Kinetic behaviour of zymogen activation processes in the presence of an inhibitor

Ramón VARÓN,*† Maria Carmen MANJABACAS,* Manuela GARCÍA-MORENO,* Edelmira VALERO* and Francisco GARCIA-CANOVAS†

* Departamento de Química-Física, Escuela Universitaria Politécnica, Universidad de Castilla-La Mancha, Avda. de España, s/n. Campus Universitario, 02071 Albacete, Spain, and † Departamento de Bioquímica y Biología Molecular, Facultad de Veterinaria, Universidad de Murcia, 30100 Espinardo, Murcia, Spain

A global kinetic analysis of a general zymogen activation model, where not only the activating but also the activated enzyme suffer an irreversible inhibition is presented. A reaction in which the enzyme acts upon a substrate is coupled to monitor the process. In addition, we determined the corresponding kinetic equations

for a number of particular cases of the general model studied. Finally, a kinetic data analysis and a procedure to discriminate among the different mechanisms considered, which are based on the kinetic equations obtained, are suggested.

INTRODUCTION

A number of proteolytic enzymes are synthesized as inactive precursors, named proenzymes or zymogens, to protect the cells which produce them. These zymogens must undergo an activation process, usually a limited proteolysis, to change into the active form.

Zymogen activation is a phenomenon which is involved in processes such as complement activation [1], the transformation of angiotensinogen into angiotensin [2], proinsulin–insulin conversion [3], the digestion of proteins in intestine [4], blood clotting [2,5–8] and protyrosinase activation [9], among others.

These important physiological processes are controlled by the different proteinase inhibitors found in cells and body fluids [10]. Such inhibitors include the α_1 and α_2 -macroglobulins involved in the regulation of fibrinolysis [11]; antithrombin III, involved in blood clotting, which inhibits the activated Factors IX–XII and the action of the thrombin [12], trypsin inhibitor, which

comprises 2% of the protein content of pancreatic juice and whose function is to regulate the cascades of activations of the pancreatic zymogens [4], etc. Different real examples of systems to which the kinetic study here presented may be applied are shown in detail in Table 1.

One incentive in inhibitor research is the control of limited proteolysis, as this constitutes a valuable pharmacological tool. Proteinase inhibitors have been proved effective in human therapy [10–12,27–29]. A pathological increase in fibrinolysis, e.g. in leukaemia or in operations involving organs with a high fibrinolysis activator content such as the uterus, prostate or lungs, can be controlled by the use of inhibitors such as 6-aminohexanoic acid, *p*-aminomethylbenzoic acid or aprotinin. In addition to inhibiting plasmin, they also inhibit trypsin, chymotrypsin and kallikrein, the last being the most important protein responsible for the release of bradykinin from kininogen [12].

The effect of an inhibitor on the kinetic behaviour of a zymogen activation process has been analysed in a simple, but

Table 1 Real examples of systems to which our kinetic study may be applied

Zymogen	Activating enzyme	Activated enzyme	Inhibitors	Substrate	References
Plasminogen		Plasmin	Plasminogen-activator inhibitor*, 6-aminohexanoic acid†	D-Val-Leu-Lys-4-Nan	[13–16]
Trypsinogen	Enteropeptidase	Trypsin	Gly-Gly-L-Phe-L-Meth*, soybean trypsin inhibitor†	Tos-Arg methyl ester	[17,18]
Pro-carboxy peptidase A	Trypsin	Carboxypeptidase A	Hydrocinnamic acid†, <i>p</i> -aminobenzamidine*	Hippuryl-L-phenylalanine	[16,18–20]
Prekallikrein	Coagulation Factor XIIa	Plasma kallikrein	C ₁ -inhibitor‡, α_2 -antiplasmin†	<i>N</i> -Benzoyl-L-arginine ethyl ester	[21–24]
Prothrombin (II)	Coagulation Factor Xa	Thrombin (IIa)	Antithrombin III‡, α_2 -antiplasmin†	<i>N</i> -Benzoyl-L-arginine ethyl ester	[12,24,25]
Protyrosinase	Trypsin	Tyrosinase	Trypsin inhibitor*, glutathione†	3,4-Dihydroxyphenylalanine	[4,9,26]

* The inhibitor acts only upon the activating enzyme.

† The inhibitor acts only upon the activated enzyme.

‡ The inhibitor acts upon both of the enzymes.

Abbreviations used: D-Val-Leu-Lys-Nan, D-valyl-L-leucyl-L-lysine 4-nitroanilide; t-PA, tissue plasminogen activator; i.u., international units established by World Health Organization for plasminogen and t-PA.

‡ To whom correspondence should be sent.

important, autocatalytic zymogen activation scheme [30]. Nevertheless, the quantitative influence of the inhibitors on the kinetics of the equally important non-autocatalytic zymogen activation processes has not, to our knowledge, been studied.

A zymogen activation model, overlapping the irreversible inhibition of the enzymes involved, can be obtained by adding the steps corresponding to the inhibition to a general model previously studied in the literature [31]. The resulting scheme is:

Scheme 1

where E is the activating proteinase, Z the inactive precursor of E, E_a is the activated enzyme, W is one or more peptides released from Z during the formation of E_a, S is a chromogenic substrate and P is the chromophoric product of the E_a reaction on S. In Scheme 1a, zymogen activation and inhibition of the enzymes take place simultaneously, whereas the coupled reaction shown in Scheme 1b acts as monitor.

The aim of the present paper is to carry out a complete kinetic study of the enzyme activation process, including the transient-phase overlapping irreversible inhibition described by Scheme 1. This is done under conditions of excess inactive precursor, zymogen-inhibitor, activated-enzyme-inhibitor and chromogenic substrate with respect to the activating enzyme. This mechanism is general enough to include, as particular cases, many important simpler processes.

MATERIALS AND METHODS

D-Valyl-L-leucyl-L-lysine 4-nitroanilide di-hydrochloride (D-Val-Leu-Lys-Nan·2HCl), 6-aminohexanoic acid, plasminogen and plasmin from bovine plasma and tissue plasminogen activator (single chain) from human melanoma-cell culture were from Sigma and used without further purification.

Plasminogen activation kinetics were measured at 37 °C by spectrophotometrically monitoring, at 405 nm, the appearance of the nitroaniline product of the reaction curves ($\epsilon \ 1 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$) [5]. The experimental progress curves thus obtained were fitted in eqn. (C20) of Appendix C by linear regression.

KINETIC EQUATIONS

The kinetic analysis carried out here assumes that only the species E, Z, I and S are present at the onset of the reaction and that their initial concentrations are as follows:

$$[\text{Z}]_0, [\text{I}]_0, [\text{S}]_0 \gg [\text{E}]_0 \quad (1)$$

Under condition (1), [Z], [I] and [S] can be considered constant during the whole course of the reaction and approximately equal to [Z]₀, [I]₀ and [S]₀.

Taking into account the above conditions, the kinetic behaviour of the species involved in Scheme 1 is described by the

following homogeneous, linear set of differential equations with constant coefficients:

$$\left. \begin{aligned}
 d[\text{E}]/dt &= -(k_{+1}[\text{Z}]_0 + k_{+3}[\text{I}]_0)[\text{E}] \\
 &\quad + (k_{-1} + k_{+2})[\text{EZ}] + k_{-3}[\text{EI}] \\
 d[\text{EZ}]/dt &= k_{+1}[\text{Z}]_0[\text{E}] - (k_{-1} + k_{+2})[\text{EZ}] \\
 d[\text{E}_a]/dt &= k_{+2}[\text{EZ}] - (k'_{+1}[\text{S}]_0 + k'_{+3}[\text{I}]_0)[\text{E}_a] \\
 &\quad + k'_{-3}[\text{E}_a\text{I}] + (k'_{-1} + k'_{+2})[\text{E}_a\text{S}] \\
 d[\text{EI}]/dt &= k_{+3}[\text{I}]_0[\text{E}] - (k_{-3} + k'_i)[\text{EI}] \\
 d[\text{EI}^*]/dt &= k'_i[\text{EI}] \\
 d[\text{E}_a\text{I}]/dt &= k'_{+3}[\text{I}]_0[\text{E}_a] - (k'_{-3} + k'_i)[\text{E}_a\text{I}] \\
 d[\text{E}_a\text{I}^*]/dt &= k'_i[\text{E}_a\text{I}] \\
 d[\text{E}_a\text{S}]/dt &= k'_{+1}[\text{S}]_0[\text{E}_a] - (k'_{-1} + k'_{+2})[\text{E}_a\text{S}] \\
 d[\text{P}]/dt &= k'_{+2}[\text{E}_a\text{S}]
 \end{aligned} \right\} \quad (2)$$

From the above system we have derived:

$$[\text{P}] = \beta + \sum_{h=1}^6 \gamma_h \exp(\lambda_h t) \quad (3)$$

where

$$\beta = \frac{k_{+1}k'_{+1}k_{+2}k'_{+2}(k_{-3} + k'_i)(k'_{-3} + k'_i)[\text{E}]_0[\text{Z}]_0[\text{S}]_0}{(k_{-1} + k_{+2})(k'_{-1} + k'_{+2})k_{+3}k'_{+3}k'_i k'_i [\text{I}]_0^2} \quad (4)$$

and λ_h ($h = 1, 2, \dots, 6$) are the roots of the equation:

$$\sum_{i=0}^6 F_i \lambda^{6-i} = 0 \quad (5)$$

where $F_0 = 1$; the expressions of the other coefficients F_i ($i = 1, 2, \dots, 6$) are summarized in the Appendix A. Hence, γ_h in eqn. (3) is:

$$\gamma_h = - \frac{k_{+1}k'_{+1}k_{+2}k'_{+2}(k_{-3} + k'_i + \lambda_h)(k'_{-3} + k'_i + \lambda_h)[\text{E}]_0[\text{Z}]_0[\text{S}]_0}{\lambda_h \prod_{\substack{p=1 \\ p \neq h}}^6 (\lambda_p - \lambda_h)} \quad (6)$$

Steady-state equations

Eqn. (3) is valid during the whole course of the reaction, i.e., for both the transient phase and the steady state. For sufficiently high enough t -values, the exponential terms of the product equation can be ignored, because they have much smaller values than the remaining constant term on the right side of this equation (because the roots λ_h ($h = 1, 2, \dots, 6$) are negative or, if complex, have a negative real part) and the behaviour of the system in the steady state ($t \rightarrow \infty$) becomes:

$$[\text{P}] = \beta \quad (\text{at the steady state}) \quad (7)$$

i.e. the concentration of the product in the steady state remains constant according to eqn. (7). This concentration depends on [E]₀, [Z]₀, [I]₀ and [S]₀, according to eqn. (4).

RESULTS AND DISCUSSION

In the literature, several kinetic analyses of reactions have been carried out where an inhibitor competes irreversibly with a non-zymogen substrate of a proteinase [32,33]. Nevertheless, the kinetic behaviour of an enzyme system, in which the activation of a zymogen overlaps the inhibition of the involved enzymes, remains to be studied. In the present paper the time course of the product, P, involved in Scheme 1 [eqn. (3) *et seq.*] has been

derived using only condition (1), which gives the obtained results wider validity. Moreover, this condition can easily be performed experimentally.

Reaction (b) of Scheme 1 was coupled to process (a) of Scheme 1, firstly to approximate our model to the physiological conditions under which the enzyme E_a acts on one of its possible substrates simultaneously with its release and, secondly, to facilitate the monitoring of the reaction (a) of Scheme 1.

Eqn. (3) is greatly simplified if rapid-equilibrium conditions prevail, and this circumstance is considered in detail below.

Rapid equilibrium conditions

If, in the reversible steps of Scheme 1, the rapid equilibrium conditions prevail, then it becomes:

Scheme 2

where $K_1 = k_{-1}/k_{+1}$, $K_3 = k_{-3}/k_{+3}$, $K'_1 = k'_{-1}/k'_{+1}$ and $K'_3 = k'_{-3}/k'_{+3}$.

The requirements for the rapid equilibrium conditions in Scheme 1 to prevail and, therefore, for it to become Scheme (2) are as in other studies concerning rapid equilibrium (6,31,34–36):

$$\begin{array}{c}
 k_{+1}[Z]_0, k_{-1}, k_{+3}[I]_0, k_{-3}, k'_{+3}[I]_0, k'_{-3}, k'_{+1}[S]_0, k'_{-1} \rightarrow \infty \\
 k_{+1}[Z]_0, k_{-1}, k_{+3}[I]_0, k_{-3}, k'_{+3}[I]_0, k'_{-3}, k'_{+1}[S]_0, k'_{-1} \\
 \text{mutually} \\
 \text{not} \\
 \text{very} \\
 \text{different}
 \end{array} \quad (8)$$

The time course of P in Scheme 2 can be easily obtained by inserting the condition (8) into eqn. (3) and related equations. In Appendix B this procedure is shown in detail. The result is:

$$[P] = \sigma + \sum_{h=1}^2 \gamma_h \exp(\lambda_h t) \quad (9)$$

where the expressions for σ , γ_1 , γ_2 and those which relate λ_1 and λ_2 are given by the eqns. (B17), (B19), (B20), (B13) and (B14) of Appendix B. Since at $t = 0$, $[P] = 0$, it is verified that:

$$\gamma_1 + \gamma_2 = -\sigma \quad (10)$$

Both eqns. (3) and (9) for the accumulation of P in Schemes 1 and 2 respectively correspond to the curves whose form is schematically indicated in Figure 1.

Particular cases of Scheme 2

Different processes can be considered as particular cases of that shown in Scheme 2 (and, therefore, of that shown in Scheme 1) if one or more of the rate constants are much higher than the others, or null. In Table 2, eight particular cases of Scheme 2 are listed. So as not to be repetitive, we have chosen the following notation. In each one of the Schemes the only aspects that

Figure 1 Schematic progress curves of P

(a) corresponds to Schemes 1 and 2 (according to eqns. 3 and 9). The constant value of P at the steady state is β in Scheme 1 and σ in Scheme 2; (b) Schemes 3–6 (according to eqn. C1); (c) Schemes 7–10 (according to eqn. C20).

Table 2 Particular cases of Scheme 2

In the third column we detail the rate constants which in Scheme 2 must go to infinity or be null in order for it to become the corresponding particular case.

Scheme no.	Scheme	Particular values of the rate and/or equilibrium constants
3	$(E + I \xrightleftharpoons{K_3} EI; E_a + I \xrightleftharpoons{K'_3} E_a I \xrightarrow{k'_i} E_a I^*)$	$k_i = 0$
4	$(E + I \xrightleftharpoons{K_3} EI \xrightarrow{k_i} EI^*; E_a + I \xrightleftharpoons{K'_3} E_a I)$	$k'_i = 0$
5	$(-; E_a + I \xrightleftharpoons{K'_3} E_a I \xrightarrow{k'_i} E_a I^*)$	$K_3 \rightarrow \infty$
6	$(E + I \xrightleftharpoons{K_3} EI \xrightarrow{k_i} EI^*; -)$	$K'_3 \rightarrow \infty$
7	$(E + I \xrightleftharpoons{K_3} EI; E_a + II \xrightleftharpoons{K'_3} E_a I)$	$k_i, k'_i = 0$
8	$(E + I \xrightleftharpoons{K_3} EI; -)$	$k_i = 0, K'_3 \rightarrow \infty$
9	$(-; E_a + I \xrightleftharpoons{K'_3} E_a I)$	$k'_i = 0, K_3 \rightarrow \infty$
10	$(-; -)$	$K_3, K'_3 \rightarrow \infty$

change with respect to Scheme 2 are the steps corresponding to the inhibition reactions, and therefore, we denote in Table 2 each of these particular cases, pointing out the two inhibition reactions only and dividing these by a semicolon (;). We denote by a dash (-), those cases in which either the activating or the activated enzyme is not inhibited.

The time course of P for each of the Schemes in Table 2 can be obtained from the corresponding system of differential equations describing its kinetics. However, the same result can be obtained more easily by setting the changes indicated on the third column in Table 2 into eqn. (9). The time course equations for P in Schemes 3–10 are summarized in Appendix C. To illustrate this procedure, we solve the following two examples.

Example 1: time-course equation for P in Scheme 3

Since in this case $k_1 \rightarrow 0$, using eqns. (B14) and (B10) we obtain:

$$\lambda_1 \lambda_2 \rightarrow 0 \quad (11)$$

whereas, according to eqns. (B13) and (B9), we have:

$$\lambda_1 + \lambda_2 = -D/G_4 \quad (12)$$

where G is as defined in Appendix B and

$$D = K'_1 K_3 k'_1 [Z]_0 [I]_0 + k'_1 K_1 K'_1 [I]_0^2 + K_1 K'_1 k'_1 K_3 \quad (13)$$

From eqns. (11) and (12) one deduces that only one of the roots, namely λ_2 , goes to zero, i.e.:

$$\lambda_2 \rightarrow 0 \quad (14)$$

Hence, from eqns. (14), (12) and (B14) we obtain:

$$\lambda_1 \approx -D/G_4 \quad (15)$$

$$\lambda_2 \approx -G_6/D \quad (16)$$

(note that $\lambda_2 \rightarrow 0$ because $G_6 \rightarrow 0$). From eqns. (16) and (14) results:

$$\lambda_1 - \lambda_2 \approx \lambda_1 \quad (17)$$

Inserting this equation into eqns. (B19) and (B20) we have:

$$\gamma_1 = -\sigma \quad (18)$$

where σ is given by eqn. (C4) in Appendix C, and

$$\gamma_2 = -\frac{k_{+2} k'_{+2} K_3 K'_3 [E]_0 [Z]_0 [S]_0}{\lambda_1 \lambda_2 G_4} \quad (19)$$

We now use the Maclaurin series expansion:

$$\exp(\lambda_2 t) = 1 + \lambda_2 t + (\lambda_2 t)^2/2! + (\lambda_2 t)^3/3! + \dots \quad (20)$$

Inserting this equation into eqn. (9) and taking eqn. (10) into account, one obtains:

$$[P] = -\gamma_1 + \gamma_2 \lambda_2 t + \gamma_1 \exp(\lambda_1 t) + \gamma_2 (\lambda_2 t)^2/2! + \gamma_2 (\lambda_2 t)^3/3! + \dots \quad (21)$$

Neglecting the terms which follow the exponential term and taking eqns. (18), (19), (C2) and (C3) into account, one obtains eqn. (C1) in Appendix C. Proceeding in the same way as in this example, the time dependence of P for Schemes 4–6 in Table 1 are derived. The result is summarized in Appendix C.

Example 2: time course equation for P in Scheme 7

Since in this case both k_1 and $k'_1 \rightarrow 0$, from eqns. (B13) $\rightarrow$ (B14), (B9) and (B10) we obtain:

$$\lambda_1, \lambda_2 \rightarrow 0 \quad (22)$$

since none of the λ_1 or λ_2 can be neglected in comparison with the

other terms, the expressions for γ_1 and γ_2 are still given by eqns. (B19) and (B20) with no possible simplification.

We now use the Maclaurin series expansion (eqn. 21) and another expansion in which λ_1 is used instead of λ_2 . Inserting these two expansions into eqn. (9) and taking eqn. (10) into account, one has:

$$[P] = (\gamma_1 \lambda_1 + \gamma_2 \lambda_2) t + (1/2!) (\gamma_1 \lambda_1^2 + \gamma_2 \lambda_2^2) t^2 + (1/3!) (\gamma_1 \lambda_1^3 + \gamma_2 \lambda_2^3) t^3 + \dots \quad (23)$$

From eqns. (B19) and (B20), we have:

$$\gamma_1 \lambda_1 + \gamma_2 \lambda_2 = 0 \quad (24)$$

and

$$(1/2!) (\gamma_1 \lambda_1^2 + \gamma_2 \lambda_2^2) = A \quad (25)$$

where A is given by eqn. (C21) in Appendix C. From eqns. (B19), (B20) and (22), we obtain:

$$(1/j!) (\gamma_1 \lambda_1^j + \gamma_2 \lambda_2^j) t^j \rightarrow 0 \quad (j = 3, 4, \dots) \quad (26)$$

Therefore, eqn. (23) becomes eqn. (C20) in Appendix C. Proceeding in the same way as in this example, the time dependence of P for Schemes (8–10) in Table 2 are derived. The result is summarized in Appendix C.

Note that the time-course equations for P in Schemes 3–6 (in which only one of the two enzymes is irreversibly inhibited) and in Schemes 7–10 (in which none of the enzyme species is irreversibly inhibited) are formally identical. In Figure 1 the progress curves of P corresponding to these schemes are schematically plotted. The progress curve of P at the steady state in Schemes 3–6 becomes a straight line with slope α (i.e., the steady-state initial rate) and an intercept with the [P]-axis σ . The intercept of these straight lines with the t -axis, i.e. the induction period, is given by:

$$\tau = -\sigma/\alpha \quad (27)$$

The progress curves of P for Schemes 7–10 are parabolic from $t = 0$, as indicated by eqn. (C20), and the rate of product formation never reaches a constant value but, on the contrary, increases linearly with t ; i.e., in these Schemes the steady state is not strictly reached.

In Figure 2 we show the experimental product accumulation curves as well as the corresponding ones fitted by linear regression to eqn. (C20), for the plasminogen activation reactions with t-PA in the absence (Scheme 10, curve i) and presence (Scheme 7, curve ii) of a reversible inhibitor of both the activating and the activated enzyme, 6-aminohexanoic acid. From linear regression of the above experimental curves, the values of the parameter A were 0.9317×10^{-2} and $3.4214 \times 10^{-2} \mu\text{M} \cdot \text{min}^{-2}$ respectively. Note the agreement between the experimental data and the expected parabolic behaviour for the Schemes 10 and 7.

The activation of plasminogen catalysed either by urokinase or t-PA (according to Scheme 10), as well as the same activations in which the plasmin is irreversibly inhibited by bovine pancreatic trypsin inhibitor (according to Scheme 5) have also been experimentally carried out [14], the results agreeing with those foreseen in our theoretical analysis.

Schemes 2–10 are particular cases of Scheme 1. Many other schemes of zymogen activation reactions are also particular cases of Scheme 1, if one, two or three of its reversible steps are in a rapid-equilibrium condition and/or if one or both inhibition reactions are not irreversible or do not exist. Nevertheless, from a kinetic point of view, Schemes 2–10, in which all the reversible steps are in rapid equilibrium, are those of interest. The kinetic equations for those particular cases of Scheme 1 other than Schemes 2 and 3 can be obtained as for the previous ones.

Figure 2 Product accumulation curves of plasminogen activation kinetics monitored by means of the plasmin activity with D-Val-Leu-Lys-Nan in the absence (i) and in the presence (ii) of 400 mM 6-aminohexanoic acid.

The continuous lines represent the experimental data and the broken lines the corresponding fittings to eqn. (C20). The reaction medium contained D-Val-Leu-Lys-Nan (0.3 mM), 0.375 i.u. of plasminogen and 100 i.u. of tissue plasminogen activator (t-PA) in a 0.05 M Tris/0.1 M NaCl buffer, pH 7.4, in a final volume of 1 ml. The reaction was started by the addition of t-PA.

Discrimination among Schemes 2–10

Before discriminating among Schemes 2–10 we must be sure that the enzyme reaction evolves according to one of these schemes. If the experimental recording of P fits a two-exponential equation, eqn. (9) (Figure 1a), a uniexponential equation, eqn. (C1) (Figure 1b) or a parabola, eqn. (C20) (Figure 1c), then the rapid-equilibrium conditions prevail in all the reversible steps, as occurs in Schemes 2–10. Any other result corresponds to a situation in which not all the reversible steps, or none, are in rapid-equilibrium conditions. Data-fitting must be made by non-linear regression [37,38].

We have limited the discrimination to Schemes 2–10. To discriminate among other possible schemes, which are particular cases of Scheme 1, including itself, we followed analogous procedures based on the form of the time-course equations of P and in the form of their corresponding progress curves.

Once we know that the scheme is one of the Schemes 2–10, the following procedure, based on the form of the time-dependence equation of P and on the corresponding progress curves, is suggested.

(1) A concentration of P which remains constant at the steady state is only compatible with Scheme 2.

(2) A behaviour at the steady state, which supposes that the progress curve becomes a straight line with positive slope not passing through the origin, is only compatible with Schemes 3–6. Hence, to discriminate among these four Schemes, we proceed as follows.

(2.1) We obtain different progress curves of P at fixed values of $[Z]_0$, $[E]_0$ and $[S]_0$, but varying the $[I]_0$ value used in each curve. From eqn. (C6) in Appendix C, we see that a plot of $\{(1/\alpha) - (1/\alpha_0)\}/[I]_0$ versus $[I]_0$ to give a straight line with positive slope and intercept, namely m and n respectively, is compatible with either Scheme 3 or 4 whereas, according to eqn. (C7) such a plot giving a straight line parallel with the $[I]_0$ -axis with positive intercept, namely n , is compatible with either Schemes 5 or 6.

(2.2) To discriminate between Schemes 3 and 4: a plot of n

versus $1/[S]_0$ (at fixed $[Z]_0$ and $[E]_0$ values) yielding a straight line through the origin with a positive slope (according to eqn. C10), is compatible with Scheme 3, and if the same plot gives a straight line parallel with the $1/[S]_0$ axis, according to eqn. (C13) the result is compatible with Scheme 4.

(2.3) To discriminate between Schemes 5 and 6: we plot the corresponding n versus $1/[S]_0$ (but maintaining the same fixed values of $[E]_0$ and $[Z]_0$). According to eqn. (C16), obtaining a straight line through the origin with a positive slope is compatible with Scheme 5, whereas obtaining a straight line parallel with the $1/[S]_0$ axis, according to eqn. (C19), is compatible with Scheme 6.

(3) Because of eqn. (C20) in Appendix C, a plot of $[P]/t$ versus t giving a straight line through the origin is only compatible with Schemes 7–10. Hence, to discriminate among these four Schemes we use the slope, A , of the above plot.

(3.1) To discriminate between Schemes 7–10 we use a set of different progress curves of P obtained at fixed $[E]_0$, $[S]_0$ and $[Z]_0$ values, but using different $[I]_0$ values for each curve. According to eqns. (C21), (B8), (C18), (C15) and (C22), a plot of $\{(1/A) - (1/A_0)\}/[I]_0$ versus $[I]_0$ (A_0 means the expression of A when $[I]_0 = 0$) resulting in a straight line with positive slope and intercept is compatible with Scheme 7; a plot resulting in a straight line parallel with the $[I]_0$ axis and positive intercept is compatible with both Schemes 8 and 9; a plot resulting in a straight line coincident with the $[I]_0$ axis is compatible with Scheme 10.

(3.2) To discriminate between Schemes 8 and 9, we determine the above-mentioned intercept for different sets of progress curves of P. In each set of progress curves, the $[E]_0$ and $[Z]_0$ values are the same, but in each set the value of $[S]_0$ is different from that of any other set, but fixed in each curve of a same set. From eqns. (C21), (C18) and (C15) we see that a replot of the intercept value (obtained for each set of progress curves) versus $1/[S]_0$ resulting in a straight line not passing through the origin is compatible with Scheme 8, whereas such a replot yielding a straight line passing through the origin is compatible with Scheme 9.

Kinetic data analysis of Schemes 2–10

To evaluate the equilibrium and rate constants involved in Schemes 2–10, we begin by classifying these schemes into the following four groups; Group (a) includes Scheme 10, i.e., the only one in Table 1 without inhibition; Group (b) includes Schemes 2, 5 and 6, i.e., those in which the inhibitions are irreversible; Group (c) includes Schemes 3 and 4, i.e., those where both irreversible and reversible inhibition exists simultaneously; and Group (d) includes Schemes 7 and 9, i.e. those Schemes in which the inhibitions are reversible.

The kinetic data analysis used for any of the above mechanisms depends on the group in which it is included. Below we indicate the procedure suggested for each case.

The Scheme belonging to Group (a)

In this case the Scheme is 10. We proceed as follows. In an assay where there are only the activated enzyme, E_a , and the substrate, S, the Scheme is simplified to Scheme 2b, which is a simple Michaelis–Menten mechanism, and therefore the constants K'_1 and k'_{+2} are easy to obtain from the steady-state rate, α . Since in this case $\alpha = k'_{+2}[S]_0/(K'_1 + [S]_0)$, a plot of $1/\alpha$ versus $1/[S]_0$ gives immediately K'_1 and k'_{+2} . Considering the complete mechanism and taking into account eqns. (C21) and (C22), from a plot of $[E]_0/A$ versus $1/[Z]_0$, maintaining $[S]_0$ constant, we obtain K_1 and k_{+2} .

The Scheme belonging to Group (b)

An assay without inhibitor must be carried out, and then we proceed in the same way as for Scheme 10 to determine the constants, K_1 , k_{+2} , K'_1 and k'_{+2} . The rate constants corresponding to the inhibitions are evaluated, taking into account the procedure for proteinase inhibitors [32].

The Scheme belonging to Group (c)

The constants K_1 , k_{+2} , K'_1 and k'_{+2} and those corresponding to the irreversible inhibition step (K'_3 and k'_i in Scheme 3 and K_3 and k_i in Scheme 4) are determined as described previously. To determine the equilibrium rate of the reversible inhibition (K_3 in Scheme 3 and K'_3 in Scheme 4), the progress curve of [P] of the global process and the corresponding experimental steady-state rate and induction period must be used together with the corresponding theoretical equation for these parameters (eqns. C2 and C5). Hence, taking into account eqns. (C2), (C5), (B8) and (C8) for Scheme 3 and eqns. (C2), (C5), (B8) and (C11) for Scheme 4, a plot of τ (see Appendix C)/ α versus $[I]_0$ allows us to determine K_3 in Scheme 3 and K'_3 in Scheme 4.

The Scheme belonging to Group (d)

An assay without inhibitor must be realized and subsequently, we proceed as for Scheme 10 to obtain the constants K_1 , k_{+2} , K'_1 and k'_{+2} . Hence, to determine the constants K_3 and/or K'_3 we use the progress curve of [P] for the global process, and the corresponding value of A obtained either from a plot of [P]/ t versus t or by fitting the experimental [P] and t^2 data to eqn. (C20) by linear regression. The latter fitting implies that the sum $\Sigma[(P) - At^2]^2$, extended to all the pairs of experimental data, must be minimum, i.e. $A = \Sigma[P \cdot t^2]/(\Sigma t^4)$.

Therefore, for Schemes 7, 8 and 9, and taking eqns. (C21), (B8), (C18) and (C15) into account, a plot of $1/A$ versus $[I]_0$ maintaining $[E]_0$, $[Z]_0$ and $[S]_0$ constant allows us to determine K_3 and/or K'_3 in each one of the mentioned schemes.

This work was partially supported by a grant number ALI89-0674 of the Comisión Interministerial de Ciencia y Tecnología (Spain).

REFERENCES

- Havsteen, B. and Varón, R. (1990) *J. Theor. Biol.* **145**, 47–64
- Holzer, H. and Heinrich, P. C. (1980) *Annu. Rev. Biochem.* **49**, 63–91
- Mihalyi, E. (1972) *Application of Proteolytic Enzymes to Protein Structure Studies*, CRC Press, Cleveland
- Cohen, P. (1976) *Control of Enzyme Activity*, pp. 20–31, John Wiley and Sons, New York
- Wohl, R. C., Summaria, L. and Robbins, K. C. (1980) *J. Biol. Chem.* **255**, 2005–2013
- Varón, R., Román, A., García-Cánovas, F. and García-Carmona, F. (1986) *Bull. Math. Biol.* **48**, 149–166
- Tankersley, D. L. and Finlayson, J. S. (1984) *Biochem. J.* **24**, 273–279
- Fisher, B. I. (1988) *Ann. Med. Lab. Diagn.* **29**, 327–332
- Galindo, J. D., Peñafiel, R., Varón, R., Pedreño, E., García-Carmona, F. and García-Cánovas, F. (1983) *Int. J. Biochem.* **15**, 633–637
- Rappay, G. (1989) *Proteinases and Their Inhibitors in Cells and Tissues* (Fischer, ed.), pp. 1–61, Gustav Fischer Verlag, Stuttgart and New York
- Karlson, P. (1988) *Kurzes Lehrbuch der Biochemie für Mediziner und Naturwissenschaftler*, 13th edn., pp. 435–437, Georg Thieme Verlag, Stuttgart
- Löffler, G. and Petrides, P. E. (1988) *Physiologische Chemie*, 4th edn., pp. 837–841, Springer-Verlag, Berlin
- Berrettini, M., Schleef, R. R., España, F., Loskutoff, D. J. and Griffin, J. H. (1989) *J. Biol. Chem.* **264**, 11738–11743
- Petersen, L. C., Brender, J. and Suenson, E. (1985) *Biochem. J.* **225**, 149–158
- Goldfarb, R. H., Quigley, J. P. (1980) *Biochemistry* **19**, 5463–5471
- Kosow, D. P. (1975) *Biochemistry* **14**(20), 4459–4464
- Luthy, J. A., Praissman, M., Finkenshtadt, W. R. and Laskowski, M., Jr. (1973) *J. Biol. Chem.* **248**, 1760–1771
- Walsh, K. A. (1970) *Methods Enzymol.* **19**, 41–63
- Elkins-Kaufman, E., Neurath, H. (1949) *J. Biol. Chem.* **178**, 645–654
- Uren, J. R., Neurath, H. (1972) *Biochemistry* **11**, 4483–4492
- Forbes, C., Pensky, J., Ratnoff, O. (1970) *J. Lab. Clin. Med.* **76**, 809–815
- Tans, T., Rosing, J., Berrettini, M., Lammle, B. and Griffin, J. H. (1987) *J. Biol. Chem.* **262**, 11308–11314
- Colman, R. W. (1984) *J. Clin. Invest.* **73**, 1249–1253
- Wiman, B. (1981) *Methods Enzymol.* **80**, 395–408
- Scott, C. F., Schapira, M., James, H. L., Cohen, A. B., Colman, R. W. (1982) *J. Clin. Invest.* **48**, 2014–2152
- Robb, D. A. (1984) *Copper Protein*, vol. 2 (R. Lontie, ed.), pp. 207–241, CRC Press, Boca Raton, FL
- Sweet, F. and Murdock, G. L. (1987) *Endocrine Rev.* **8**, 154–184
- Ferguson, R. K., Vlasses, P. H. and Rotmensch, H. H. (1984) *Am. J. Med.* **77**, 690–698
- Scharpe, S., De Meester, I., Hendriks, D., Vanhoof, G., van Sande, M. and Vriend, G. (1991) *Biochimie* **73**, 121–126
- Manjabacas, M. C., Valero, E., García-Moreno, M., García-Cánovas, F., Rodríguez, J. N. and Varón, R. (1992) *Biochem. J.* **282**, 583–587
- Varón, R., García-Cánovas, F., García-Carmona, F., Tudela, J., García-Moreno, M., Vázquez, A. and Valero, E. (1987) *Math. Biosci.* **87**, 31–45
- Leytus, S. P., Toledo, D. L. and Mangel, W. F. (1984) *Biochim. Biophys. Acta* **788**, 74–86
- Liu, W. and Tsou, C. I. (1986) *Biochim. Biophys. Acta* **870**, 185–190
- Varón, R., Havsteen, B. H., Vázquez, A., García-Moreno, M., Valero, E., García-Cánovas, F. (1990) *J. Theor. Biol.* **145**, 123–131
- Varón, R., García-Cánovas, F., García-Carmona, F., Tudela, J., Román, A. and Vázquez, A. (1988) *J. Theor. Biol.* **132**, 51–59
- García-Cánovas, F., Varón, R., Gálvez, J., García-Carmona, F., Tudela, J. and García, M. (1987) *An. Quim.* **83**(C), 219–223
- Wilkinson, G. N. (1961) *Biochem. J.* **80**, 324–332
- Marquardt, D. W. (1963) *J. Soc. Ind. Appl. Math.* **11**, 431–441

APPENDIX A

Expressions of the coefficients F_i ($i = 1, 2, \dots, 6$) in eqn. (5)

$$F_1 = k_{+1}[Z]_0 + k_{+3}[I]_0 + k_{-1} + k_{+2} + k'_{+3}[I]_0 + k'_{+1}[S]_0 + k_{-3} + k_i + k'_{-3} + k'_i + k'_{-1} + k'_{+2} \quad (A1)$$

$$F_2 = k_{+1}k'_{+1}[S]_0[Z]_0 + k_{+1}k'_{+3}[I]_0[Z]_0 + k_{+3}k'_{+3}[I]_0^2 + k_{+1}k_{+3}[S]_0[I]_0 + k_{+1}(k_{-3} + k_i + k'_{-3} + k'_i + k'_{-1} + k'_{+2})[Z]_0 + [(k_{+3} + k'_{+3})(k_{-1} + k_{+2} + k_i + k'_i + k'_{-1} + k'_{+2}) + k_{+3}k'_{-3} + k'_{+3}k_{-3}][I]_0 + k'_{+1}(k_{-1} + k_{+2} + k_{-3} + k_i + k'_{-3} + k'_i)[S]_0 + (k_{-1} + k_{+2})(k_{-3} + k_i + k'_{-3} + k'_i + k'_{-1} + k'_{+2}) + (k_{-3} + k_i)(k'_{-3} + k'_i + k'_{-1} + k'_{+2}) + (k'_{-3} + k'_i)(k'_{-1} + k'_{+2}) \quad (A2)$$

$$F_3 = k_{+1}k'_{+3}(k_{-3} + k_i + k'_i + k'_{-1} + k'_{+2})[Z]_0[I]_0 + k_{+1}k'_{+1}(k_{-3} + k_i + k'_{-3} + k'_i)[Z]_0[S]_0 + k_{+3}k'_{+3}(k_{-1} + k_{+2} + k_i + k'_i + k'_{-1} + k'_{+2})[I]_0^2 + k_{+3}k'_{+1}(k_{-1} + k_{+2} + k_i + k'_{-3} + k'_i)[I]_0[S]_0 + k_{+1}[(k_{-3} + k_i)(k'_{-3} + k'_i + k'_{-1} + k'_{+2}) + (k'_{-3} + k'_i)(k'_{-1} + k'_{+2})][Z]_0 + \{k_{+3}[(k_{-1} + k_{+2} + k_i)(k'_{-3} + k'_i + k'_{-1} + k'_{+2}) + (k_{-1} + k_{+2})k_i + (k'_{-3} + k'_i)(k'_{-1} + k'_{+2})] + k'_{+3}[(k_{-1} + k_{+2})(k_{-3} + k_i + k'_i + k'_{-1} + k'_{+2}) + (k_{-3} + k_i)(k'_i + k'_{-1} + k'_{+2}) + k'_i(k'_{-1} + k'_{+2})]\}[I]_0 + k'_{+1}[(k_{-1} + k_{+2})(k_{-3} + k_i + k'_{-3} + k'_i) + (k_{-3} + k_i)(k'_{-3} + k'_i)][S]_0 + (k_{-1} + k_{+2})[(k_{-3} + k_i)(k'_{-3} + k'_i + k'_{-1} + k'_{+2}) + (k'_{-3} + k'_i)(k'_{-1} + k'_{+2})] + (k_{-3} + k_i)(k'_{-3} + k'_i)(k'_{-1} + k'_{+2}) \quad (A3)$$

$$\begin{aligned}
F_4 = & k_{+1} k'_{+3} [(k'_{-1} + k'_{+2}) (k_{-3} + k_1 + k'_1) + k'_1 (k_{-3} + k_1)] [Z]_0 [I]_0 + k_{+1} k'_{+1} (k_{-3} + k_1) (k'_{-3} + k'_1) [Z]_0 [S]_0 \\
& + k_{+3} k'_{+3} [(k'_{-1} + k'_{+2}) (k_{-1} + k_{+2} + k_1 + k'_1) + k'_1 (k_{-1} + k_{+2} + k_1) + k_1 (k_{-1} + k_{+2})] [I]_0^2 \\
& + k_{+3} k'_{+1} [(k_{-1} + k_{+2}) (k_1 + k'_{-3} + k'_1) + k_1 (k_{-3} + k'_1)] [I]_0 [S]_0 \\
& + k_{+1} (k_{-3} + k_1) (k'_1 + k'_{-3}) (k'_{-1} + k'_{+2}) [Z]_0 + k_{-1} k'_{+1} (k_{-3} + k_1) (k'_{-3} + k'_1) [S]_0 \\
& + \{k_{+3} [k_1 (k_{-1} + k_{+2}) (k'_{-3} + k'_1 + k'_{+2}) + (k_{-1} + k_{+2} + k_1) (k'_{-3} + k'_1) (k'_{-1} + k'_{+2})] \\
& + (k_{+2} + k_{-1}) [k'_{+3} (k_{-3} + k_1) (k'_1 + k'_{-1} + k'_{+2}) + k'_{+3} k'_1 (k'_{-1} + k'_{+2})] + k'_{+3} k'_1 (k_{-3} + k_1) (k'_{-1} + k'_{+2})\} [I]_0 \\
& + (k_{-1} + k_{+2}) [k_{-3} (k'_{-3} + k'_1) (k'_{-1} + k'_{+2}) + k_1 (k'_{-3} + k'_1) (k'_{-1} + k'_{+2})]
\end{aligned} \quad (A4)$$

$$\begin{aligned}
F_5 = & k_{+1} k'_{+3} k'_1 (k_{-3} + k_1) (k'_{-1} + k'_{+2}) [Z]_0 [I]_0 + k_{+3} k'_{+3} [(k_1 + k'_1) (k_{-1} + k_{+2}) (k'_{-1} + k'_{+2}) + k_1 k'_1 (k_{-1} + k_{+2} + k'_{-1} + k'_{+2})] [I]_0^2 \\
& + k'_{+1} k_{+3} k_1 (k_{-1} + k_{+2}) (k'_1 + k'_{-3}) [I]_0 [S]_0 + (k_{-1} + k_{+2}) (k'_{-1} + k'_{+2}) [k_{+3} k'_1 (k'_{-3} + k'_1) + k'_{+3} k'_1 (k_{-3} + k_1)]
\end{aligned} \quad (A5)$$

$$F_6 = (k_{-1} + k_{+2}) (k'_{-1} + k'_{+2}) k_{+3} k'_{+3} k_1 k'_1 [I]_0^2 \quad (A6)$$

APPENDIX B

Derivation of the time course of the product P, taking into account rapid equilibrium conditions

If we insert condition (11) into eqns. (A1–A6), we obtain:

$$F_1 \approx k_{+1} [Z]_0 + k_{+3} [I]_0 + k_{-1} + k'_{+3} [I]_0 + k'_{+1} [S]_0 + k_{-3} + k'_{-3} + k'_{-1} \quad (B1)$$

$$\begin{aligned}
F_2 \approx & k_{+1} k'_{+1} [Z]_0 [S]_0 + k_{+1} k'_{+3} [Z]_0 [I]_0 + k_{+3} k'_{+3} [I]_0^2 + k'_{+1} k_{+3} [S]_0 [I]_0 + k_{+1} (k_{-3} + k'_{-3} + k'_{-1}) [Z]_0 \\
& + [(k_{+3} + k'_{+3}) (k_{-1} + k'_{-1}) + k_{+3} k'_{-3} + k'_{+3} k_{-3}] [I]_0 + k'_{+1} (k_{-1} + k_{-3} + k'_{-3}) [S]_0 + (k_{-1} + k_{-3}) (k'_{-3} + k'_{-1}) + k_{-1} k_{-3} + k'_{-1} k'_{-3}
\end{aligned} \quad (B2)$$

$$\begin{aligned}
F_3 \approx & k_{+1} k'_{+3} (k_{-3} + k'_{-1}) [Z]_0 [I]_0 + k_{+1} k'_{+1} (k_{-3} + k'_{-3}) [Z]_0 [S]_0 + k_{+3} k'_{+3} (k_{-1} + k'_{-1}) [I]_0^2 + k_{+3} k'_{+1} (k_{-1} + k'_{-3}) [I]_0 [S]_0 \\
& + k_{+1} [k_{-3} (k'_{-3} + k'_{-1}) + k'_{-3} k'_{-1}] [Z]_0 + k_{+3} [k_{-1} (k'_{-3} + k'_{-1}) + k'_{-3} k'_{-1} + k'_{+3} k_{-1} (k_{-3} + k'_{-1}) + k_{-3} k'_{-1}] [I]_0 \\
& + k'_{+1} [k_{-1} (k_{-3} + k'_{-3}) + k_{-3} k'_{-3}] [S]_0 + k_{-1} [k_{-3} (k'_{-3} + k'_{-1}) + k'_{-3} k'_{-1}] + k_{-3} k'_{-3} k'_{-1}
\end{aligned} \quad (B3)$$

$$\begin{aligned}
F_4 \approx & k_{+1} k'_{+3} k'_{-1} k_{-3} [Z]_0 [I]_0 + k_{+1} k'_{+1} k_{-3} k'_{-3} [Z]_0 [S]_0 + k_{+3} k'_{+3} k'_{-1} k_{-1} [I]_0^2 + k_{+3} k'_{+1} k_{-1} k'_{-3} [I]_0 [S]_0 + k_{+1} k_{-3} k'_{-3} k'_{-1} [Z]_0 \\
& + k_{-1} k'_{+1} k_{-3} k'_{-3} [S]_0 + k_{-1} k'_{-1} (k_{+3} k'_{-3} + k'_{+3} k_{-3}) [I]_0 + k_{-1} k_{-3} k'_{-3} k'_{-1}
\end{aligned} \quad (B4)$$

$$\begin{aligned}
F_5 \approx & k_{+1} k'_{+3} k'_1 k_{-3} k'_{-1} [Z]_0 [I]_0 + k_{+3} k'_{+3} k'_{-1} k_{-1} (k_1 + k'_1) [I]_0^2 + k'_{+1} k_{+3} k'_1 k_{-1} k'_{-3} [S]_0 [I]_0 \\
& + k_{-1} k'_{-1} (k_{+3} k'_1 k'_{-3} + k'_{+3} k'_1 k_{-3})
\end{aligned} \quad (B5)$$

$$F_6 \approx k_{-1} k'_{-1} k_{+3} k'_{+3} k_1 k'_1 [I]_0^2 \quad (B6)$$

According to eqns. (B1)–(B6), the following simplifications are possible:

$$\left. \begin{aligned} F_i & \rightarrow \infty \quad (i = 1, 2, 3, 4, 5, 6) \\ \frac{F_j}{F_4} & \rightarrow 0 \quad (j = 1, 2, 3) \\ \frac{F_5}{F_4} & = \frac{G_5}{G_4} \\ \frac{F_6}{F_4} & = \frac{G_6}{G_4} \end{aligned} \right\} \quad (B7)$$

where:

$$\begin{aligned}
G_4 = & K'_1 K_3 [Z]_0 [I]_0 + K_3 K'_3 [Z]_0 [S]_0 + K_1 K'_1 [I]_0^2 + K_1 K'_3 [S]_0 [I]_0 + K'_1 K_3 K'_3 [Z]_0 + K_1 K_3 K'_3 [S]_0 \\
& + K_1 K'_1 (K_3 + K'_3) [I]_0 + K_1 K'_1 K_3 K'_3
\end{aligned} \quad (B8)$$

$$G_5 = K'_1 K_3 k'_1 [Z]_0 [I]_0 + K_1 K'_1 (k_1 + k'_1) [I]_0^2 + K_1 K'_3 k_1 [S]_0 [I]_0 + K_1 K'_1 (K'_3 k_1 + K_3 k'_1) \quad (B9)$$

$$G_6 = K_1 K'_1 k_1 k'_1 [I]_0^2 \quad (B10)$$

We divide both the left and the right sides of eqn. (5) by F_4 to obtain:

$$\frac{1}{F_4} \lambda^6 + \frac{F_1}{F_4} \lambda^5 + \frac{F_2}{F_4} \lambda^4 + \frac{F_3}{F_4} \lambda^3 + \frac{F_4}{F_4} \lambda^2 + \frac{F_5}{F_4} \lambda + \frac{F_6}{F_4} = 0 \quad (B11)$$

Since $1/F_4$ and F_i/F_4 ($i = 1, 2, 3$) are small, the first four terms in the left side may be ignored and (B11) converts into:

$$\lambda^2 + F_5/F_4 \lambda + F_6/F_4 = 0 \quad (B12)$$

Therefore, two finite roots are obtained, and taking the last of eqns. (B7) into account, it is verified that:

$$\lambda_1 + \lambda_2 = -G_5/G_4 \quad (B13)$$

$$\lambda_1 \lambda_2 = G_6/G_4 \quad (B14)$$

The roots of eqn. (B11) are the same as those of eqn. (5). Hence, if condition (8) prevails:

$$|\lambda_h| \rightarrow \infty \quad (h = 3, 4, 5, 6) \quad (\text{B15})$$

$$\lambda_h - \lambda_i \approx \lambda_h \quad (h = 3, 4, 5, 6; i = 1, 2) \quad (\text{B16})$$

Eqn. (B15) allows us to ignore the exponential terms in eqn. (3) because they are smaller in comparison with the others; therefore, the product concentration, $[P]$, is given by eqn. (9) in the main paper.

To obtain the parameters involved in this equation, we must take into account the following: if in eqn. (4) we consider condition (8) and divide both the numerator and denominator by $k_{+1}k'_{+1}k_{+3}k'_{+3}$, we obtain:

$$\sigma = \frac{k_{+2}k'_{+2}K_3K'_3[E]_0[Z]_0[S]_0}{K_1K'_1k_1k'_1[I]_0^2} \quad (\text{B17})$$

Hence:

$$G_4 = \frac{\lambda_3\lambda_4\lambda_5\lambda_6}{k_{+1}k'_{+1}k_{+3}k'_{+3}} \quad (\text{B18})$$

And inserting condition (8) and eqns. (B15), (B16) and (B18) into eqn. (6), we obtain:

$$\gamma_1 = -\frac{k_{+2}k'_{+2}K_3K'_3[E]_0[Z]_0[S]_0}{\lambda_1(\lambda_2 - \lambda_1)G_4} \quad (\text{B19})$$

$$\gamma_2 = -\frac{k_{+2}k'_{+2}K_3K'_3[E]_0[Z]_0[S]_0}{\lambda_2(\lambda_1 - \lambda_2)G_4} \quad (\text{B20})$$

APPENDIX C

Time-course equation of the product P for each of the particular cases (Schemes 3–10)

In Schemes 3–6, α_0 means the expression for α when $[I]_0 = 0$ and τ is the induction period, i.e. $-\sigma/\alpha$. Note that K_3 or/and K'_3 are cancelled when the general expression is applied to some concrete schemes.

Schemes 3–6:

$$[P] = \alpha t + \sigma[1 - \exp(\lambda_1 t)] \quad (\text{C1})$$

$$\alpha = k_{+2}k'_{+2}K_3K'_3[E]_0[Z]_0[S]_0/H \quad (\text{C2})$$

$$\lambda_1 = -H/G_4 \quad (\text{C3})$$

$$\sigma = -k_{+2}k'_{+2}K_3K'_3[E]_0[Z]_0[S]_0/\lambda_1^2 G_4 \quad (\text{C4})$$

$$\tau = G_4/H \quad (\text{C5})$$

Schemes 3 and 4

$$[(1/\alpha) - (1/\alpha_0)]/[I]_0 = m[I]_0 + n \quad (\text{C6})$$

Schemes 5 and 6

$$[(1/\alpha) - (1/\alpha_0)]/[I]_0 = n \quad (\text{C7})$$

where, in Scheme 3:

$$H = K'_1K_3k'_1[Z]_0[I]_0 + k'_1[I]_0^2K_1K'_1 + K_1K'_1K_3k'_1 \quad (\text{C8})$$

G_4 is given by eqn. (B8).

$$m = (K_1K'_1k'_1)/(k_{+2}k'_{+2}K_3K'_3[E]_0[Z]_0[S]_0) \quad (\text{C9})$$

$$n = (K'_1k'_1)/(k_{+2}k'_{+2}K_3K'_3[E]_0[S]_0) \quad (\text{C10})$$

Scheme 4

$$H = K_1K'_1k_1[I]_0^2 + K_1K'_3k_1[S]_0[I]_0 + K_1K'_1K'_3k_1 \quad (\text{C11})$$

G_4 is given by eqn. (B8).

$$m = (K_1K'_1k_1)/(k_{+2}k'_{+2}K_3K'_3[E]_0[Z]_0[S]_0) \quad (\text{C12})$$

$$n = (K_1k_1)/(k_{+2}k'_{+2}K_3K'_3[E]_0[Z]_0) \quad (\text{C13})$$

Scheme 5:

$$H = (K'_1k'_1[Z]_0[I]_0 + K_1K'_1k'_1)K_3 \quad (\text{C14})$$

$$G_4 = (K'_1[Z]_0[I]_0 + K'_3[Z]_0[S]_0 + K'_1K'_3[Z]_0 + K_1K'_3[S]_0 + K_1K'_1[I]_0 + K_1K'_1K'_3)K_3 \quad (\text{C15})$$

$$n = (K'_1k'_1)/(k_{+2}k'_{+2}K_3K'_3[E]_0[S]_0) \quad (\text{C16})$$

Scheme 6:

$$H = (K_1k_1[S]_0[I]_0 + K_1K'_1k_1)K'_3 \quad (\text{C17})$$

$$G_4 = (K_3[Z]_0[S]_0 + K_1[I]_0[S]_0 + K_3K'_1[Z]_0 + K_1K_3[S]_0 + K_1K'_1[I]_0 + K_1K'_1K_3)K'_3 \quad (\text{C18})$$

$$n = (K_1k_1)/(k_{+2}k'_{+2}K_3K'_3[E]_0[Z]_0) \quad (\text{C19})$$

Schemes 7–10:

$$[P] = At^2 \quad (\text{C20})$$

with

$$A = \frac{k_{+2}k'_{+2}K_3K'_3[E]_0[Z]_0[S]_0}{2G_4} \quad (\text{C21})$$

and G_4 for Schemes 7, 8 and 9 is given by eqns. (B8), (C18) and (C15) respectively and for Scheme 10:

$$G_4 = K_3K'_3([Z]_0[S]_0 + K'_1[Z]_0 + K_1[S]_0 + K_1K'_1) \quad (\text{C22})$$