Mathematical Analysis of a Proteolytic Positive-Feedback Loop: Dependence of Lag Time and Enzyme Yields on the Initial Conditions and Kinetic Parameters†

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ABSTRACT: A model of a proteolytic positive-feedback loop, similar in general terms to feedback loops that occur in blood coagulation and other systems, has been examined by both explicit and numerical analysis. In this loop, modeled as a closed system, each enzyme (E1, E2) catalyzes the formation of the other from its respective zymogen (Z1, Z2), and both enzymes are subject to irreversible inhibition. The system shows three major characteristics. (1) No significant Z1 or Z2 activation occurs unless the combination of initial conditions and kinetic parameters is above a threshold level. This threshold occurs when the product of the enzyme generation rates equals the product of their inhibition rates. When the formation-rate product is less than the inhibition-rate product, there is no response: E1 and E2 generation is minimal and the lag time is effectively infinite. Conversely, when the generation-rate product exceeds the inhibition-rate product, explosive formation of both E1 and E2 is seen. For responses exceeding the threshold, the following obtain. (2) The lag time in E1 and E2 generation is a highly nonlinear function of the zymogen concentrations and the enzyme generation and inhibition rates. In contrast, there is a simple logarithmic relationship between the lag time and the initial trace concentration of the enzyme that is responsible for initiating the system; in this model, E1. (3) The extent of Z1 and Z2 activation is similarly a nonlinear function of the conditions and parameters but is independent of the initiating trace level of E1. Comparison of the predictions of explicit analysis with numerical simulation show that lag time and enzyme yield are acccurately described by the analytical functions, even to a large degree under conditions where the assumptions made in the mathematical analysis may not hold. Explicit expressions for the response threshold and the stimulus–response relationship hold great promise for our understanding of the role of positive-feedback loops in physiological systems.

A number of the homeostatic systems of higher organisms involve proteolytic cascades. In such cascades, each step involves the action of a proteolytic enzyme (protease) on a precursor molecule (zymogen) to generate another protease. The classic cascade is that of blood coagulation (Davie & Ratnoff, 1964; Macfarlane, 1964), but others exist—for example, in fibrinolysis and complement. As Macfarlane (1964) pointed out, one feature of such cascades is their potential for amplification; indeed, he likened the clotting cascade to a photomultiplier, in which each stage multiplies the gain of the device. The capacity for amplification, particularly in systems of many steps, is a significant liability in a system where tight control of the response is required. Maximal amplification in coagulation would be disastrous, causing the response (clot formation) to be inappropriate to the level of stimulus (extent of damage of blood vessels).

A second feature of the architecture of the clotting system was known before the cascade was identified: the plasma inhibitors of the clotting proteases. The major targets of these inhibitors—the chief one is antithrombin (antithrombin III)—are the final two proteases involved in the clotting scheme, factor Xa and thrombin.

In addition to cascade amplification and protease inhibition, two types of feedback control are seen in clotting and other systems. As one might expect when a response must be tightly controlled, there are negative feedbacks by which products, once formed, act to block their own formation. In coagulation, for example, thrombin acts through a complex system involving protein C, thrombomodulin, and protein S to inactivate two cofactors involved in its formation: factors VIIIa and Va (Esmon, 1989). Another negative feedback in coagulation involves the combination of tissue factor pathway inhibitor (TFPI) with factor Xa to form an inhibitory complex that inactivates the initiating complex of coagulation: the complex of tissue factor (TF) and factor VIIa (fVIIa) (Broze *et al.*, 1991).

The more unexpected controls in coagulation are the positive feedbacks. They occur throughout the system and number at least six. In them, a protease formed toward the end of the sequence—factor Xa or thrombin—acts to accelerate its own formation. For example, the tissue factor–fVII complex (TF–fVII), which is formed when plasma factor VII combines with tissue factor, is activated in a feedback loop by factor Xa, generating the active TF–fVIIa complex. Similarly, factors VIII and V, which have a cofactor role in clotting, require feedback activation to form the actual active cofactors. More recently, it has been reported that factor XI is subject to feedback activation by both thrombin and the immediate product of its activation, factor XIa (Gailani & Broze, 1991; Naito & Fujikawa, 1991). Finally, there is the activation of platelets by thrombin. Separate from their structural role in the formation of a blood clot, activated platelets probably

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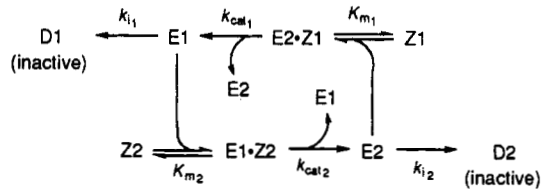
Scheme I. Model of a Proteolytic Feedback Loop[a]



[a] The loop consists of the activation of two zymogens (Z1 and Z2) to proteolytic enzymes (E1 and E2), the activation of each zymogen being catalyzed by the other enzyme. Activations are assumed to obey Briggs–Haldane steady-state assumptions, defined by a K_m and k_{cat}. Both enzymes are subject to first-order inhibition.

provide most of the requirement of the clotting system for negatively charged phospholipid. Normal circulating platelets have no significant negative phospholipid on their membrane surface. Upon activation by a number of agonists, including thrombin, their surface phospholipid gains a negative charge, chiefly through the appearance of phosphatidylserine on the outer membrane leaflet [see Schroit and Zwaal (1991)]. In addition to this, thrombin-activated platelets provide factor Va, and platelet-bound factor Va is almost certainly the major functional form in hemostasis (*e.g.*, Tracy & Mann, 1983). Since thrombin is—perhaps arguably, but likely—the major agonist of platelets, these effects constitute a potent positive feedback.

From this listing it is clear that positive feedbacks play a major role in the clotting scheme. But despite our having known of several of these feedbacks for many years, it has not yet been possible to specify the benefits that they confer. Where, for instance, is the benefit in synthesizing factor VII as an inactive precursor rather than an active protease? If factor VII circulated in the plasma in its active form, on the appearance of tissue factor it could instantly form the active proteolytic complex that activates factors IX and X. As it is, activation of the TF-fVII complex requires the feedback action of factor Xa. Where is the benefit in this?

In this report we examine a model of a prototypical proteolytic feedback loop, we derive analytical expressions that specify its major properties, and we relate them explicitly to the kinetic parameters and the initial conditions. Further, we test the validity of this analysis by comparison with numerical simulation of the feedback-loop model.

The Model. A feedback loop of the type shown in Scheme I confers three important characteristics on the generation of the product enzyme (E2) as a function of the stimulus Z1+E1. If neither E1 nor E2 is initially present in such a system, a stimulus of Z1 cannot generate any product. In this report we consider the stimulus as Z1 plus a trace of E1. The ratio of the two, $E1/(E1 + Z1)_0$, may be considered as the initial activation state, which is responsible for initiating the loop. The three major characteristics follow.

(1) As long as both enzymes are inhibited, *i.e.*, k_{i_1} and k_{i_2} are more than zero, there is a threshold stimulus [of $(E1+Z1)_0$] below which the feedback loop does not operate. The threshold dose, or stimulus, is a function of all reactant concentrations, of the kinetic parameters of both activations, and of the rates of enzyme inhibition.

(2) A lag phase in E2 generation is always seen as long as the stimulus is above the threshold and $E1_0/(E1_0+Z1_0) < 1$. Below the threshold, because there is no response, the lag time is effectively infinite. As one might expect, the lower the initial trace concentration of E1, the longer the lag phase. It should be noted that lags occur even in the absence of E1 and/or E2 inhibition, when there is no threshold. Thus even in systems where there is no inhibition of the enzymes, a delay in E1 and E2 production will still occur.

(3) As long as k_{i_1} and $k_{i_2} > 0$, the final yields of E1 and E2—the total amounts of Z1 and Z2 activated—are also controlled by the reactant concentrations and the kinetic parameters. And just as the lag time is controlled in a threshold manner, so are the yields of the enzymes.

For all three characteristics, it was our goal to derive analytical expressions that relate them to the kinetic parameters of enzyme generation and inhibition and to the initial conditions, *i.e.*, the concentrations of Z1, E1, and E2. The study hinges on the use of Scheme I as a prototype feedback loop: it does not correspond exactly with any of the feedback loops of the clotting system, nor possibly with other real feedback loops. We nevertheless consider the analysis of its general behavior to be applicable to many similar systems.

With those provisos in mind, let us consider Scheme I. First, it is formally symmetrical: the top line (E1 generation and inhibition) is identical to the bottom (E2 generation and inhibition). Although we initially hoped to develop an analysis for the general case, of any combination of kinetic parameters and conditions, this can be solved only by numerical methods. For explicit analysis it has been necessary to restrict the conditions to the nonsymmetrical case. We have assumed that the level of Z1 is small relative to Z2 and less than K_{m_1}. Since we are considering Z1 as the initial small stimulus, this seems reasonable. It is certainly true in coagulation, where, for instance, the level of TF–fVII complex must be substantially lower than the factor X concentration, and in the following step even the maximal level of factor Xa generated during clotting can be only a small fraction of the concentration of prothrombin. A second important assumption is that the activation of Z1 largely precedes major activation of Z2. In light of the relative concentrations, this too is reasonable.

Enzyme generation in both cases is assumed to involve the steady-state formation of an enzyme–substrate (EZ) complex, defined by a K_m, and the generation of product according to a k_{cat} (see Experimental Procedures). The steady-state assumption means that we ignore the complications of the approach to steady state, even though it may well be significant in systems where the reactant concentrations are in the nanomolar or subnanomolar range. As justification, we note that for almost all the reactions in clotting, at least, only K_m and k_{cat} are known, rather than the individual rate constants of EZ formation, dissociation, and breakdown to product.

It has also been necessary to assume simplifying conditions in order to linearize various rate expressions; these are described in the analysis itself. In contrast, numerical simulation, which we use to test and validate the explicit analysis, although it assumes a steady state in EZ formation, requires no linearizing assumptions.

We first present the analysis and derive expressions that relate (1) the threshold, (2) the lag time, and (3) enzyme (E1 and E2) yields to the initial conditions and kinetic parameters. These are then compared with the results determined by numerical solution of the model.

NUMERICAL PROCEDURES

In Scheme I, Z1 and Z2 represent inactive precursors, or zymogens, which are activated by proteolytic cleavage to form proteolytic enzymes E1 and E2. In the numerical simulation we assume a simple kinetic mechanism for both activations: the approximately steady-state (i) formation of an enzyme–substrate (EZ) complex, according to a Michaelis constant (K_{m_1}, K_{m_2}), followed by (ii) breakdown of the EZ complex to

produce the active product enzyme, according to a first-order rate constant (k_{cat_1}, k_{cat_2}). The reader should note that K_m is defined in the normal way and not necessarily as the true Michaelis–Menten equilibrium constant for formation of the E·Z complex that is implied by the formulation of Scheme I. For example, if we define the rate constants of formation and dissociation of the E2·Z1 complex as k_{+1} and k_{-1}, and assume Briggs–Haldane steady-state conditions, then $K_{m_1} = (k_{-1} + k_{cat_1})/k_{+1}$. In either case, K_m is defined as $(E)(Z)/EZ$ at steady state. Inhibition or decay of the enzymes produced, E1 and E2, is assumed to obey first-order, or pseudo-first-order, kinetics (k_{i_1}, k_{i_2}).

Symbols. Throughout this report we use *italics* rather than square brackets to denote concentrations of the various molecular species. A center dot is used to denote enzyme–substrate complexes (*e.g.*, E·Z), and a prime denotes the first derivative with respect to time, dx/dt.

Numerical Integration. The model was solved for the concentrations of four variables as a function of time. These variables are the total concentrations of each zymogen and active enzyme species, defined as follows. At any time, t, (1) $Z1_{tot,t} = Z1 + E2{\cdot}Z1$; (2) $E1_{tot,t} = E1 + E1{\cdot}Z2$; (3) $Z2_{tot,t} = Z2 + E1{\cdot}Z2$; and (4) $E2_{tot,t} = E2 + E2{\cdot}Z1$, the center dot here denoting enzyme–substrate (E·Z) complexes. To calculate the derivatives of these variables in the numerical integration routine we first determine, at the start of each iteration of the integration routine, the steady-state (ss) concentrations of E2·Z1 and E1·Z2, using for each the quadratic expression

$$E{\cdot}Z_{ss} = {}^1/_2(E_{tot} + Z_{tot} + K_m - [(E_{tot} + Z_{tot} + K_m)^2 - 4E_{tot}Z_{tot}]^{1/2})$$

where E_{tot} and Z_{tot} are defined above and K_m is the Michaelis constant for the appropriate reaction, K_{m_1} or K_{m_2}, defined (in terms of free concentrations) by $(E)(Z)/EZ$. The use of the expression requires no assumption that either E or Z is in excess over the other. Knowing $E1{\cdot}Z2_{ss}$ and $E2{\cdot}Z1_{ss}$, we obtain the concentrations of free enzyme:

$$E1 = E1_{tot,t} - E1{\cdot}Z2_{ss}$$

$$E2 = E2_{tot,t} - E2{\cdot}Z1_{ss}$$

The derivatives of the four variables are then obtained as follows:

$$Z1_{tot}' = -k_{cat_1}E2{\cdot}Z1$$

$$E1_{tot}' = k_{cat_1}E2{\cdot}Z1 - k_{i_1}E1$$

$$Z2_{tot}' = -k_{cat_2}E1{\cdot}Z2$$

$$E2_{tot}' = k_{cat_2}E1{\cdot}Z2 - k_{i_2}E2$$

With the steady-state calculation of the concentrations of the E·Z complexes, this problem is not numerically stiff. Integration was done using the fourth-order Runge–Kutta method with adjustable-step-size driver, as described by Press *et al.* (1986). ϵ, the maximum relative error per iteration, was set to 10^{-6} or smaller. Integration errors in $Z1_{tot}$ and $E1_{tot}$ were scaled (in the step-size driver) relative to the initial concentration of Z1, and those in $Z2_{tot}$ and $E2_{tot}$ were scaled to the initial concentration of Z2.

Initial conditions were specified as (1) the initial E1 + Z1 concentration, $(E1 + Z1)_0$; (2) the initial activation state of Z1, *i.e.*, the $E1_0/(E1 + Z1)_0$ ratio; (3) the initial Z2 concentration, $Z2_0$; and (4) the initial E2 concentration, $E2_0 = 0$.

Determination of t_{lag}, $E1_{Yield}$, and $E2_{Yield}$ in Simulations. (1) Lag time, t_{lag}, was determined from numerical simulations of E1 generation just as we define it in the analytical derivation (see Results): t_{lag} is the time at which the concentration of E1 attains 5% of the initial level of Z1. In other words, $E1_{(t=t_{lag})} = Z1_0/20$. Linear interpolation was used to determine t_{lag} values that fell between time points returned by the integration routine. The maximum error of these determinations is of the order of 0.05 time units (tu). (2) Enzyme yields, $E1_{Yield}$ and $E2_{Yield}$, were determined from extended simulations, up to a maximum of 500 tu. Yields are defined as the final extent of Z1 or Z2 activation, *plus* any enzyme present at $t = 0$ (see Determination of Enzyme Yield). This definition produces a small problem under conditions that are very close to the threshold: in this case enzyme generation may still continue even at 500 tu. We have not corrected for this source of error. Under all other conditions the maximum error in enzyme yield estimates is 0.01% of the respective initial zymogen concentrations.

Units. Kinetic parameters and concentrations in the model can be specified in any consistent set of units, and specific units have therefore been omitted in graphs. Readers may, however, prefer to relate results to a real system: if one assumes the concentration unit to be nanomolar and the time unit to be minutes, the parameter values we have used correspond very approximately—within an order of magnitude—with the parameters known or estimated for factor X activation by the TF–fVII complex and its feedback loop.

RESULTS

Analysis of Threshold Response

We derive here an explicit function that relates the response threshold to the initial conditions and kinetic parameters. Below the threshold stimulus, $(E1 + Z1)_{0,thr}$, no response occurs because the E1 and E2 inhibition rates exceed their generation rates. In contrast, above the threshold, the feedback loop operates, producing explosive generation of E1 and E2.

In analyzing the threshold we assume that at levels of stimulus below and up to the threshold, any E1 or E2 generation is small relative to the initial concentrations of Z1 and Z2, *i.e.*,

$$E1 \ll Z1;\quad D1 \ll Z1;\quad E2 \ll Z2;\quad D2 \ll Z2$$

$Z1$ and $Z2$ are therefore considered as constants, equal to $Z1_0$ and $Z2_0$, respectively. We also assume that substrates (Z1, Z2) are in large excess over the respective activating enzymes (E2, E1) during the early phases of the response and that Michaelis–Menten kinetics therefore hold; *i.e.*; $Z' = (-E)(Z)k_{cat}/(K_m + Z)$. This assumption is valid while

$$E2 \ll (K_{m_1} + Z1_0) \quad \text{and} \quad E1 \ll (K_{m_2} + Z2_0)$$

The first holds only in the very earliest phases of E2 generation and is therefore only applicable to the lag phase. The second, however, will hold throughout E1 generation under the specified conditions. We can then express E1 and E2 formation as

$$E1' = \mu_1 E2 \quad \text{and} \quad E2' = \mu_2 E1 \qquad (1)$$

where $E1$ and $E2$ are the concentrations of the respective enzymes (including enzyme present as enzyme–substrate

complexes, *E1·Z2* and *E2·Z1*), and the rate constants, μ, are defined as

$$\mu_1 = \frac{k_{cat_1} Z1_0}{K_{m_1} + Z1_0} \quad \text{and} \quad \mu_2 = \frac{k_{cat_2} Z2_0}{K_{m_2} + Z2_0} \tag{2}$$

E1 and E2 inhibition are assumed to be first-order, but we correct for the fact that enzyme present as an enzyme–substrate complex is not subject to inhibition, *i.e.*, the substrates Z1 and Z2 are assumed to reduce the inhibition rates of E2 and E1 in a competitive manner. Thus inhibition of the two enzymes is expressed as

$$E1' = -\kappa_1 E1 \quad \text{and} \quad E2' = -\kappa_2 E2 \tag{3}$$

where the rate constants for inhibition, κ, are

$$\kappa_1 = \frac{k_{i_1} K_{m_2}}{K_{m_2} + Z2_0} \quad \text{and} \quad \kappa_2 = \frac{k_{i_2} K_{m_1}}{K_{m_1} + Z1_0} \tag{4}$$

The differential equations for E1 and E2 are therefore

$$E1' = \mu_1 E2 - \kappa_1 E1 \quad \text{and} \quad E2' = \mu_2 E1 - \kappa_2 E2 \tag{5}$$

This system has the form

$$\mathbf{E}' = (\mathbf{A})(\mathbf{E})$$

where

$$\mathbf{A} = \begin{pmatrix} -\kappa_1 & \mu_1 \\ \mu_2 & -\kappa_2 \end{pmatrix} \quad \text{and} \quad \mathbf{E} = \begin{pmatrix} E1 \\ E2 \end{pmatrix}$$

Using standard techniques for the solution of systems of differential equations (*e.g.*, Beltrami, 1987), we obtain

$$E_t = c_1 e^{\lambda_1 t} v_1 + c_2 e^{\lambda_2 t} v_2$$

where E_t is the concentration of enzyme (*E1* or *E2*) at time t, λ_1 and λ_2 are eigenvalues of $\mathbf{A}$, v_1 and v_2 are the corresponding eigenvectors, and c_1 and c_2 are constants dependent on the initial conditions and parameter values. The eigenvalues of this system are obtained as

$$\lambda_1, \lambda_2 = \left(\frac{\kappa_1 + \kappa_2}{2}\right) \pm \left[\left(\frac{\kappa_1 + \kappa_2}{2}\right)^2 - (\kappa_1\kappa_2 - \mu_1\mu_2)\right]^{1/2} \tag{6}$$

λ_1 being the sum and λ_2 the difference. In terms of the eigenvectors,

$$v_i = \begin{pmatrix} v_{i1} \\ v_{i2} \end{pmatrix}_{i=1,2}$$

the relation $\mathbf{A}v_i = \lambda_i v_i$ becomes

$$-\kappa_1 v_{i1} + \mu_1 v_{i2} = \lambda_i v_{i1}$$

$$-\mu_2 v_{i1} - \kappa_2 v_{i2} = \lambda_i v_{i2}$$

By letting $v_{i1} = 1$, we obtain the general solution describing the earliest generation of E1 and E2, during the lag phase:

$$E1_t = E1_0\left\{\frac{\lambda_1 + \kappa_2}{\lambda_1 - \lambda_2} e^{\lambda_1 t} + \frac{\lambda_2 + \kappa_2}{\lambda_2 - \lambda_1} e^{\lambda_2 t}\right\} \tag{7}$$

and

$$E2_t = E1_0\left\{\frac{\mu_2}{\lambda_1 - \lambda_2} e^{\lambda_1 t} + \frac{\mu_2}{\lambda_2 - \lambda_1} e^{\lambda_2 t}\right\}$$

Control of the Threshold by λ_1. Since λ_2 is always negative, the $e^{\lambda_2 t}$ terms rapidly become very small, and we can neglect them. Enzyme generation is thus controlled by λ_1. When $\lambda_1 < 0$, there is no significant generation of either enzyme: the E1 originally present and the minuscule amount of generated E2 decay exponentially. When $\lambda_1 > 0$, sufficient E2 is generated to support the feedback loop. The result is explosive generation of E1 and E2. The threshold between these two conditions occurs when $\lambda_1 = 0$, and it is seen from eq 6 that this occurs when $\mu_1\mu_2 = \kappa_1\kappa_2$, *i.e.*, when

$$\frac{k_{cat_1}}{K_{m_1}} Z1_0 \frac{k_{cat_2}}{K_{m_2}} Z2_0 = k_{i_1} k_{i_2} \tag{8}$$

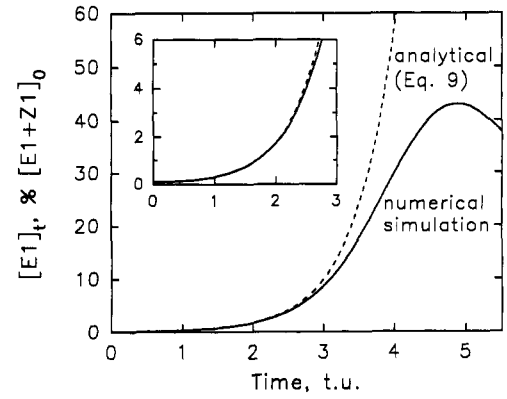


FIGURE 1: Analytical approximation of the early phase of E1 generation. The dotted line shows the solution of eq 9 under the following conditions: K_{m_1} = 10 cu; k_{cat_1} = 10 tu^{-1}; k_{i_1} = 1 tu^{-1}; $(E1+Z1)_0$ = 0.1 cu; $E1_0/(E1 + Z1)_0$ = 0.001; K_{m_2} = 200 cu; k_{cat_2} = 200 tu^{-1}; k_{i_2} = 1 tu^{-1}; $Z2_0$ = 100 cu. The solid line shows numerical simulation of E1 generation in Scheme I under the same conditions. The inset shows the same data with an expanded ordinate scale.

Lag Time. When $\mu_1\mu_2 > \kappa_1\kappa_2$, significant E1 and E2 generation occurs, and it is characterized by a lag phase. Since the assumptions we made above break down when Z1 activation becomes significant, we have not been able to derive analytical expressions for the whole course of E1 and E2 generation. We cannot determine, for instance, the inflection point of E1 generation, or other objective measure. We can, however, derive an expression for the very early time course of E1 generation. We define the lag time, t_{lag}, as the time at which *E1* attains a certain small fractional value of $Z1_0$, x. Thus $E1(t_{lag}) = Z1_0 x$. Since $e^{\lambda_2 t}$ is neglected in eq 7, the early time course of E1 generation is described by

$$E1_t = E1_0 \frac{\lambda_1 + \kappa_2}{\lambda_1 - \lambda_2} e^{\lambda_1 t} \tag{9}$$

In Figure 1 we compare the time course of E1 generation calculated from this expression with that obtained by numerical simulation of the model, and we confirm the validity of our main assumption: in the initial period, when $E1_t \ll Z1$, the error in $E1_t$ remains very small. We have set x = 0.05. Rearranging eq 9, we get the following explicit expression for the lag time,

$$t_{lag,x} = \frac{1}{\lambda_1} \ln\left(\frac{\lambda_1 - \lambda_2}{\lambda_1 + \kappa_2} \frac{Z1_0 x}{E1_0}\right) \tag{10}$$

which is valid for all $\lambda_1 > 0$.

Determination of Enzyme Yield

Another major characteristic of the feedback-loop model is regulation of the yield of both enzymes. Just as the lag time is a threshold function of dose, so are the enzyme yields. In this section we derive this relationship and show that Z1 and Z2 activation are controlled by all kinetic parameters and initial conditions except one: as long as $E1_0 > 0$, the initial Z1 activation state, or $E1_0/(E1 + Z1)_0$ ratio, does not affect the final yields of E1 or E2.

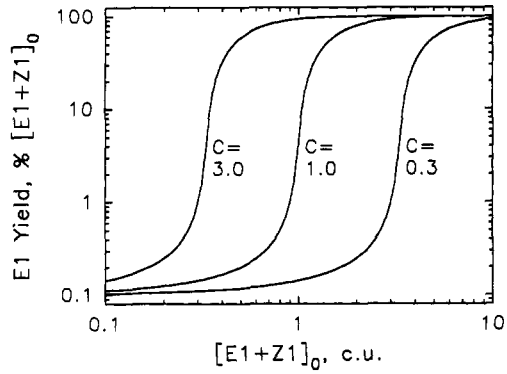


FIGURE 2: Threshold function for $E1_{Yield}$. Equation 20 was solved for three values of the lumped kinetic constant C (units are cu^{-1}) as a function of $(E1 + Z1)_0$, from 0.1 to 10 cu, with a fixed initial activation state, $E1_0/(E1 + Z1)_0 = 0.001$. Other parameters and conditions were as described for Figure 1.

Definition of Enzyme Yield. We define enzyme yield as the total amount of zymogen activated, plus any enzyme initially present at $t = 0$. This definition includes enzyme that is subsequently inhibited. Specifically,

$$E1_{Yield} = E1_0 + Z1_0 - Z1_\infty; \quad E2_{Yield} = E2_0 + Z2_0 - Z2_\infty \quad (11)$$

As we have previously shown (Jesty, 1990), enzyme yield, as defined here, is related to the integrated area under the enzyme-generation curve by the rate of inhibition, κ (defined in eq 4):

$$E_{Yield} = \kappa \int_0^\infty E \, dt$$

Determination of $E1_{Yield}$. In contrast to analysis of the threshold and lag phase, we obviously cannot assume here that E1 generation is small. In this analysis we stipulate the following conditions:

$$E1_0 > 0; \quad E2_0 = 0; \quad k_{i_1} > 0; \quad k_{i_2} > 0$$

The reader should note that the initiating enzyme is E1; we have not analyzed models in which $E2 > 0$. Because both k_{i_1} and k_{i_2} are nonzero, all enzyme initially present or generated is eventually inhibited, so that

$$E1_\infty = 0; \quad E2_\infty = 0; \quad E1_{Yield} = D1_\infty; \quad E2_{Yield} = D2_\infty \quad (12)$$

Like the previous analysis, the analysis of enzyme yield requires linearizing assumptions. (1) We consider that for most physiological systems it is reasonable to assume that $Z2_0 \gg Z1_0$. We also assume, with somewhat less evidence, that the activation of Z1—the target of the feedback—substantially precedes Z2 activation. To determine $E1_{Yield}$, therefore, we assume that $Z2 = Z2_0$, and remains constant during E1 generation. (2) In order to linearize the expression for E1 generation, another assumption is required: that $Z1 \ll K_{m_1}$. This is reasonable in systems where Z1 is a trace stimulus, and it is this that is our major focus.

We use four differential equations in the analysis:

$$Z1' = -\alpha_1 E2 \cdot Z1 \quad (13)$$

$$E1' = \alpha_1 E2 \cdot Z1 - \kappa_1 E1 \quad (14)$$

$$E2' = \mu_2 E1 - \kappa_2 E2 \quad (15)$$

$$D2' = \kappa_2 E_2 \quad (16)$$

where $\alpha_1 = k_{cat_1}/K_{m_1}$ and μ_2, κ_1, and κ_2 are as described earlier (eqs 1–4). Since we assume that $Z1 \ll K_{m_1}$, $\kappa_2 \simeq ki_2$. Adding eqs 13–16 and rearranging, we obtain

$$\frac{d}{dt}\left\{Z1 + E1 + \frac{\kappa_1}{\mu_2}(E2 + D2)\right\} = 0$$

The expression within braces is therefore constant. In the model we stipulate $E2$ and $D2$ to be zero at $t = 0$. It follows that at any time

$$Z1 + E1 + \frac{\kappa_1}{\mu_2}(E2 + D2) = Z1_0 + E1_0$$

But at $t = \infty$, $E1_\infty = 0$. Recalling that $E1_{Yield} = (Z1_0 + E1_0 - Z1_\infty)$, we get

$$E1_{Yield} = \frac{\kappa_1}{\mu_2} D2_\infty \quad (17)$$

However, $D2_\infty = E2_{Yield}$, so that under the conditions assumed,

$$E1_{Yield} = \frac{\kappa_1}{\mu_2} E2_{Yield} \quad (18)$$

From eq 13 we see that

$$\frac{Z1'}{Z1} = \frac{d}{dt}(\ln Z1) = -\alpha_1 E2$$

Integration from $t = 0$ to ∞ gives

$$\ln\left(\frac{Z1_\infty}{Z1_0}\right) = -\alpha_1 \int_0^\infty E2_t \, dt \equiv -\alpha_1 \frac{E2_{Yield}}{\kappa_2}$$

Substituting eq 18 and rearranging, we get

$$Z1_\infty = Z1_0 \, e^{-(\alpha_1\mu_2/\kappa_1\kappa_2)E1_{Yield}}$$

Defining a constant,

$$C = \frac{\alpha_1\mu_2}{\kappa_1\kappa_2} = \frac{k_{cat_1}k_{cat_2}Z2_0}{K_{m_1}K_{m_2}k_{i_1}k_{i_2}} \quad (19)$$

we obtain the following expression for $E1_{Yield}$:

$$E1_{Yield} \equiv E1_0 + Z1_0 - Z1_\infty = E1_0 + Z1_0(1 - e^{(-C)E1_{Yield}}) \quad (20)$$

We reiterate that this analysis is based on the assumption that when $E1_{Yield} < (E1 + Z1)_0$, $E2_{Yield} \ll Z2$; *i.e.*, μ_2 is a constant. Equation 20 is easily solved using a root-finding routine. The form of the function—the dependence of $E1_{Yield}$ on dose $[(E1 + Z1)_0]$ and C—is shown in Figure 2. Its threshold nature is clear, with a threshold height equal to the $E1_0/(E1 + Z1)_0$ ratio (fixed here at 0.001). The threshold dose, $(E1 + Z1)_{0,thr}$, is equal to $1/C$.

Determination of $E2_{Yield}$. In contrast with the analysis for $E1_{Yield}$, consideration of $E2_{Yield}$ clearly requires us to take changes in Z2 concentration into account: we cannot assume that μ_2 is a constant. We therefore start with the following differential equations:

$$Z2' = -E1\frac{k_{cat_2}Z2}{K_{m_2} + Z2}; \quad D1' = E1\frac{k_{i_1}K_{m_2}}{K_{m_2} + Z2} \quad (21)$$

Hence

$$Z2' = -\frac{k_{cat_2}Z2}{k_{i_1}K_{m_2}}D1'$$

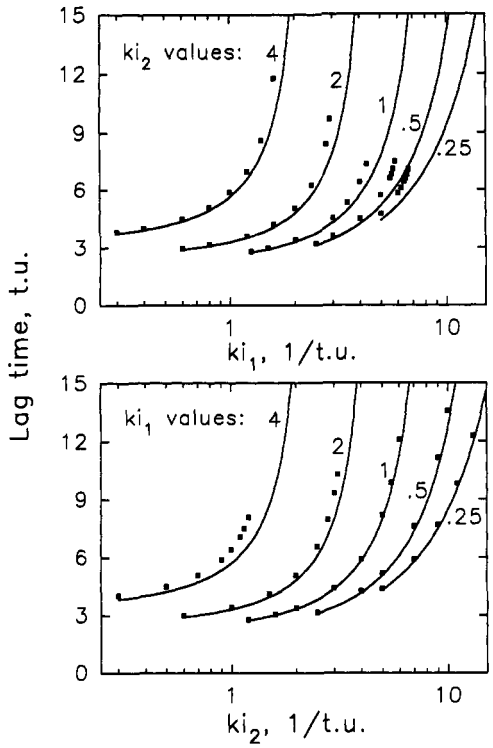


FIGURE 3: Threshold control of lag time by enzyme inhibition rates. Top: lag times were determined from eq 10 (lines) and by numerical simulation of Scheme I (points), as a function of k_{i_1}, at the k_{i_2} values shown. Bottom: lag times were similarly estimated, as a function of k_{i_2}, at the k_{i_1} values shown. Other kinetic parameters and conditions were as described for Figure 1.

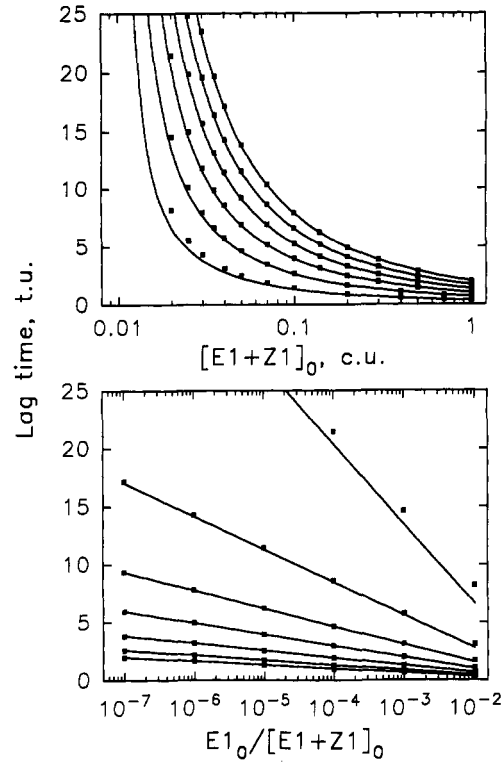


FIGURE 4: Threshold control of lag time by dose; logarithmic control by initial activation state. Lag times were determined from eq 10 (lines) and by numerical simulation of Scheme I (points). Top: lag time as a function of stimulus size, $(E1 + Z1)_0$, at varying values of the initial activation state, $E1_0/(E1 + Z1)_0$, from 10^{-2} (left) to 10^{-8} (right) in 10-fold decrements. Bottom: lag time as a function of initial activation state at $(E1 + Z1)_0$ values of (top to bottom) 0.02, 0.04, 0.08, 0.15, 0.3, 0.6, and 1.2 cu. Other parameters and conditions were as described for Figure 1.

Integrating, we obtain

$$\ln\left(\frac{Z2}{Z2_0}\right) = -\frac{k_{\text{cat}_2}}{k_{i_1}K_{m_2}}D1$$

For $t = \infty$, we then get

$$Z2_\infty = Z2_0\, e^{-(k_{\text{cat}2}/k_{i1}K_{m2})D1_\infty}$$

Recalling that $D1_\infty = E1_{\text{Yield}}$, and $E2_{\text{Yield}} = (Z2_0 - Z2_\infty)$, we obtain the following expression:

$$E2_{\text{Yield}} = Z2_0(1 - e^{-(k_{\text{cat}2}/k_{i1}K_{m2})E1_{\text{Yield}}}) \qquad (22)$$

It may be noted that in the case $E1_{\text{Yield}}$ is small, we may approximate eq 22 using the relationship that for small x, $x \simeq (1 - e^{-x})$. This gives

$$E2_{\text{Yield}} \simeq \frac{Z2_0 k_{\text{cat}_2}}{k_{i_1}K_{m_2}}E1_{\text{Yield}} = \frac{\mu_2}{\kappa_1}E1_{\text{Yield}}$$

which is identical to eq 18.

Properties of the System

In this section we examine the behavior of the system and its important features. We also compare the behavior predicted by the explicit analysis with that obtained from numerical simulation of Scheme I, emphasizing that since simulation requires no linearizing assumptions it reflects the "real" behavior of the model. Simulation results may therefore be considered as a test, or validation, of the functions derived by explicit analysis. Before considering the results, let us note that the concentration unit (cu) and time unit (tu) are undefined: the analyses are valid for any consistent set of units.

Threshold, Lag Times. In Figure 3 we show the dependence of lag time on the rates of E1 and E2 inhibition, and compare results derived from the analytical function under selected conditions with those from numerical analysis. Comparison of the top and bottom panels of Figure 3 shows that the function (eq 10, lines) is essentially symmetrical for k_{i_1} and k_{i_2} save at the highest values, where slight differences appear. There are, however, significant differences between the analytical estimates and the results of numerical simulation (points). These errors are largely confined to the threshold region and the combination of high k_{i_1} and low k_{i_2} values. In this region the function (eq 10) underestimates the lag time.

One of the assumptions involved in the analysis was that the $e^{\lambda_2 t}$ term in the general solution for $E1$ (eq 7) was insignificant relative to the $e^{\lambda_1 t}$ term, and the former was ignored in deriving eqs 9 and 10. Examination of the $e^{\lambda_2 t}$ term under varying conditions shows that the assumption is valid: the term indeed becomes very small very quickly.

Scrutiny of the time courses of E1 and E2 generation in simulations has demonstrated a more likely cause of the error (data not shown). Under the conditions analyzed and simulated, we set $(E1 + Z1)_0 = Z2_0/1000$, which is a severe test of the assumption that $E2 < (K_{m_1} + Z1)$ during the lag phase. Simulations confirm that at low k_{i_2} values $E2$ can significantly exceed $Z1$ during the lag phase, producing errors in estimates of E1 generation rate and E2 inhibition rate. We reiterate, however, that the errors in t_{lag} estimation under these difficult conditions are significant only in the immediate region of the threshold.

The marked similarity between the data of the two panels of Figure 3 allows another important conclusion: there is no large difference between k_{i_1} and k_{i_2} in their effects on the lag

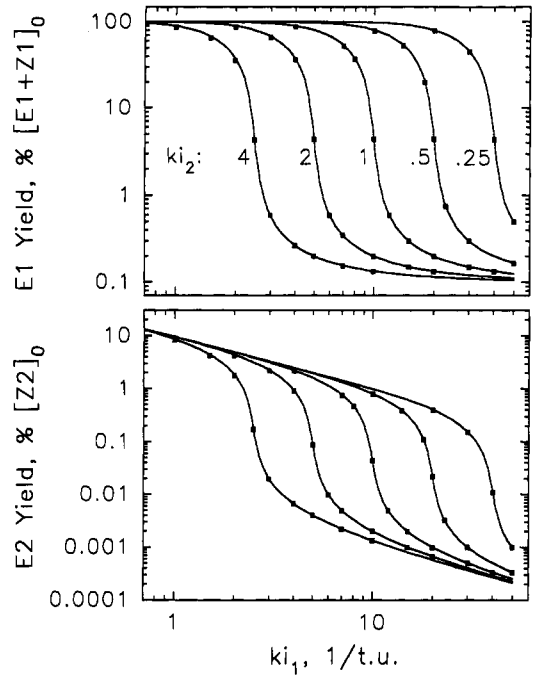


FIGURE 5: Threshold control of $E1_{Yield}$ and $E2_{Yield}$ by enzyme inhibition rates. Final yields of E1 (top) and E2 (bottom), as defined by eq 11, were determined by solution of eq 20 (lines) and by numerical simulation of Scheme I (points), as a function of k_{i_1}, at the values of k_{i_2} shown in the top panel. Other parameters and conditions were as described for Figure 1.

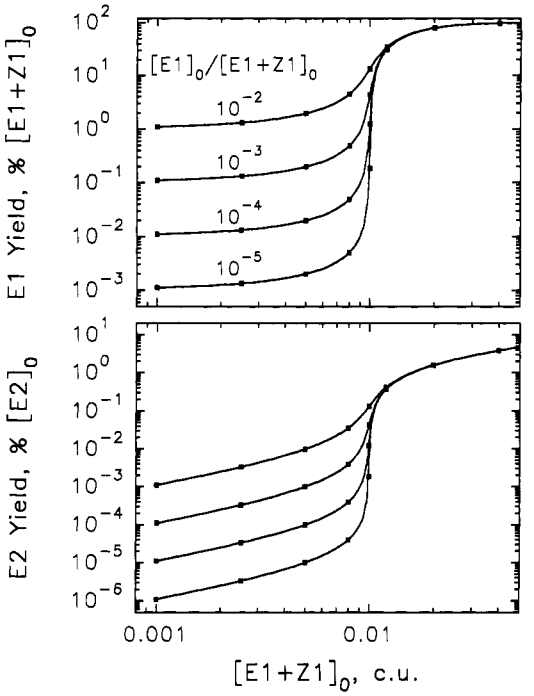


FIGURE 6: Threshold control of $E1_{Yield}$ and $E2_{Yield}$ by dose. E1 yields (top) and E2 yields (bottom) were determined from eq 20 (lines) or by numerical simulation of Scheme I (points), as a function of stimulus size, $(E1 + Z1)_0$, at the $E1_0/(E1 + Z1)_0$ ratios shown in the top panel. Other parameters and conditions were as described for Figure 1.

time. A similar conclusion will appear when we consider the control of enzyme yield.

In Figure 4 we show the dependence of lag time on two of the initial conditions: the stimulus size, $(E1 + Z1)_0$, and the initial activation state of Z1, the $E1_0/(E1 + Z1)_0$ ratio. As we may expect from the analysis, the lag time is a threshold function of $(E1 + Z1)_0$ as it is of inhibition rate (top panel). Under the conditions shown, the predicted threshold dose ($t_{lag} \rightarrow \infty$) is 0.01 for all $E1_0/(E1 + Z1)_0$ ratios and therefore independent of the initial Z1 activation state.

In contrast to the threshold relationship between lag time and stimulus, there is a simple logarithmic relationship between the lag time and the initial activation state (bottom panel). The lag time is relatively insensitive to the initial activation state. For example, reducing the $E1_0/(E1 + Z1)_0$ ratio from 10^{-2} to 10^{-4} under any particular conditions merely doubles the lag time (see Discussion).

Control of Enzyme Yields. The analytical approach to the determination of lag times is applicable only to the earliest phases of E1 generation: it accounts neither for changes in $Z1$ nor for $E2$ rising in excess over $Z1$. To determine the control of enzyme yield we therefore use a different approach (eqs 13–16). Figure 2 shows that eq 20, which describes the dependence of $E1_{Yield}$ on the kinetic parameters and initial conditions, is also a threshold function and that C is the reciprocal of the threshold dose, $1/(E1 + Z1)_{0,thr}$. This analysis therefore confirms independently our earlier conclusion that the threshold occurs when $\mu_1\mu_2 = \kappa_1\kappa_2$ (eq 6).

The control of $E1_{Yield}$ by inhibition rate (Figure 5, top) is essentially the inverse of the control of the lag time (Figure 3) but the agreement between analytical function and simulation here is much better. Figure 5 (bottom) shows the corresponding data for the control of $E2_{Yield}$. Here the shape of the function is different: above the threshold, $E2_{Yield}$, unlike $E1_{Yield}$, is incomplete and is inversely proportional to inhibition rate. The reason is that $E2_{Yield}$ is controlled by $\int_0^\infty E1\ dt$, which is inversely proportional to k_{i_1} (eq 22). The converse is not true under the conditions we have analyzed and simulated: $E1_{Yield}$ above the threshold is always essentially complete and therefore little affected by k_{i_2} (Figure 5, top).

A similar, but mirror-image, result is seen in Figure 6, where we show the effect of varying the initial stimulus, $(E1 + Z1)_0$, and the initial activation state, $E1_0/(E1 + Z1)_0$. Once again, because we chose $(E1 + Z1)_0 \ll Z2_0$, $E2_{Yield}$, but not $E1_{Yield}$, is controlled by the initial stimulus above the threshold. The apparent difference in the subthreshold behavior in this figure is not particularly meaningful and reflects the fact that some E1 is always initially provided, in proportion to total dose. In the top panel, because $E1_0$ is included in the definition of $E1_{Yield}$ (eq 11), the subthreshold values essentially equal the level of E1 initially present (see eq 11). Correspondingly, in the bottom panel, $E2_{Yield}$ in the subthreshold region is proportional to $E1_0$. The more important result is the fact that neither the threshold itself nor the yield of either enzyme above the threshold is affected by the initial Z1 activation state.

Effect of Varying Enzyme Generation Rates. In the preceding figures we have shown the dependence of lag time and enzyme yield on two kinetic parameters—k_{i_1} and k_{i_2}—and two initial conditions—the dose of $(E1 + Z1)_0$, and the initial activation state, $E1_0/(E1 + Z1)_0$. Simulations and analytical predictions have also been done at varying K_m and k_{cat} values and $Z2_0$. The results are not shown because they are essentially as predicted (eqs 10, 21, and 23).

DISCUSSION

The model we have analyzed (Scheme I) is a prototype, related in varying degrees to several positive feedback loops in physiological systems. Although it does not strictly correspond with many real feedback loops, the present analysis gives a clear indication to their general behavior. To meet the

requirements of analogy with Scheme I, a system must include (1) two, coupled, enzyme-catalyzed steps, whereby each enzyme catalyzes the production of the other, and (2) inhibition of both enzymes. The key property in these systems' behavior is the threshold response: below the threshold stimulus, the response dies away and no significant generation of either enzyme is observed. Once the threshold is exceeded, the feedback loop occurs and we see explosive generation of E1 and E2. Under these conditions the response is characterized by two properties: a lag time in the generation of both enzymes and control of their yield. The threshold itself, the lag time, and the enzyme yields are all controlled by the initial conditions and the kinetic parameters, and we can now specify the nature of that control using the analytical solutions we have obtained.

Although other modeling studies have been done in coagulation (*e.g.*, Liniger *et al.*, 1980; Naski & Shafer, 1991), no analysis has been made of the general properties of the positive feedback loops. Compared with the few studies on proteolytic systems, there is a large literature on the controls of metabolic systems, such as the cascade role of phosphatases and kinases in glycogen metabolism. While such cascades do, like coagulation, involve the generation of enzymes by enzymes, we would point out a fundamental difference: proteolytic systems, including their feedbacks, are fundamentally unidirectional and irreversible. Z1, for example, once activated to E1, cannot be regenerated.

Feedback Analogies. In considering Scheme I we have considered Z1 to be the controlling stimulus since it is the major variable in regulating the response. In some real systems it may indeed be a zymogen; the tissue factor–factor VII complex, which initiates coagulation, is a case in point. In other cases it may not be. For example, in the activation of factor X by factor IXa, the essential stimulus is factor IXa, which technically is an enzyme; but until its cofactor, factor VIII, is activated, factor IXa has no activity on factor X. In that sense, factor IXa in the presence of unactivated factor VIII can be considered to have the properties of a zymogen: generation of proteolytic activity requires activation of factor VIII and the formation of a factor IXa–VIIIa complex. It is this complex that is equivalent to E1.

A similar situation exists in prothrombin activation by factor Xa, where activated factor V is required as a cofactor. Initially, when factor V is in its unactivated state, factor Xa has little or no activity on prothrombin and may likewise be considered as a pseudozymogen. Not until factor V is activated do we generate the active enzyme (E1), the factor Xa–Va complex. Prothrombin activation, however, is made considerably more complicated by several things that do not have a parallel in Scheme I. First, factor Xa itself, which in the absence of factor Va we are equating with Z1, is subject to inhibition; second, the factor Xa–Va complex (E1) is inhibited more slowly than factor Xa; and third, factor Xa activates factor V (Monkovic & Tracy, 1990). The lag-phase behavior of this system has been recently analyzed by one of us (Willems, 1991), and the results do indeed show general properties similar to Scheme I—as long as we consider the initiating factor Xa as equivalent to Z1 rather than E1.

Feedback Targets. Analysis of the present model predicts the existence of a threshold in the activation of the feedback target Z1. Below the threshold Z1 remains unactivated, and above the threshold it is fully activated. The major targets of feedback action in clotting are the TF–fVII complex, factor VIII, and factor V, and a superthreshold stimulus might thus be expected to produce the fully activated TF–fVIIa, IXa–VIIIa, and Xa–Va complexes. We are not aware of experimental data that yet confirm or refute the suggestion of this all-or-none phenomenon.

The Threshold. Both explicit analysis and numerical simulation of Scheme I show that the threshold dose, $(E1 + Z1)_{0,thr}$, is a linear function of all the kinetic parameters of the loop and of the initial conditions. It is proportional to the product of the rates of enzyme inhibition, $(k_{i_1}k_{i_2})$, and inversely proportional to the product of the rates of enzyme generation, $(k_{cat_1}k_{cat_2})/(K_{m_1}K_{m_2})$. This result is of major importance. The key is the control of the system by the products of rate constants.

Enzyme Generation Rates. On the enzyme generation side, this adds to our understanding of the effect of changes in the kinetics of enzyme generation. For instance, it is well accepted that the provision of a negative phospholipid surface vastly increases the catalytic efficiency, k_{cat}/K_m, in all the reactions of the vitamin K-dependent factors, and we note that these are the central reactions in the main feedback loops of the clotting system. Conversely, in the absence of negative phospholipid the catalytic efficiency of these reactions is extremely low. We postulate that, in addition to the fact that catalytic efficiencies determine rates of zymogen activation, they also control feedback-loop thresholds. Imagine, for example, that the rates of E1 and E2 generation in Scheme I are both increased 20-fold in the presence of a source of negative phospholipid such as activated platelets. We may then expect the threshold stimulus—the minimum stimulus that will generate a response—to be reduced as much as 400-fold. The converse is also important: in the absence of anionic phospholipid, *e.g.*, in the absence of activated platelets, we may expect the threshold stimulus to be very high. We suggest that this may be an important consideration in situations where a source of anionic phospholipid exists in the absence of a sizable coagulation stimulus.

Enzyme Inhibition Rates. On the inhibition side, the picture is the exact inverse, but the focus is still the product of the rate constants. One particularly important prediction that follows from our analysis is that to attain a certain threshold, lag time, or enzyme yield, it does not much matter which enzyme in a feedback loop is better inhibited. A 100-fold increase in the threshold, for instance, can be attained by increasing either k_{i_1} or k_{i_2} 100-fold; but the same result should obtain if k_{i_1} and k_{i_2} are both increased just 10-fold.

Anticoagulation. We see this as particularly relevant to strategies of anticoagulant therapy. Heparin is known to increase the inhibition rates (by antithrombin) of several enzymes at once, chief among them three enzymes involved in the major feedback loops of clotting—factors IXa and Xa and thrombin. Because of the multiple targets it is probably not required—nor indeed is it observed—that the inhibition rates of the individual enzymes in the presence of therapeutically effective levels of heparin be extraordinarily high.

In contrast, we would propose that a very different approach is required if anticoagulation is directed at a single enzyme. If the feedback loops of clotting behave in a generally equivalent manner to the system we have analyzed here, we would predict that anticoagulant therapy directed at single enzymes will require very much higher enzyme inhibition rates (in the plasma) to attain the same effects on lag time, enzyme yields, etc. Examples include the inhibition of thrombin by hirudin and its analogues and of factor Xa by specific inhibitors from leeches and ticks. Judging by the results of the present theoretical study, and judging solely on a kinetic rather than a pharmacological basis, we suggest that selective targeting of thrombin or factor Xa, both of which participate in feedback

loops, might be a significantly less efficient approach than targeting of multiple enzymes.

Idling. Threshold behavior is relevant to our understanding of how the clotting system copes with baseline, or idling, levels of coagulant enzymes in the circulation. We have mentioned that two major clotting enzymes—factors IXa and Xa—gain activity on their substrates only when their cofactors (factors VIII and V) are activated in feedback loops, and we have suggested that these enzymes, before feedback activation of their cofactors, are analogous to Z1 of the current model. Bauer *et al.* (1989) have reported evidence that factors IXa and Xa are both present at low idling levels in the plasma. Our results suggest that as long as the levels of these enzymes remain significantly below the thresholds for activation of their cofactors, the system is protected against a significant response.

Lag Time. Although the lag phase of positive-feedback loops has long been recognized, we have until now been unable to relate the lag time in any quantitative fashion to the kinetic parameters and conditions of a feedback loop. Much has been made in recent years of the initial activation state of clotting zymogens and cofactors, particularly factor VII. In terms of the present model, this relates directly to the trace level of E1 that we provide in the model to initiate the system. Both explicit analysis and numerical simulation suggest that the effect of variations in this is minor when compared with the large effect of other variables. (1) As long as $E1_0 > 0$, neither the threshold nor the final enzyme yields are affected by the initial activation state, which we have expressed as $E1_0/(E1 + Z1)_0$. (2) Although the lag time is indeed a function of the $E1_0/(E1 + Z1)_0$ ratio, this relationship is logarithmic. For example, the predicted lag times for ratios of 0.1 and 0.001 differ only by a factor of 3. Compared with the threshold behavior (Figures 2, 5, and 6), where the response may differ by several hundredfold over a 5–10-fold range of stimulus, this is not a large effect.

The prediction that even at extremely low levels of initial activity the system is fully capable of generating a response suggests the possibility that some systems might require no active enzyme at all to initiate the feedback loop; rather, the zymogen itself (Z1 in Scheme I) might be responsible. It is known that the zymogens of serine proteases do possess very low levels of catalytic activity, although we should note that the level of activity involved depends on the particular substrate [see, *e.g.*, Lonsdale-Eccles *et al.* (1978)]. And factor VII is known to be active in just this way, at least on small substrates and on small inhibitors such as diisopropyl phosphofluoride, in its zymogen state (Zur *et al.*, 1982).

Assumptions. We emphasize here the conditions we have assumed in our analysis. In particular, the conditions are nonsymmetrical and reflect the idea that Z1 is a small stimulus relative to Z2. The major assumptions arising from this are that (1) $Z1 < K_{m_1}$ and (2) Z1 activation largely precedes Z2 activation. Numerical analysis of the model, which did not involve any linearizing assumptions, allowed us to show that while errors do occur even under the stipulated conditions, they are reasonably small and largely confined to the estimates of the lag time. Even then they are significant only under conditions close to the threshold. Estimates of threshold dose and enzyme yield are not significantly affected.

Conclusion. In the introduction we asked where is the benefit in a feedback loop? What is the advantage of plasma containing factor VII rather than factor VIIa, or factor VIII rather than factor VIIIa? We suggest that proteolytic feedback loops of this general type confer at least one major advantage: they can sustain minor insult—a subthreshold stimulus—and generate no significant response. Yet if the stimulus exceeds a threshold, determined by the initial conditions and parameters, they will support the generation of a substantial and appropriate response.

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