend the analysis of the kinetic behaviour of a modifier as inhibitor or nonessential activator to the transient phase of the reaction, i.e. to the whole course of the reaction, including both the transient phase and the steady state. Moreover, concerning to the behaviour of a modifier at the transient phase, we suggest its classification as activator or inhibitor based on the concentration and activator or inhibitor based on the rate. We have studied the behaviour of the modifier involved in the general modifier mechanisms of Botts and Morales in which the reversible bindings of the modifier to the enzyme forms are assumed in rapid equilibrium. The result is that depending on the values of the rate constants, equilibrium constants and the initial concentrations of both the involved substrate and modifier, the latter can act during the whole reaction course only as an activator, only as an inhibitor, first as an activator and then, from a determined reaction time, as inhibitor, or vice versa. Therefore, it is possible that a modifier showing an activating behaviour at the steady state behaves as an inhibitor in the transient phase, or vice versa. Novel indices pointing to the conditions under which the modifier can show any of the behaviours indicated above are suggested. The goodness of the analytical results is tested by comparison with the simulated curves obtained by numerical integration. From these results, those corresponding to several reaction mechanisms involving a modifier, and which can be regarded as particular cases of the general case analysed here, can be directly and easily obtained.

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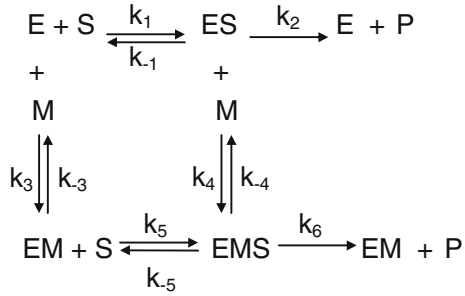
KEY WORDS: modifier, activator, inhibitor, enzyme kinetics, transient phase, steady state, rapid equilibrium

1. Introduction

Reversible modifiers (inhibitors or activators) represent a useful tool for studying enzyme mechanisms and metabolic routes [1–7]. Moreover, they have applications in pharmacology, toxicology, industry and agriculture [8–10]. To date, inhibitors and activators are defined as modifiers that decrease or increase, respectively, the steady state rate of an enzyme-catalysed reaction [11,12].

As it is known, modifiers are best used according to their kinetic characterisation through an evaluation of their corresponding kinetic parameters. Most enzyme reaction mechanisms involving a modifier reversibly acting on Michaelis type enzymes can be considered as particular cases of the general modifier mechanism of Botts and Morales depicted in scheme 1, whose steady state and transient phase kinetics have been widely discussed in the literature [12–24].



Scheme 1.

More recently, kinetic analyses of the transient phase have been made of simple enzyme reaction mechanisms in which a competitive [25] or uncompetitive [26] inhibitor acts reversibly on Michaelis type enzymes, whose substrate is suicide. Nevertheless, these simple mechanisms can be considered as particular cases of a more general reaction scheme consisting of the general modifier mechanism of Botts and Morales, with the substrate involved being a suicide substrate [23].

The action of the modifier involved in scheme 1 as activator or inhibitor has only been discussed, as far as we know, with relation to its effect on the product rate at the steadystate [11,12,15,27].

Segel [11] and Segel and Martin [15] studied fully the steady-state rate of the general unireactant modifier mechanism described by scheme 1. This author yielded a velocity equation of second degree in both [*S*] and [*M*] which was later

reduced to one of first degree in [*S*] and [*M*] after some assumptions. These equations are valid for M being an inhibitor as well as an activator.

Laidler [27] carried out an extensive contribution about the action of M as activator or inhibitor at the steady state in enzyme catalysed reactions evolving according to scheme 1. This author determined the conditions under which there is activation or inhibition at the steady state and that sometimes there is a transition from activation to inhibition as the substrate concentration is varied. He found a classification of the modification involved in scheme 1 as overall activation or inhibition, initial activation or inhibition or terminal activation or inhibition. Laidler also suggested definitions of competitive, uncompetitive and noncompetitive activation, by analogy with the generally accepted definitions for inhibition.

More recently, Fontes et al. [12] made a combined analysis of enzyme inhibition and activation of scheme 1 based on rapid equilibrium model assumptions, also at the steady state of the reaction. They determined that the modifier acts as activator, as inhibitor (total or partial) or has no effect on the reaction rate, depending on the values of the equilibrium constants, the rate constants of the limiting velocity steps and the concentration of the substrate.

Segel's, Laidler's and Fontes et al.'s contributions limit their respective analysis to the steady state. The main difference between the analyses of these authors is the set of simplifying assumptions made about the steady state reached by enzyme systems which fit to scheme 1 or any of its particular cases.

A transient phase analysis similar to those above commented for the steady state has not been carried out yet. It is in principle interesting to study if when a modifier, M, will act as activator or inhibitor during the whole course of the reaction or if, on the contrary, it may happen a transition of activator to inhibitor or vice versa at a time of the reaction course. The study of the action of a modifier during the transient phase of the reaction would give completeness to the general analysis of the modifier as activator or inhibitor and will extend the definition of activator or inhibitor.

The aims of the present contribution are (1) to analyse the kinetic behaviour as an activator or as an inhibitor of the modifier, M, involved in scheme 1, at each time of the reaction course, i.e. during both the transient phase and the steady state; (2) to test the goodness of the analytical solutions by comparison with the results from the simulated curves obtained by numerical integration and (3) to suggest the use of indices of the action of the modifier in order to know if it acts as an activator or as an inhibitor at any time of the reaction course.

2. Materials and methods

Kinetic equations for scheme 2 were obtained using the computer program TRAPHAER developed by Varón et al. [22] for obtaining the symbolic

expressions for both transient phase and steady state equations of enzyme reactions. The simulated progress curves were obtained by numerical solution of the non-linear set of differential equations corresponding to scheme 1, using arbitrary sets of rate constants and initial concentration values. This numerical solution was found by the Runge–Kutta–Fehlberg algorithm [28,29] using the computer program WES implemented in Visual C++ 6.0 [30]. The above program was run on a PC compatible computer based on a Pentium III/450 MHz processor with 128 Mbytes of RAM. Figures were carried out using the SigmaPlot Scientific Graphing System for Windows version 8.02.

3. Kinetic analysis

3.1. Notation

We indicate here part of the notation used in this paper. The remaining notation either has been already introduced or will be introduced later in the text across according it is needed.

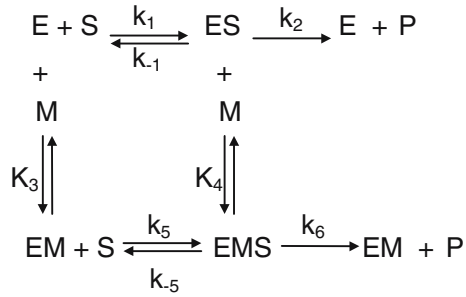
$[E]$, $[S]$, $[M]$ and $[P]$: Concentrations of E, S, M and P at any reaction time, t, of the reaction course.

$[E]_0$, $[S]_0$ and $[M]_0$: Concentrations, at $t = 0$, of E, S and M.

K_3 and K_4: Equilibrium constants of the reversible bindings of M to E and ES in scheme 1, i.e.:

$$K_j = \frac{k_{-j}}{k_j} \quad (j = 3, 4) \tag{1}$$

To indicate that the mentioned binding steps are at equilibrium we rewrite scheme 1 as the following scheme 2:



Scheme 2.

3.2. Initial and final conditions

We assume that at the onset of the reaction only are present the free enzyme, E, the substrate, S, and the modifier, M and that $[S]_0$, $[M]_0 \gg [E]_0$. We also assume that the assayed reaction time is thus that the product concentration reached at this time is much less than the initial concentration of the substrate, $[S]_0$. The above conditions are easy to be reached experimentally and these ensure that during the whole course of the assayed reaction time it is observed that $[S] \approx [S]_0$ and $[M] \approx [M]_0$.

3.3. Time course equations

The product accumulation equation corresponding to the transient phase of scheme 2 can be obtained either manually or easier by using the software TRAPHAER corresponding to the contribution of Varón et al. [22] concerning with the derivation of the transient phase and steady state equation of enzyme reactions. The result is:

$$[P] = \alpha t + \beta \left(e^{-\lambda t} - 1\right) \tag{2}$$

where t is the time and the parameters α, β and λ are given by:

$$\alpha = \left\{ \left\{ k_1 k_2 K_3 K_4 [S]_0 + (k_2 k_5 K_4 + k_1 k_6 K_3)[S]_0 [M]_0 + k_5 k_6 [S]_0 [M]_0^2 \right\} \Big/ \left\{ (k_{-1} + k_2) K_3 K_4 + k_1 K_3 K_4 [S]_0 + \left\{ K_3 (k_{-5} + k_6) + K_4 (k_{-1} + k_2) \right\} [M]_0 + (k_5 K_4 + k_1 K_3)[S]_0 [M]_0 + (k_{-5} + k_6)\ [M]_0^2 \right\} \right\} [E]_0 \tag{3}$$

$$\beta = \left\{ \left\{ K_3 K_4 + (K_3 + K_4)[M]_0 + [M]_0^2 \right\} \Big/ \left\{ (k_{-1} + k_2) K_3 K_4 + k_1 K_3 K_4 [S]_0 + \left\{ K_3 (k_{-5} + k_6) + K_4 (k_{-1} + k_2) \right\} [M]_0 + (k_5 K_4 + k_1 K_3)[S]_0 [M]_0 + (k_{-5} + k_6)[M]_0^2 \right\} \right\} \alpha \tag{4}$$

$$\lambda = \left\{ (k_{-1} + k_2) K_3 K_4 + k_1 K_3 K_4 [S]_0 + \left\{ K_3 (k_{-5} + k_6) + K_4 (k_{-1} + k_2) \right\} [M]_0 + (k_5 K_4 + k_1 K_3)[S]_0 [M]_0 + (k_{-5} + k_6)[M]_0^2 \right\} \Big/ \left\{ K_3 K_4 + (K_3 + K_4)[M]_0 + [M]_0^2 \right\} \tag{5}$$

Note that the following relationship between these parameters is observed:

$$\beta \cdot \lambda = \alpha \tag{6}$$

If $[P]$, given by equation (2), is derivated with respect to t, and relationship (6) is taken into account, then we have for the instantaneous rate, v, of product formation:

$$v = \alpha \left(1 - e^{-\lambda t}\right). \tag{7}$$

In figure 1 the variation of $[P]$ and v with t, according to equations (2) and (7) are schematically shown.

3.3.1. Time course equations at the onset of the reaction (i.e. when $t \to 0$)

If we use Maclaurin's series for $\exp(-\lambda t)$ in equations (2) and (7), and we take into account equation (6), it results that near $t = 0$ it is observed, if we neglect (because $t \to 0$) all of the terms of the series except the three first ones, that:

$$[P] \approx \frac{\alpha \lambda}{2} t^2 \quad \text{(in presence of the modifier and } t \to 0) \tag{8}$$

and

$$v \approx \alpha \lambda t \quad \text{(with modifier and } t \to 0) \tag{9}$$

Note that equation (9) could also have been derived merely by derivating equation (8).

3.3.2. Time course equations at the steady state of the reaction (i.e. when $t \to \infty$)

At high enough reaction time values, $t (t \to \infty)$, i.e. at the steady state, the exponential term in equation (2) can be neglected and the later ones becomes:

$$[P] \approx \alpha t - \beta \quad \text{(steady state)} \tag{10}$$

Equation (10) corresponds to a straight line with the slope α, origin ordinate $-\beta$ and an intercept, τ, with the time axis given by

$$\tau \approx \frac{\beta}{\alpha} \tag{11}$$

If in equation (10), equation (11) is taken into account, the first one can be rewritten as

$$[P] \approx \alpha (t - \tau) \quad \text{(steady state)} \tag{12}$$

Since at the steady state the time takes high values and therefore much greater than τ, equation (12) can be approached to:

$$[P] \approx \alpha t \quad \text{(steady state)} \tag{13}$$

Analogously, at high enough reaction times, $t(t \to \infty)$, i.e., at the steady state, the exponential term in equation (7) can be neglected so that:

$$v \approx \alpha \quad \text{(steady state)} \tag{14}$$

Note that equation (14) could also have been derived merely by derivating equation (13).

4. Action of the modifier as an instantaneous activator or an inhibitor

In order to know the behaviour of the modifier, M, as an activator or an inhibitor we must compare the kinetics of scheme 2 in the presence and absence of M. If in the reaction mechanism shown in scheme 2 we suppress the presence of the modifier, M, the mechanism becomes the well known Michaelis–Menten one whose kinetic equations corresponding to $[P]$ and v are the same equations (2) and (7) but replacing in them α, β, and λ by α_0, β_0, and λ_0, respectively, obtained from equations (3)–(5) setting in them $[M]_0 = 0$. Next, we summarise the kinetic equations corresponding to $[M]_0 = 0$:

$$[P] = \alpha_0 t + \beta_0 \left(e^{-\lambda_0 t} - 1\right) \tag{15}$$

$$v = \alpha_0 \left(1 - e^{-\lambda_0 t}\right) \tag{16}$$

$$\alpha_0 = \frac{k_1 k_2 [S]_0}{k_{-1} + k_2 + k_1 [S]_0} [E]_0 \tag{17}$$

$$\beta_0 = \frac{1}{k_{-1} + k_2 + k_1 [S]_0} \alpha_0 \tag{18}$$

$$\lambda_0 = k_{-1} + k_2 + k_1 [S]_0 \tag{19}$$

$$\beta_0 \cdot \lambda_0 = \alpha_0 \tag{20}$$

$$[P] \approx \frac{\alpha_0 \lambda_0}{2} t^2 \quad (t \to 0) \tag{21}$$

$$v_0 \approx \alpha_0 \lambda_0 t \quad (t \to 0) \tag{22}$$

$$[P] \approx \alpha_0 t \quad \text{(steady state)} \tag{23}$$

$$v \approx \alpha_0 \quad \text{(steady state)} \tag{24}$$

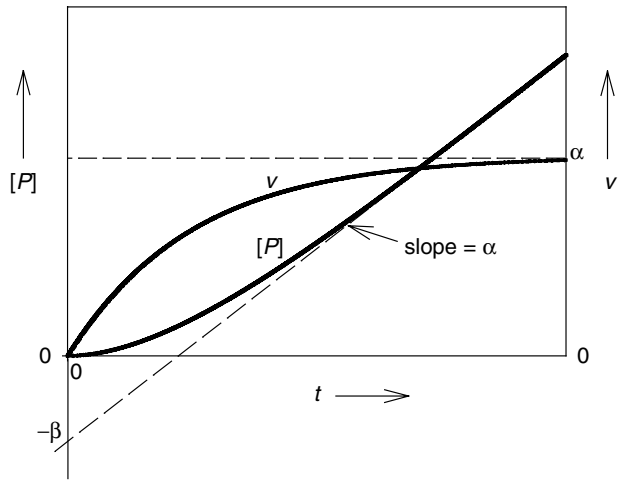


Figure 1. Schematic plot of $[P]$ and v versus t, according to equation (2) and (7).

The dependence of $[P]$ and v upon t retains the same schematic sharp as in figure 1.

The instantaneous actions of the modifier can be analysed using criteria of either product concentration or rate of product formation.

4.1. Instantaneous action of the modifier based on the product concentration

If at a determined reaction time the product concentration is higher or less than the corresponding one (at the same reaction time) in absence of the modifier, this one will behave, at that reaction time, as activator or inhibitor, respectively, with regard to its concentration. From the uni-exponential character of equations (2) and (15) the progress curves of P either will intersect in an unique point corresponding to a reaction time, t_c and to a concentration of P, $[P]_i$, or they do not intersect and, therefore, exist the four different possible cases denoted as cases (a), (b), (c) and (d) and which are summarised in figures 2(a) and (b).

The conditions in order that the two time progress curves corresponding to equations (7) and (16) behave according to cases (a)–(b) can be easily obtained. Let us consider the case in figure 2(a). Obviously, in order to act the modifier as activator at any reaction time anterior to t_c (from $t = 0$) and as inhibitor at any time reaction posterior to t_c (until $t \to \infty$) it must be fulfilled that at any reaction time anterior and posterior to t_c, the corresponding $[P]$-value in presence of the modifier must be higher and less, respectively, than in its absence. To establish the conditions for this change of the modifier from activator to inhibitor it is enough to know the behaviour of the progress curves of P in presence and

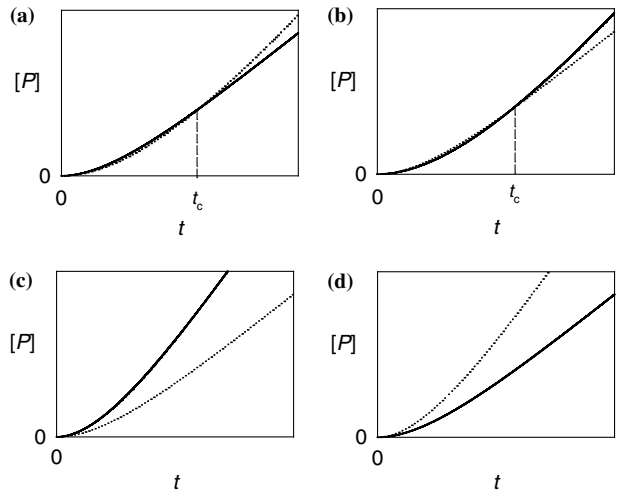


Figure 2. The four possible cases for the relative behaviour with regard to the instantaneous product concentration of a same enzyme system evolving according to the mechanism shown in Scheme 2 and that only differs in the presence (——) and the absence (.....) of the modifier M (i.e. the $[E]_0$- and $[S]_0$-values are the same in each case). (a) The time progress curves of the product intercept in a determined time, which we will denote as t_c, acting the modifier as activator before t_c and as inhibitor after t_c. (b) As in (a), but acting the modifier as inhibitor before t_c and as activator after t_c. (c) The progress curves do not intercept and the modifier acts as activator during the whole course of the reaction. (d) The two progress curves do not intercept and the modifier acts as inhibitor during the whole course of the reaction.

absence of the modifier near $t = 0$ ($t \to 0$) and at high reaction times ($t \to \infty$), i.e.: at the steady state. Effectively, the relative position of the progress curves at $t \to 0$ remains until the reaction time t_c. On the other hand, the relative position of the progress curves at $t \to \infty$ is the same than from t_c.

From equations (8), (13), (21) and (23) we have that the conditions for the modifier changes, in the reaction course, from concentration-based activator to concentration based inhibitor are

$$\alpha\lambda > \alpha_0\lambda_0 \tag{25}$$

and

$$\alpha < \alpha_0. \tag{26}$$

From equations (6) and (20), condition (25) can also be written as the equivalent one:

$$\frac{\alpha^2}{\beta} > \frac{\alpha_0^2}{\beta_0}. \tag{27}$$

Table 1
Conditions that must be observed to become the modifier involved in scheme 2 the kinetic behaviour shown in each of cases (a)–(b) in figures 2 and 3.

Case	Conditions
(a)	$\frac{\alpha^2}{\beta} > \frac{\alpha_0^2}{\beta_0}$ and $\alpha < \alpha_0$
(b)	$\frac{\alpha^2}{\beta} < \frac{\alpha_0^2}{\beta_0}$ and $\alpha > \alpha_0$
(c)	$\frac{\alpha^2}{\beta} > \frac{\alpha_0^2}{\beta_0}$ and $\alpha > \alpha_0$
(d)	$\frac{\alpha^2}{\beta} < \frac{\alpha_0^2}{\beta_0}$ and $\alpha < \alpha_0$

The conditions are the same for a criterium of modifier based on the product concentrations as well as on the rate of the product rate.

Relationships (25) and (26) are the conditions so that the modifier involved in scheme 2 changes (at $t = t_c$) from concentration-based activator (i.e. activator with regard to the product concentration) to concentration-based inhibitor (i.e. inhibitor with regard to the product concentration) in the time reaction course. Reasoning analogously for cases in figure 2(b), (c) and (d) the conditions summarised in table 1 are obtained.

4.2. Instantaneous action of the modifier based on the product formation rate

If at a determined reaction time the product formation rate, v, is higher or less than the corresponding one (at the same reaction time) in absence of the modifier, this one will behave, at that reaction time, as activator or inhibitor, respectively, with regard to the rate. Due to the uni-exponential character of equations (7) and (16), the progress curves of v either these curves will intersect in an unique point corresponding to a reaction time, t_v and to a rate of P, v_i, or they do not intersect and, therefore, there exist the four different possible cases we denote as cases (a), (b), (c) and (d) shown in figure 3(a), (b), (c) and (d).

The conditions for the two time progress curves corresponding to equations (2) and (16) behave according to cases (a)–(b) can be easily obtained. Let us focus on case in figure 3(a). If the modifier acts as activator at any reaction time anterior to t_v (from $t = 0$) and as inhibitor at any reaction time posterior to t_v (until $t \to \infty$) it must be fulfilled that at any reaction time anterior and posterior to t_v, the corresponding v-value in presence of the modifier must be higher and less, respectively, than in its absence. To establish the conditions for this change of the modifier from rate-based activator to rate-based inhibitor it is enough to know the behaviour of the time progress curves of v in presence and absence of the modifier near $t = 0$ ($t \to 0$) and at high reaction time ($t \to \infty$), i.e., at the steady state. Effectively, the relative position of the progress curves at $t \to 0$

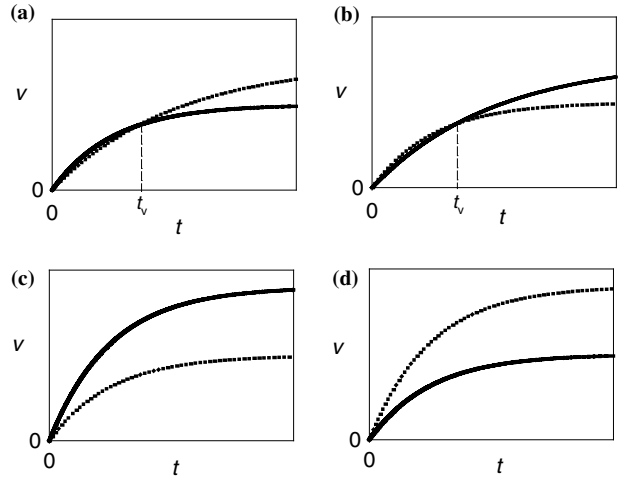


Figure 3. The four possible cases for the relative behaviour with regard to the instantaneous product rate of a same enzyme system evolving according to the mechanism shown in scheme 2 and that only differs in the presence (——) and the absence (.....) of the modifier, M (i.e. the $[E]_0$- and $[S]_0$-values are the same in each case). (a) The time progress curves of the product rate intercept in a determined time, which we will denote as t_v, acting the modifier as activator before t_v and as inhibitor after t_v. (b) As in (a), but acting the modifier as inhibitor before t_c and as activator after t_c. (c) The progress curves do not intercept and the modifier acts as activator during the whole course of the reaction. (d) The two progress curves do not intercept and the modifier acts as inhibitor during the whole course of the reaction. Figs. (a), (b), (c) and (d) correspond, respectively, to figures 2(a), (b), (c) and (d).

remains until the reaction time t_v. On the other hand, the relative position of the progress curves at $t \to \infty$ is the same than from t_c.

From equations (9), (14), (22) and (24), we see that relationships (26) and (27) are also the conditions in which the modifier involved in scheme 2 changes (at $t = t_v$) from rate-based activator (i.e. with regard to the product rate) to rate-based inhibitor (i.e. with regard to the product rate) in the time reaction course. Reasoning analogously for the cases in figure 3(b), (c) and (d) conditions summarised in table 1 are obtained.

5. Results and discussion

When the analysis of the action of a modifier is limited to the steady state of an enzyme-catalysed reaction there is no ambiguity in its characterisation as activator or inhibitor for a given reaction mechanism and concrete values of the rate constants, equilibrium constants (if any) and initial concentrations. Nevertheless, if we consider the action of the modifier during the whole reaction time it is necessary: (i) to define the *instantaneous* action of the modifier as activator or inhibitor and (ii) to characterise each of these two actions considering their

effect on the instantaneous values of the product concentration and the product rate both in presence and absence of the modifier. This task is that we carried out in this contribution.

In the same way allowing to obtain the time progress curve v–t from the time progress curve $[P]$–t (from the derivative value at each time) it is possible to obtain the time progress curve $[P]$–t from the time progress curve v–t because $[P]$ at any reaction time, t, coincides with the area between the progress curve v–t, the time axis and the straight line parallel to the v-axis containing the point $(t, 0)$.

In figure 4, we indicate schematically and simultaneously the behaviour of the modifier which acts according to figure 2(a) and 3(a). At reaction time t_c, corresponding to $[P]_i$, the two areas shown in figure 4(b) are equal because their values must coincide with the $[P]_i$-value. Note that the t_c-value is necessarily higher than the t_v-value because at the reaction time t_v the equality between the two areas has not yet been reached what only will happen at a reaction time posterior to t_v concretely at $t = t_c$. This simple consideration allows us to state that $t_c > t_v$.

Briefly, in the example shown in figure 4, in the rank between $t = 0$ and $t = t_v$ the modifier behaves as *a concentration-based activator* and *a rate-based activator*. Between $t = t_v$ and $t = t_c$ the modifier acts simultaneously as a *concentration-based activator* and as a *rate-based inhibitor*. From $t = t_c$ the modifier is always a *concentration-based inhibitor and a rate-based inhibitor*. The same happens for a modifier acting according to figures 2(b) and 3(c) but interchanging the words activator and inhibitor.

In turn, a modifier which is a *concentration-based activator* during the whole time course of the reaction [see figure 2(c)] is also a rate-based *activator* during the whole time course of the reaction [see figure 3(c)]. Finally, a modifier which is a *concentration-based inhibitor* during the whole time course of the reaction [see figure 2(c)] is also a *rate-based inhibitor during the whole course of the reaction* [see figure 3(c)].

5.1. *Validity of the results*

The analytical results obtained in this contribution are accomplished whenever the assumptions used in their derivation are fulfilled. Thus, the initial concentration of both the substrate and the inhibitor must be in excess with respect to that of free enzyme, i.e.:

$$[S]_0 , [M]_0 \gg [E]_0 \tag{28}$$

so that the substrate and inhibitor concentrations can be assumed constant during the reaction time assayed. Therefore, since the substrate is tranformed into P, the reaction time assayed (which we will denoted below by "rt") should suppose

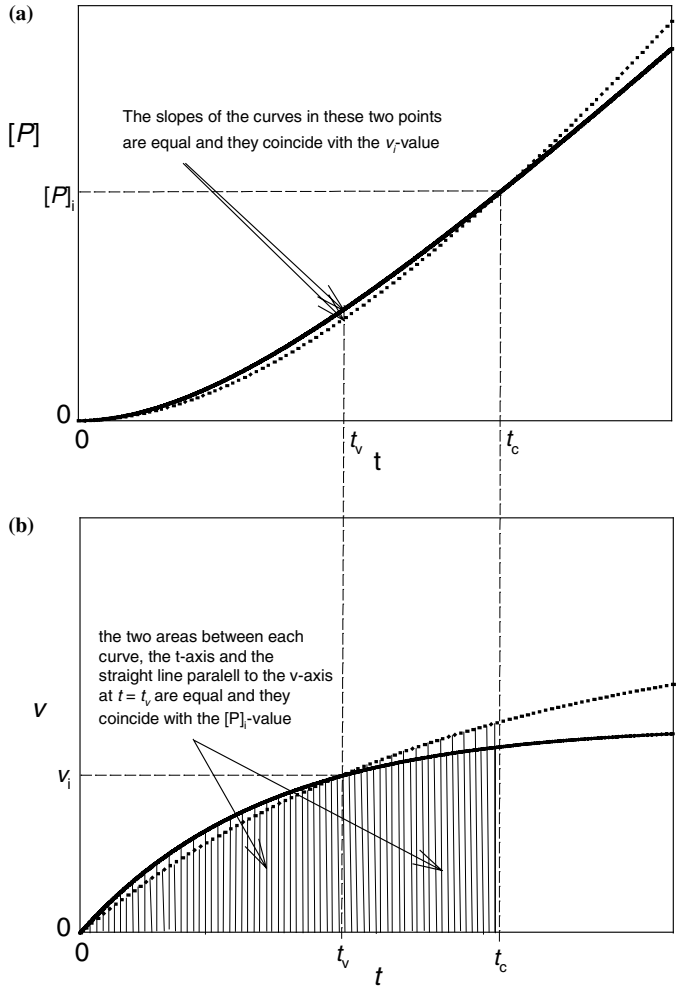


Figure 4. (a) (——) Schematic representation of the kinetic behaviour of a modifier which acts firstly as a concentration-based activator and then as a concentration-based inhibitor. (.....) Schematic representation of $[P]$ versus t in absence of the modifier. This (a) corresponds with figure 2(a). (b) (——) Schematic representation of the kinetic behaviour of a modifier which acts firstly as rate-based activator and then as rate-based inhibitor. (.....) Schematic representation of $[P]$ versus t in absence of the modifier. This (b) corresponds with figure 3(a).

a low product accumulation compared with the initial product concentration, i.e.

$$[P]\,(\text{at time} = \text{rt}) \ll [P]_0 \tag{29}$$

The experimental fulfilment of condition (29) is better to reach, the less the initial concentration of the free enzyme is.

Moreover, the analytical kinetic equations have been derived assuming that the bindings of the modifier to the corresponding enzyme forms are in rapid equilibrium from the onset of the reaction. The necessary and sufficient condition to these equilibrium approach is [22]

$$\left.\begin{array}{l} k_3[M]_0, k_{-3}, k_4[M]_0, k_{-4} \gg k_1[S]_0, k_{-1}, k_2, k_5[S]_0, k_{-5}, k_6 \\ k_3[M]_0, k_{-3}, k_4[M]_0, k_{-4} \text{ mutually not very different} \end{array}\right\} \quad (30)$$

Note that in the kinetic equations derived the rate constants k_3, k_{-3}, k_4 and k_{-4}, are not involved but the equilibrium constants K_3 and K_4, defined by equation (1). But by deriving these equations we have assumed rapid equilibrium in the bindings of the modifier, M, and, therefore, we have implicitely accepted condition (30).

Briefly, the more conditions given by equations (28)–(30) are fulfilled, the more approached will be the theoretical results to the experimental ones.

5.2. *Comparison of the kinetic behaviour of the modifier at the steady state and during the reaction course*

The condition to act the modifier M as activator or inhibitor at the steady state is that the corresponding rate of product formation at this state in presence of the modifier is higher or less, respectively, than the same rate constant in the absence of the modifier, i.e.:

$$\text{M is an activator at the steady state if } \alpha > \alpha_0 \quad (31)$$

$$\text{M is an inhibitor at the steady state if } \alpha < \alpha_0 \quad (32)$$

$$\text{M has no effect if } \alpha = \alpha_0 \text{ (an unlikely situation)} \quad (33)$$

Situation (33) above has been included for completeness but its occurrence can be neglected.

Since the steady-state rate α (with modifier) depends on $[S]_0$, $[M]_0$ and $[E]_0$ [see equation (3)] and the steady-state rate α_0 (with modifier) depends only of $[S]_0$, and $[E]_0$ [see equation (17)], the kinetic behaviour of M as activator or inhibitor for a concrete enzyme reaction fitting scheme 2 in concrete experimental conditions (fixed values of the rate and equilibrium constants) will depend on the relative values of $[S]_0$ and $[M]_0$ (note that $[E]_0$ is cancelled by using conditions in table 1). Thus, it may happen that depending on these values, a same modifier can act as activator or inhibitor. Note that both types of modifier defined in this paper, i.e. the concentration-based modifier (activator or inhibitor) and the rate-based modifier (activator or inhibitor) coincide at the steady state.

These considerations made above about the action of the modifier at the steady state are only a particular case of our more wide analysis covering the

whole course of the reaction. Specific kinetic analysis of the kinetic behaviour of a modifier at the steady state have been already carried out extensively [11,12,15,27]. Both, Segel and Martin [15] and Laidler [27] analysed scheme 1 considering strict steady state, i.e. without assuming rapid equilibrium in the bindings of M and S to the corresponding enzyme forms whereas Fontes et al. [12] assumed total rapid equilibrium, i.e. that all of the reversible steps in scheme 1 are in rapid equilibrium. Our analysis, and therefore its particular application to the steady state, are referred to scheme 1 when the bindings of the modifier are in rapid equilibrium, i.e. to scheme 2 (partial equilibrium approach).

Before the attainment of the steady state, i.e. at the transient phase of the reaction, the kinetic behaviour of the modifier for a given mechanism (rate and equilibrium constants fixed) depends on the four relationships summarised in table 1. By finding the fulfilment or not of these conditions $[E]_0$ is cancelled and, therefore, the kinetic behaviour of the modifier as activator or inhibitor will depend, as at the steady state, on the relative values of $[S]_0$ and $[M]_0$, but not only. Effectively, for those values of $[S]_0$ and $[M]_0$ yielding cases (a) or (b), the time, t, is another variable on which depends the behaviour of the modifier, so that, depending on the concrete case, for $t < t_c$ the modifier acts as concentration-based activator [case (a)] or inhibitor [case (b)] and for $t > t_c$ the modifier acts as concentration-based inhibitor [case (a)] or activator [case (b)]. Analogously, for $t < t_v$ the modifier acts as rate-based activator [case (a)] or inhibitor [case (b)] and for $t > t_c$ the modifier acts as rate-based inhibitor [case (a)] or activator [case (b)]. The time between t_v and t_c the modifier acts as concentration-based activator and rate-based inhibitor [case(a)] or vice versa [case (b)]. In cases (c) and (d) the modifier acts during the whole course of the reaction as concentration- and rate-based activator [case (c)].

Examples: In table 2 we summarised four different examples (examples 1–4) characterised by their values of the rate constants, equilibrium constants and initial concentrations. From these values and equations (3), (4), (17) and (18) the values of α, β, α_0 and β_0, respectively, have been obtained. The above values as

Table 2
Examples of different sets of values of the rate constants, equilibrium constants and initial concentrations yielding each of the kinetic behaviours in cases (a)–(d) indicated in figure 2 and table 1.

Example	K_3 (M)	K_4 (M)	k_5 ($M\,s^{-1}$)	k_{-5} (s^{-1})	k_6 (s^{-1})	$[M]_0$
1	10^{-4}	10^{-1}	10^{5}	10^{2}	10^{-4}	10^{-3}
2	10^{-4}	10^{-2}	1	10^{-4}	10^{-2}	10^{-4}
3	10^{-2}	10^{-2}	10^{5}	10^{-1}	10^{-2}	10^{-3}
4	10^{-2}	10^{-2}	10^{3}	10^{-3}	10^{-2}	10^{-3}

In all of the cases $k_1 = 10^4\,M\,s^{-1}$, $k_{-1} = 0.01\,s^{-1}$, $k_2 = 0.001\,s^{-1}$ $[E]_0 = 10^{-8}$ M and $[S]_0 = 10^{-4}$ M.

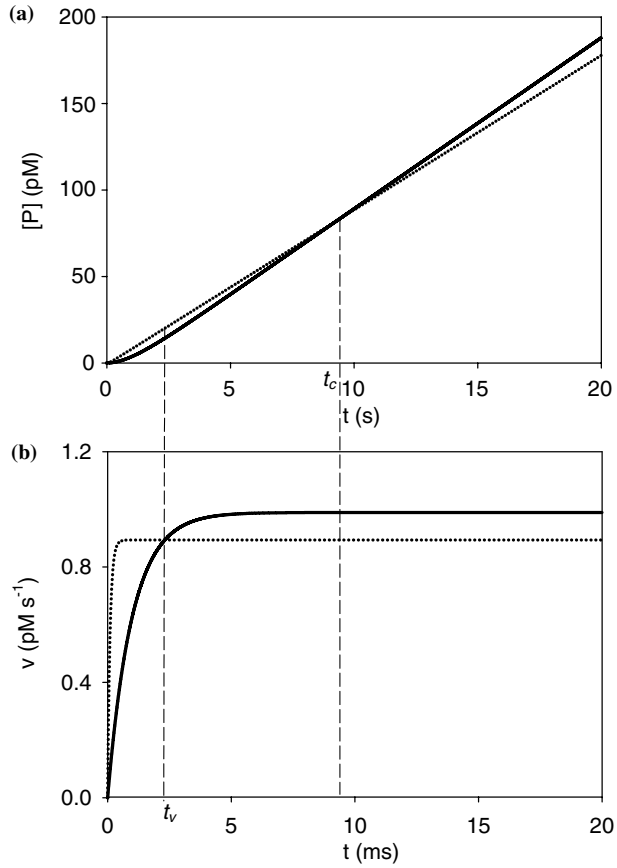


Figure 5. (a) Simulated progress curves corresponding to the product accumulation for the case (a) indicated in table 4 in the absence (.....) and in the presence of the modifier, M. Note the action of the modifier first as concentration-based activator and then, from $t = t_c$ as concentration-based inhibitor. (b) Simulated progress curves corresponding to the rate product accumulation for the case (a) indicated in table 4 in absence (.....) and in presence of the modifier M obtained under the same conditions as the curves in (a). Note the action of the modifier first as rate-based activator and then, from $t = t_v$ as rate-based inhibitor. From time t_v to t_c the modifier M acts simultaneously both as an rate-based inhibitor and as an concentration-based activator.

well as those of α^2/β and α_0^2/β_0 are summarised in table 3. The comparison of α with α_0 and of α^2/β with α_0^2/β_0 allows, according to table 1, to classify the examples as belonging to cases (a), (b), (c) or (d) in this table. Note that examples 1,2,3 and 4 in table 2 correspond to cases (a), (b), (c) and (d), respectively, in tables 1 and 3. The t_c and t_v-values corresponding to cases (a) and (b) are also given in table 3.

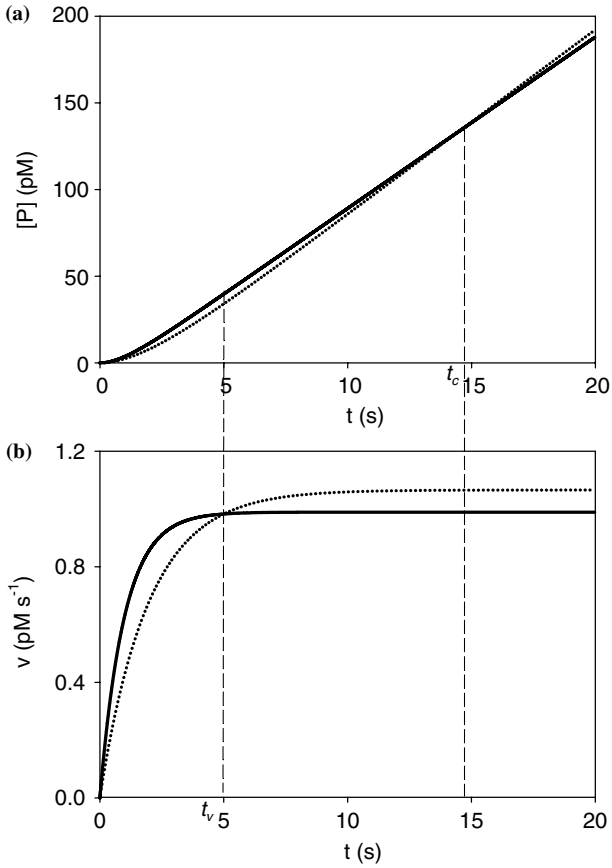


Figure 6. (a) Simulated progress curves corresponding to the time course of the product accumulation for the case (b) indicated in table 4 in absence (.....) and in presence of the modifier, M. Note the action of the modifier first as concentration-based inhibitor and then, from $t = t_c$ as concentration-based activator. (b) Simulated progress curves corresponding to the rate product accumulation for the case (a) indicated in table 4 in absence (.....) and in presence of the modifier M obtained under the same conditions as the curves in (a). Note the action of the modifier first as rate-based inhibitor and then, from $t = t_v$ as rate-based activator. From time t_v to t_c the modifier M acts simultaneously both as rate-based activator and as concentration-based inhibitor.

5.3. *Goodness of our analytical results*

In order to test the validity of our above analytical considerations we simulated both the concentration and the rate formation of the product for the cases (a), (b), (c) and (d) in table 3. The simulated curves have been obtained by numerical integration of the non-linear set of differential equations in Appendix A. The rate constants k_3, k_{-3}, k_4 and k_{-4} instead of the equilibrium constants K_3 and K_4 used for analytical considerations are necessary for the numerical

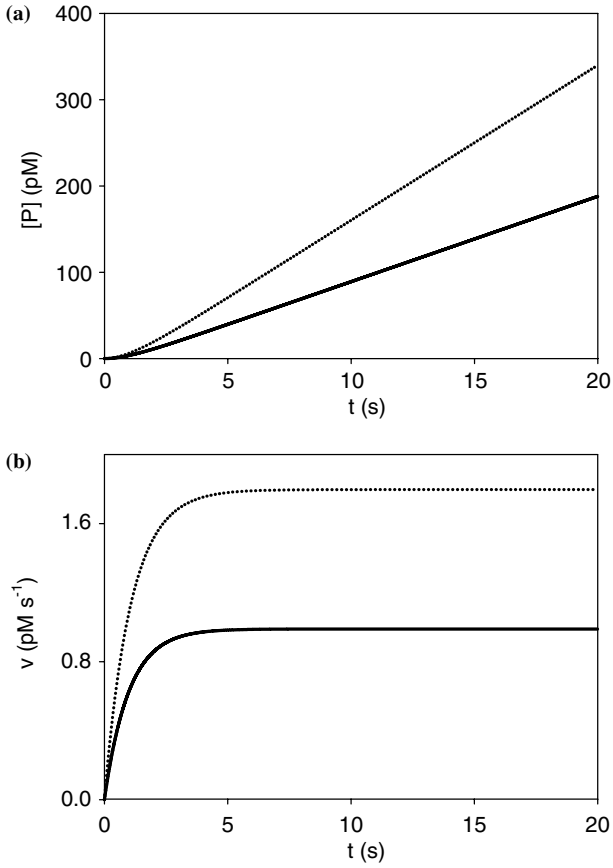


Figure 7. (a) Simulated progress curves corresponding to the time course of the product accumulation for the case (c) indicated in table 4 in absence (.....) and in presence of the modifier M. (b) Simulated progress curves corresponding to the rate product accumulation for the case (a) indicated in table 4 in absence (.....) and in presence of the modifier M obtained under the same conditions as the curves in (a). Note the action of the modifier as both concentration- and rate-based activator during the whole course of the reaction.

integration. These rate constants have been arbitrarily chosen, so that k_{-3}/k_3 and k_{-4}/k_4 coincide with the equilibrium constants K_3 and K_4 assigned to each example and that condition (30) fulfils. The values used for k_3, k_{-3}, k_4 and k_{-4} in each case are indicated in the caption of table 4 in which we also indicate the values of α, β, α_0, β_0, α^2/β and α_0^2/β_0 as well as the t_c- and t_v- values [for cases (a) and (b)] obtained from numerical integration. The reaction time, rt, used was in each of the simulations 20 s. Note the agreement with the analytical results in table 3.

For better graphic understanding of the kinetic behaviour of the modifier predicted from results in table 4, in figures 5–8 we have plotted the simulated

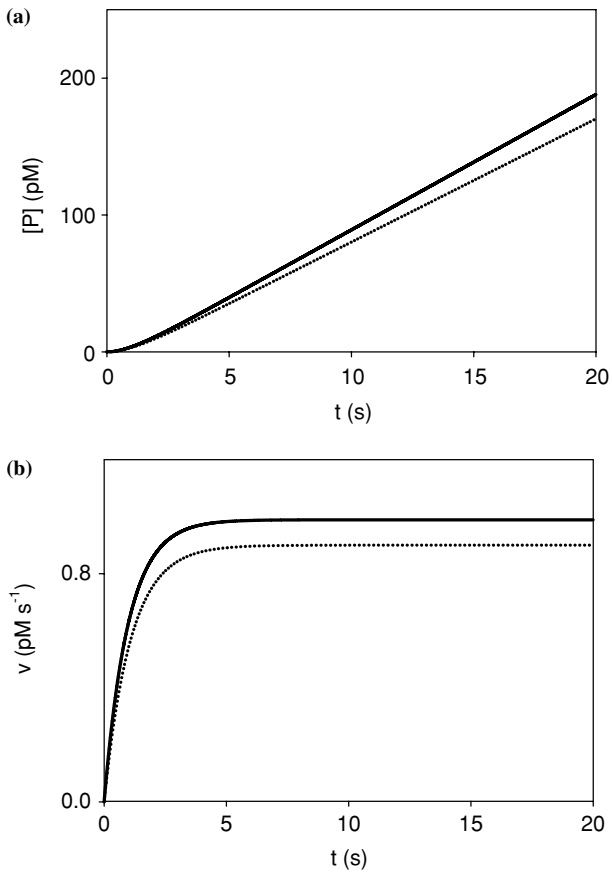


Figure 8. (a) Simulated progress curves corresponding to the time course of the product accumulation for the case (d) indicated in table 4 in absence (.....) and in presence of the modifier M. (b) Simulated progress curves corresponding to the rate product accumulation for the case (a) indicated in table 4 in absence (.....) and in presence of the modifier M obtained under the same conditions as the curves in (a). Note the action of the modifier as both concentration- and rate-based inhibitor during the whole course of the reaction.

time progress curves corresponding to the product accumulation and product rate for each of cases (a)–(d) in table 4. Each of the figures 5–7 consists in two ones (a) and (b). In (a) we have plotted the simulated time progress curves for the product accumulation with and without presence of the modifier to know the time kinetic behaviour of the latter as an concentration-based activator or inhibitor. In (b) we have plotted the simulated time progress curves for the rate of product accumulation with and without presence of the modifier to know the time kinetic behaviour of the latter as an rate-based activator or inhibitor. In figures 5–6 are also observable the values and meaning of the kinetic parameters defined here t_c and t_v.

Table 3
Values of α, β, α^2/β, α_0, β_0, and α_0^2/β_0 corresponding to examples 1–4 in table 2 obtained from the kinetic equations.

Example	Case in table 1	α (pM s^{-1})	β (pM)	α^2/β (pM s^{-2})	t_c (s)	t_v (s)
1	(a)	9.016328	0.893343	91.00000	10.16139	2.398937
2	(b)	10.65686	20.85326	5.446087	14.44073	4.997338
3	(c)	17.98257	19.37035	16.69422	–	–
4	(d)	9.000988	9.793263	8.272808	–	–

The values of α, β, α_0, β_0 were directly obtained from equations (3), (4), (17) and (18). In all of the examples it is observed: $\alpha_0 = 9.891197$ pM s^{-1}, $\beta_0 = 9.783578$ pM, $\alpha_0^2/\beta_0 = 10$ pM s^{-2}. On the second column the case in table 1 to which belongs the corresponding example is given. On the two last columns the values of the corresponding t_c- and t_v- values for cases (a) and (b) are indicated. These values were obtained from the list of t-values and the corresponding ones for $[P]$ and v with and without modifier M arising from equations (2), (7), (8) and (9) inserting in them the values of the rate constants, equilibrium constants and initial concentrations given in table 2.

Table 4
Values of α, β, α^2/β, α_0, β_0, and α_0^2/β_0 corresponding to examples 1–4 in table 2 obtained from numerical integration.

Example	Case in table 1	α (pM s^{-1})	β (pM)	α^2/β (pM s^{-2})	t_c (s)	t_v (s)
1	(a)	8.936549	0.878632	90.89347	9.343217	2.308277
2	(b)	10.65641	20.84481	5.447834	14.56309	5.004173
3	(c)	17.96654	19.33744	16.69283	–	–
4	(d)	9.000971	9.793619	8.272476	–	–

The values of α and β were directly furnished by the program WES from the numerical integration of the system of differential equations in Appendix A. The values of α_0 and β_0 were directly furnished by the program WES from numerical integration of the system of differential equations in Appendix A setting in it $[M] = 0$, i.e. when the reaction mechanism is a Michaelis–Menten one. The values of k_1, k_{-1}, k_2, $[E]_0$, $[S]_0$ and $[M]_0$ were, in all the examples, the same as in table 3. The values of k_3, k_{-3}, k_4 and k_{-4} used in each example were: example 1, $k_3 = 10^8$ M s^{-1}, $k_{-3} = 10^4$ s^{-1}, $k_4 = 10^6$ M s^{-1}, $k_{-4} = 10^5$ s^{-1}; example 2, $k_3 = 10^6$ M s^{-1}, $k_{-3} = 10^2$ s^{-1}, $k_4 = 10^5$ M s^{-1}, $k_{-4} = 10^3$ s^{-1}; example 3, $k_3 = 10^7$ M s^{-1}, $k_{-3} = 10^5$ s^{-1}, $k_4 = 10^6$ M s^{-1}, $k_{-4} = 10^4$ s^{-1}; example 4, $k_3 = 10^7$ M s^{-1}, $k_{-3} = 10^5$ s^{-1}, $k_4 = 10^6$ M s^{-1}, $k_{-4} = 10^4$ s^{-1}. In all of the examples 1–4 it is observed: $\alpha_0 = 9.891186$ pM s^{-1}, $\beta_0 = 9.784034$ pM and from these values $\alpha_0^2/\beta_0 = 9.999511$ pM s^{-2}. On the second column the case in table 1 to which belongs the corresponding example is given. The values of t_c and t_v for cases (a) and (b) are also indicated. These values were obtained from the list of t-values and the corresponding ones for $[P]$ and v with and without modifier M arising from the numerical integrations. In all cases the reaction time chosen for the simulation was 100 s for which in the four cases the attainment of the corresponding steady state has been already reached.

In all the cases (a)–(d) in table 3, the curves arising from the plot of the kinetic equtions for $[P]$ and v with and without modifier practically overlap, in the reaction time assayed, with the corresponding simulated progress curves in figures 5 and 6.

Note that the predictions based on the analytical results are completely confirmed by the numerical integration under the same assumptions under which those ones are valid.

APPENDIX A: System of differential equations describing the evolution of the species involved in schemes 1 and 2

$$\frac{\mathrm{d}[E]}{\mathrm{d}t} = -k_1[E]\ [S] - k_3[E]\ [M] + k_{-3}[EM] + (k_{-1}+k_2)[ES] \quad \text{(A.1)}$$

$$\frac{\mathrm{d}[ES]}{\mathrm{d}t} = k_1[E]\ [S] - (k_{-1}+k_2)\ [ES] - k_4[ES]\ [M] + k_{-4}[EMS] \quad \text{(A.2)}$$

$$\frac{\mathrm{d}[EM]}{\mathrm{d}t} = -k_5[EM][S] - k_{-3}[EM] + k_3[E][M] + (k_{-5}+k_6)[EMS] \quad \text{(A.3)}$$

$$\frac{\mathrm{d}[EMS]}{\mathrm{d}t} = k_5[EM]\ [S] + k_4[ES]\ [M] - (k_{-4}+k_{-5}+k_6)\ [EMS] \quad \text{(A.4)}$$

$$\frac{\mathrm{d}[S]}{\mathrm{d}t} = -k_1[E]\ [S] - k_5[EM]\ [S] + k_{-1}[ES] + k_{-5}\ [EMS] \quad \text{(A.5)}$$

$$\frac{\mathrm{d}[M]}{\mathrm{d}t} = -k_3[E]\ [M] - k_4[ES]\ [M] + k_{-3}[EM] + k_{-4}\ [EMS] \quad \text{(A.6)}$$

$$\frac{\mathrm{d}[P]}{\mathrm{d}t} = k_2[ES]\ + k_6\ [EMS] \quad \text{(A.7)}$$

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