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A Model for the Tissue Factor Pathway to Thrombin

II. A MATHEMATICAL SIMULATION*

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A mathematical simulation of the tissue factor pathway to the generation of thrombin has been developed using a combination of empirical, estimated, and deduced rate constants for reactions involving the activation of factor IX, X, V, and VIII, in the formation of thrombin, as well as rate constants for the assembly of the coagulation enzyme complexes which involve factor VIIIa-factor IXa (intrinsic *tenase*) and factor Va-Xa (*prothrombinase*) assembled on phospholipid membrane. Differential equations describing the fate of each species in the reaction were developed and solved using an interactive procedure based upon the Runge-Kutta technique. In addition to the theoretical considerations involving the reactions of the tissue factor pathway, a physical constraint associated with the stability of the factor VIIIa-factor IXa complex has been incorporated into the model based upon the empirical observations associated with the stability of this complex. The model system provides a realistic accounting of the fates of each of the proteins in the coagulation reaction through a range of initiator (factor VIIa-tissue factor) concentrations ranging from 5 pM to 5 nM. The model is responsive to alterations in the concentrations of factor VIII, factor V, and their respective activated species, factor VIIIa and factor Va, and overall provides a reasonable approximation of empirical data. The computer model permits the assessment of the reaction over a broad range of conditions and provides a useful tool for the development and management of reaction studies.

The first paper in this series (1) reports the formation of thrombin in a system containing saturating levels of phospholipid and all of the proteins necessary for initiating coagulation via the "extrinsic" pathway. The data from that paper indicate that the sequential activation of the extrinsic pathway proteins when initiated by picomolar to nanomolar concentrations of tissue factor-factor VIIa is sufficient to give stoichiometric conversion of prothrombin to α-thrombin in 30–240 s.

It is not intuitively obvious how the kinetics of the activation of each protein contributes to the overall cascade, resulting in the explosive formation of thrombin. Kinetic analyses of the individual reactions in the extrinsic pathway have been reported (2, 3), but an analysis of the complete reaction with its multiple reactants has been given less attention. In previous reports, this laboratory reported an activation study of factors IX and X with tissue factor-factor VIIa (4) and also developed a computer-based mathematical model to describe the kinetics of the *prothrombinase* complex (5). This latter model explained the sensitivity of that system to the concentrations of various reactants including the paradoxical decreases in rates observed with increasing concentrations of enzyme, substrate, or lipid surface.

These examples point out the need to identify the different kinetic fates of the individual proteins initially present when reacting mixtures are studied. Factor Xa is an enzyme with distinctly different catalytic properties in the presence or absence of its active cofactor factor Va (6). Both factor Xa-phospholipid and thrombin can activate factor V and factor VIII to their respective active cofactors (7–12). Factor VIIIa, once formed, is subject to spontaneous inactivation (13, 14). Factor IXa is virtually inactive without factor VIIIa (15). Factors IX and X serve as competitive substrates for the tissue factor-factor VIIa complex (4, 16, 17), and factor Xa-phospholipid catalyzes the formation of factor IXα (4). These complexities make interpretation of the kinetics of coagulation less than obvious. The reaction is even more difficult to analyze because of the absence of steady state conditions. The concentrations of all substrates, cofactors, and enzymes change throughout the reaction, and enzyme/activator concentrations may and do exceed substrate concentrations. In addition, the natural concentrations of the coagulation proteins in blood vary over an extremely broad range.

In this study we model the procoagulant reactions using the Runge-Kutta digital integration technique (18). In previously reported work, Lewis *et al.* (19) used a similar algorithm to successfully model the assembly of fibrin.

METHODS

In the activation of prothrombin via the extrinsic pathway, at least 18 kinetic species exist. A separate differential equation describes the changing concentration of each species. For example, the changing concentration of factor Va can be described in the mathematical shorthand:

$$d\mathrm{Va} = k_1[\mathrm{V}][\mathrm{Xa}] + k_2[\mathrm{V}][\mathrm{IIa}] - k_3[\mathrm{Va}][\mathrm{Xa}] + k_4[\mathrm{Va}\cdot\mathrm{Xa}] \quad \text{(Eq. 1)}$$

where k_1 and k_2 correspond to the second order rate constants for factor Va formation via factor Xa and thrombin as the activating enzymes, respectively. k_4 corresponds to the first order decay of the *prothrombinase* complex (Va·Xa), and k_3 corresponds to the association rate constant for factor Va with factor Xa in the *prothrombinase* complex.

Solving the complete set of 18 differential equations is not practical, but approximating the solution using a Runge-Kutta numerical technique (18) provides a useful description of the reaction progress.

The anticipated mathematical approach should allow prediction of the changing concentrations of each species. For such a complex set of reactions, several different solutions using different rate constants may match experimental results. We have restricted our model to be based, for the most part, upon published rate constants determined in earlier work.

The process of developing the mathematical model has three steps. First, identifying the enzymatic reactions that are integral to the complete coagulation cascade; second, developing empirically restricted approximations for the rate constants for each enzymatic reaction; and

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third, adjusting the rate constants to allow the model to simulate the empirical results derived from laboratory experiments. The first step in this process was developed by multiple laboratories who identified the different enzymes, proenzymes, and cofactors important in coagulation. The literature which deals with the evaluation of rate constants for each reaction, although extensive (Table I), is not complete. This shortcoming puts more importance and potential for misinterpretation upon the third step of the evaluation process. In the present analysis, we compare the results from the mathematical model with the experimental, empirical results presented by Lawson *et al.* (1) in the accompanying paper. In that paper, the changing concentration of each protein is tracked throughout the reaction under different initial conditions. The use of those results helps determine which of the rate constants can or should be adjusted in the mathematical model.

The results of the Lawson *et al.* (1) experiment are consistent with a model that includes the following reaction steps:

$$\mathrm{IX + TF \cdot VIIa} \underset{k_{16}}{\overset{k_6}{\Leftrightarrow}} \mathrm{IX \cdot TF \cdot VIIa} \xrightarrow{k_{11}} \mathrm{TF \cdot VIIa + IXa}$$

$$\mathrm{X + TF \cdot VIIa} \underset{k_{17}}{\overset{k_6}{\Leftrightarrow}} \mathrm{X \cdot TF \cdot VIIa} \xrightarrow{k_{12}} \mathrm{TF \cdot VIIa + Xa}$$

$$\mathrm{X + VIIIa \cdot IXa} \underset{k_{18}}{\overset{k_6}{\Leftrightarrow}} \mathrm{X \cdot VIIIa \cdot IXa} \xrightarrow{k_{13}} \mathrm{VIIIa \cdot IXa + Xa}$$

$$\mathrm{IX + Xa} \xrightarrow{k_{15}} \mathrm{Xa + IXa}$$

$$\mathrm{V + Xa} \xrightarrow{k_1} \mathrm{Xa + Va}$$

$$\mathrm{VIII + Xa} \xrightarrow{k_3} \mathrm{Xa + VIIIa}$$

$$\mathrm{V + IIa} \xrightarrow{k_2} \mathrm{IIa + Va}$$

$$\mathrm{VIII + IIa} \xrightarrow{k_4} \mathrm{IIa + VIIIa}$$

$$\mathrm{II + Va \cdot Xa} \underset{k_{19}}{\overset{k_6}{\Leftrightarrow}} \mathrm{II \cdot Va \cdot Xa} \xrightarrow{k_{14}} \mathrm{Va \cdot Xa + mIIa}$$

$$\mathrm{mIIa + Va \cdot Xa} \xrightarrow{k_5} \mathrm{Va \cdot Xa + IIa}$$

$$\mathrm{VIIIa + IXa} \underset{k_9}{\overset{k_7}{\Leftrightarrow}} \mathrm{VIIIa \cdot IXa}$$

$$\mathrm{Va + Xa} \underset{k_{10}}{\overset{k_8}{\Leftrightarrow}} \mathrm{Va \cdot Xa}$$

RELEVANT REACTIONS IN COAGULATION 1

Under the conditions used by Lawson *et al.* (1), sufficient lipid was added to saturate all protein-lipid binding reaction conditions; therefore, lipid binding equilibria are not included as reaction steps *per se*. Thus, in this model as well as the experiments described in Lawson *et al.* (1), the kinetic significance of the phospholipid concentration is reduced by performing each experiment with vast excesses of phospholipid. Therefore, these mathematical models do not, at present, include a factor for the concentration of lipid.

Simulated determinations of the changing concentrations of the proenzymes, pro-cofactors, and their products with time in the complex reaction were developed using the Runge-Kutta numerical technique (18). A computer program developed in the laboratory of Dr. David Ballou of the University of Michigan (20) allows a DOS-based computer to carry out calculations of the concentrations of each component in the system over time (t). The initial concentration condition (where $t = t_0$) for each component is provided by the user. The model then uses those concentrations in a set of differential equations to calculate the concentration present after the next interval (i) at time ($t_0 + i$). The process is continually repeated to calculate the concentration of each species for each interval of time.

TABLE I
Rate constants used to model the activation of thrombin

	Decription	Value
k_1	Activation of V by Xa (2nd order)	2×10^7 M^{-1} s^{-1}
k_2	Activation of V by IIa (2nd order)	2×10^7 M^{-1} s^{-1}
k_3	Activation of VIII by Xa (2nd order)	1×10^7 M^{-1} s^{-1}
k_4	Activation of VIII by IIa (2nd order)	2×10^7 M^{-1} s^{-1}
k_5	Conversion of mIIa to IIa by (Va·Xa) (2nd order)	1×10^7 M^{-1} s^{-1}
k_6	On-rate for rapidly formed complexes (2nd order)	1×10^8 M^{-1} s^{-1}
k_7	On-rate for the VIIIa·IXa complex (2nd order)	1×10^7 M^{-1} s^{-1}
k_8	On-rate for the Va·Xa complex (2nd order)	4×10^8 M^{-1} s^{-1}
k_9	Off-rate for VIIIa·IXa complex	0.005 s^{-1}
k_{10}	Off-rate for Va·Xa complex	0.4 s^{-1}
k_{11}	V_{max} for activation of IX by TF·VIIIa	0.3 s^{-1}
k_{12}	V_{max} for activation of X by TF·VIIIa	1.15 s^{-1}
k_{13}	V_{max} for activation of X by VIIIa·IXa	8.2 s^{-1}
k_{14}	$V_{max}$for mIIa formation by Va·Xa	32 s^{-1}
k_{15}	Activation of IX by Xa (2nd order)	1×10^5 M^{-1} s^{-1}
k_{16}	Off-rate for IX on TF·VIIa complex	24 s^{-1}
k_{17}	Off-rate for X on TF·VIIa complex	44 s^{-1}
k_{18}	Off-rate for X on VIIIa·IXa complex	0.001 s^{-1}
k_{19}	Off-rate for II on Va·Xa complex	70 s^{-1}
k_{20}	Constant for the slow degration of VIIIa·IXa (see comment above)	0.02 s^{-1}

Very small time intervals require more computing time but decrease the possibility of generating artifactual results due to approximating linear interpolations between intervals when nonlinear events are taking place. In order to avoid these types of artifacts, the model was subjected to repeated analyses at decreasing values of i until a convergent result was obtained.

The differential equations describing each of the components in the reaction are as follows:

$$d[\mathrm{TF \cdot VIIa}] = k_{11}[\mathrm{TF \cdot VIIa \cdot IX}] - k_6[\mathrm{TF \cdot VIIa}][\mathrm{IX}] + k_{16}[\mathrm{TF \cdot VIIa \cdot IX}] + k_{12}[\mathrm{TF \cdot VIIa \cdot X}] - k_6[\mathrm{TF \cdot VIIa}][\mathrm{X}] + k_{17}[\mathrm{TF \cdot VIIa \cdot X}] \quad \text{(Eq. 2)}$$

$$d[\mathrm{IX}] = k_{16}[\mathrm{TF \cdot VIIa \cdot IX}] - k_6[\mathrm{TF \cdot VIIa}][\mathrm{IX}] - k_{15}[\mathrm{IX}][\mathrm{Xa}] - k_{15}[\mathrm{IX}][\mathrm{Va \cdot Xa}] \quad \text{(Eq. 3)}$$

$$d[\mathrm{X}] = k_{17}[\mathrm{TF \cdot VIIa \cdot X}] - k_6[\mathrm{TF \cdot VIIa}][\mathrm{X}] - k_6[\mathrm{VIIIa \cdot IXa}][\mathrm{X}] + k_{18}[\mathrm{VIIIa \cdot IXa \cdot X}] \quad \text{(Eq. 4)}$$

$$d[\mathrm{V}] = -k_1[\mathrm{V}][\mathrm{Xa}] - k_2[\mathrm{V}][\mathrm{IIa}] - k_2[\mathrm{V}][\mathrm{mIIa}] \quad \text{(Eq. 5)}$$

$$d[\mathrm{VIII}] = -k_3[\mathrm{VIII}][\mathrm{Xa}] - k_4[\mathrm{VIII}][\mathrm{IIa}] - k_4[\mathrm{VIII}][\mathrm{mIIa}] \quad \text{(Eq. 6)}$$

$$d[\mathrm{II}] = k_{19}[\mathrm{Va \cdot Xa \cdot II}] - k_6[\mathrm{Va \cdot Xa}][\mathrm{II}] \quad \text{(Eq. 7)}$$

$$d[\mathrm{VIIIa \cdot IXa}] = k_7[\mathrm{VIIIa}][\mathrm{IXa}] - k_9[\mathrm{VIIIa \cdot IXa}] - k_6[\mathrm{VIIIa \cdot IXa}][\mathrm{X}] + k_{18}[\mathrm{VIIIa \cdot IXa \cdot X}] + k_{13}[\mathrm{VIIIa \cdot IXa \cdot X}] - (f_{abs}(\mathrm{I} - [\mathrm{VIIIa \cdot IXa}])) + (\mathrm{I} - [\mathrm{VIIIa \cdot IXa}]) \quad \text{(Eq. 8)}$$

$$d[\mathrm{Va \cdot Xa}] = k_8[\mathrm{Xa}][\mathrm{Va}] - k_{10}2[\mathrm{Va \cdot Xa}] + k_{19}[\mathrm{Va \cdot Xa \cdot II}] - k_6[\mathrm{Va \cdot Xa}][\mathrm{II}] + k_{14}[\mathrm{Va \cdot Xa \cdot II}] \quad \text{(Eq. 9)}$$

$$d[\mathrm{IIa}] = k_5[\mathrm{Va \cdot Xa}][\mathrm{mIIa}] \quad \text{(Eq. 10)}$$

$$d[\mathrm{Va \cdot Xa \cdot II}] = k_6[\mathrm{Va \cdot Xa}][\mathrm{II}] - k_{19}[\mathrm{Va \cdot Xa \cdot II}] - k_{14}[\mathrm{Va \cdot Xa \cdot II}] \quad \text{(Eq. 11)}$$

$$d[\mathrm{mIIa}] = k_{14}[\mathrm{Va \cdot Xa \cdot II}] - k_5[\mathrm{Va \cdot Xa}][\mathrm{mIIa}] \quad \text{(Eq. 12)}$$

$$d[\mathrm{TF \cdot VIIa \cdot IX}] = k_6[\mathrm{TF \cdot VIIa}][\mathrm{IX}] - k_{16}[\mathrm{TF \cdot VIIa \cdot IX}] - k_{11}[\mathrm{TF \cdot VIIa \cdot IX}] \quad \text{(Eq. 13)}$$

$$d[\mathrm{TF \cdot VIIa \cdot X}] = k_6[\mathrm{TF \cdot VIIa}][\mathrm{X}] - k_{17}[\mathrm{TF \cdot VIIa \cdot X}] - k_{12}[\mathrm{TF \cdot VIIa \cdot X}] \quad \text{(Eq. 14)}$$

$$d[\mathrm{VIIIa \cdot IXa \cdot X}] = k_6[\mathrm{VIIIa \cdot IXa}][\mathrm{X}] - k_{18}[\mathrm{VIIIa \cdot IXa \cdot X}] - k_{13}[\mathrm{VIIIa \cdot IXa \cdot X}] \quad \text{(Eq. 15)}$$

$$d[\text{IXa}] = k_9[\text{VIIIa} \cdot \text{IXa}] - k_7[\text{VIIIa}][\text{IXa}] + k_{11}[\text{TF} \cdot \text{VIIa} \cdot \text{IX}] + k_{15}[\text{IX}][\text{Xa}] + k_{15}[\text{IX}][\text{Va} \cdot \text{Xa}] \quad \text{(Eq. 16)}$$

$$d[\text{Xa}] = k_{10}[\text{Va} \cdot \text{Xa}] - k_6[\text{Xa}][\text{Va}] + k_{12}[\text{TF} \cdot \text{VIIa} \cdot \text{X}] + k_{13}[\text{VIIIa} \cdot \text{IXa} \cdot \text{X}] \quad \text{(Eq. 17)}$$

$$d[\text{Va}] = k_{10}[\text{Va} \cdot \text{Xa}] - k_6 1[\text{Xa}][\text{Va}] + k_1[\text{V}][\text{Xa}] + k_2[\text{V}][\text{IIa}] + k_2[\text{V}][\text{mIIa}] \quad \text{(Eq. 18)}$$

$$d[\text{VIIIa}] = k_9[\text{VIIIa} \cdot \text{IXa}] - k_7[\text{VIIIa}][\text{IXa}] + k_3[\text{VIII}][\text{Xa}] + k_4[\text{VIII}][\text{IIa}] + k_4[\text{VIII}][\text{mIIa}] \quad \text{(Eq. 19)}$$

where f_{abs} is the absolute value function and mIIa is meizothrombin.

From the empirical data of Lawson *et al.* (1), it is clearly established that there is a decay in the activity of the factor VIIIa-factor IXa complex with time.

In order to approximate this empirically established decay of the factor VIIIa-factor IXa complex activity, an additional equation which would cause a decreasing maximal concentration (I) of the factor VIIIa-factor IXa complex is required:

$$dI = (-(f_{abs}(I - [\text{VIIIa} \cdot \text{IXa}])) + (I - [\text{VIIIa} \cdot \text{IXa}]))k_{20} \quad \text{(Eq. 20)}$$

The reader should note a similar factor is used in determining the changing concentration of the factor VIIIa-factor IXa complex (Equation 7).

In order to execute the model, it is necessary to provide the starting concentrations and estimates of the kinetic constants appropriate for each reaction system. For most simulations described in this paper, the initial concentrations are identical with those used in the experimental conditions of Lawson *et al.* (1) at 5 nM factor VIIa-tissue factor unless specified otherwise.

Table I lists the empirical, estimated, and deduced rate constants used in the simulated models. k_{11}, k_{12}, k_{13}, and k_{14} are taken directly from previously published values (15, 21, 22). Each of these studies reported k_{cat} and K_m values for the reactions. For the purposes of this model, the k_{on} for each of the reactions (k_6) was assumed to be rapid and the k_{off} values (k_{16}, k_{17}, k_{18}, and k_{19}) were calculated from the Michaelis-Menten relationship. The kinetics of factor V activation (k_1, k_2) were described by Monkovic and Tracy (23), and the activation of factor VIII (k_3, k_4) were initially approximated by analogy and refined by comparison with the experimental results of Lawson *et al.* (1). It is known that the K_d values for the factor Va-factor Xa complex and factor VIIIa-factor IXa complex are on the order of 1–2 nM (15, 24), and the ratio of the on and off rates (k_{10}/k_8 and k_9/k_7) are consistent with those studies. The actual on-rate for the *prothrombinase* complex formation from lipid-bound proteins has been reported by Krishnaswamy *et al.* (3), and the value for k_8 (and by analogy, k_7) reflects that study. The accelerated conversion of factor IX to factor IXa shows complex kinetics in the presence of factor Xa and the tissue factor-factor VIIa complex. A previous study by Lawson *et al.* (4) reported the results of experiments in which factor IX was activated to factor IXaβ by factor VIIa-tissue factor in the presence of factor Xa. That report included data showing the changing rate as the concentration of substrate was depleted. From those data, a rough estimation of a second order rate constant (k_{15}) was calculated for the present study. The rates for meizothrombin conversion to α-thrombin (k_5) and the decay rate for factor VIIIa (a combination of k_{20} and I as described above) were determined by matching results of the computer model with experimental results reported by Lawson *et al.* (1).

The rate constants for activation of factor V, k_1 and k_2, were adjusted from the published rate constants by factors of 2 to 3. This adjustment was necessary to match the empirical results of Lawson *et al.* (1). The different reaction conditions under which the data of the laboratories were obtained may explain the small difference between the experimentally derived constants and those used in this model. Similarly, the rate constants for activation of factor VIII were determined by fitting model results to those observed by Lawson *et al.* (1).

The results of each simulation are a set of reactant concentrations predicted for each time interval selected which can be converted to a graphical form by assigning relative specific activities for each species. The graphical representations for thrombin activity correspond to the experimental observation of thrombin activity as influenced by the differing activity of α-thrombin and meizothrombin. The activity of meizothrombin is known to be about 120% that of α-thrombin (25); therefore, a relative specific activity of 1.2 is assigned to meizothrombin and 1 to α-thrombin in order to model thrombin activity in experimental results. In all other cases, a relative specific activity coefficient of 1 is assigned to the species of interest.

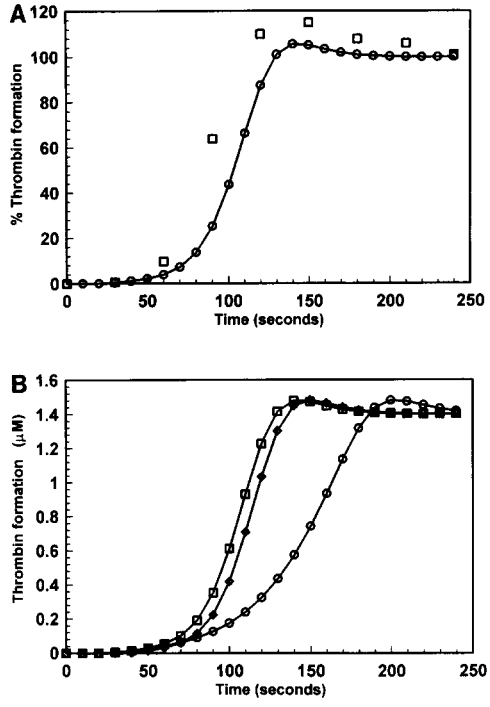

FIG. 1. *A*, a comparison of the formation of thrombin seen under experimental conditions to that modeled by mathematical simulation. The percent thrombin formation is plotted *versus* time. The empirical data (□) were taken from the results of Lawson *et al.* (1). The model (○) is as described under "Methods"; protein at plasma concentrations, tissue factor-factor VIIa at 5 pM, and the rate constants as described in Table I. Relative specific activity for αIIa = 1 and for mIIa = 1.2. 100% formation corresponds to complete formation of αIIa (1.4 μM). *B*, a test of the effect of varying enzyme complex formation rate constants on mathematical model results. The initial model (◇) represents the simulation with 5 pM tissue factor-factor VIIa and the rate constants as described in Table I. Relative specific activity for αIIa = 1 and for mIIa = 1.2. The other results show the simulated results with $k_7 = 1 \times 10^6$ M^{-1} s^{-1} (◇), $k_9 = 0.0005$ s^{-1} (□), or $k_8 = 4 \times 10^7$ M^{-1} s^{-1}, $k_{10} = 0.04$ sec^{-1} (○).

RESULTS

Fig. 1*A* shows the rate of thrombin formation predicted by the model at 5 pM factor VIIa-tissue factor complex (*open circles*), together with the empirical results obtained under the same conditions (*open squares*). The similarity in the form of the predicted reaction course to the empirical data is encouraging. The form of the reaction profile, characterized by a lag or initiation phase, a propagation phase, and the decay of meizothrombin to α-thrombin (the observed "bump" in activity) seen between 140 and 180 s, does not prove the model, nor does it validate that all of the assumptions upon which it was built are correct. However, the result suggests the model provides a reasonable approximation of the empirical result. The slight differences between the empirical result and the model are easily reconciled if one considers the many varied conditions under which the empirical rate constants used were derived and appreciates the difficulty in obtaining precise laboratory results in a reaction system as complex as the extrinsic pathway. The many different laboratory determinations of the empirical rate constants which are used in the model were collected under different conditions of temperature, metal ion and protein and lipid concentration. Each of these variables is known to have significant effects upon reaction rates. For our empirical results (1), different experiments conducted on different days using different protein preparations produced similar relative results but with the lag periods often varying as much as the difference between the model and the displayed results.

An example of the sensitivity of the model to some of the rate constant assumptions is presented in Fig. 1*B*. This figure shows how the variation of some of the approximated rate constants will cause alterations in the modeled output. These results are similar in magnitude to the difference between the model and empirical results. One set of rate constants for which there is an extended range is the k_{on} and k_{off} for the *prothrombinase* complex, k_8 and k_{10}. While the K_d for the *prothrombinase* complex is well characterized, thereby defining the ratio of k_{off}/k_{on} (k_{10}/k_8), the actual values reported for the individual rate constants could range within 1 or 2 orders of magnitude, depending upon whether the reaction requires exchange dissociation-reassociation to the membrane surface or simply the membrane facilitates two-dimensional transfer. Decreasing the k_{on} and k_{off} by a factor of 10 for the *prothrombinase* complex increases the lag period by 10%. Changing k_{off}/k_{on} (k_9/k_7) for the factor VIIIa-factor IXa complex increases the lag more noticeably. This observation reinforces the importance of the factor VIIIa-factor IXa complex in accelerating the overall reaction at the low factor VIIa-tissue factor complex concentration (5 pM) specified. Similar results are obtained by changing the individual rate constants for formation of the Michaelis complexes.

The results displayed in Fig. 1*B* illustrate two important elements of the model. First, the assignment of rate constants to those reaction steps that are not well characterized is not so critical as to either validate or invalidate the complete model. Second, the results show that reasonable changes in assumptions provide enough difference in the resulting output to explain the small differences between the model and the empirical results to which they are compared.

Despite the relative insensitivity of the model to any one rate constant, some fundamental mechanistic properties are critical. Fig. 2*A* represents the very slight acceleration in α-thrombin formation that is evident in a model that allows formation of a stable, completely active factor VIIIa-factor IXa complex. The overall effect of a model which assumes a stable factor VIIIa-factor IXa complex on the rate of α-thrombin formation, however, is insignificant. This is because during the first 80 s the level of factor VIIIa-factor IXa complex activity and, thus, factor Xa concentrations are similar whether or not a stable complex is assumed to exist. However, as seen in Fig. 2, *B* and *C*, the assumption of factor VIIIa-factor IXa complex degradation is critical after the initial 80 s (a time point *after* the initiation of maximal thrombin activation). In Fig. 2*B*, the rate of factor Xa formation is seen to be much greater in the model when it allows a stable factor VIIIa-factor IXa complex activity to be expressed than in both the empirical results (1) and in the model which includes the degradation of factor VIIIa-factor IXa complex activity. This conclusion is further reinforced when the role of factor IX activation is considered. Fig. 2*C* shows the results due to the feedback activating effect of the large concentration of factor Xa on factor IX generation when the factor VIIIa-factor IXa complex is modeled as a stable catalyst in the reaction.

Further evidence of the validity of the model is seen in Fig. 3. This figure illustrates the concentration-time dependence of thrombin, factor Xa, factor Va, factor VIIIa, and factor IXa during the course of the reaction. The model data show the sequence of events that is associated with the resulting thrombin formation. Consistent with the empirical results reported by Lawson *et al.* (1), the activation of pro-cofactors factor V and factor VIII takes place rapidly and precedes the "propagation phase" of the rapid formation of thrombin. The formation of factor IXa and factor Xa controls the ultimate levels of activity of the enzyme complexes, *prothrombinase*, and factor VIIIa-factor IXa complex. While the timely formation of sufficient

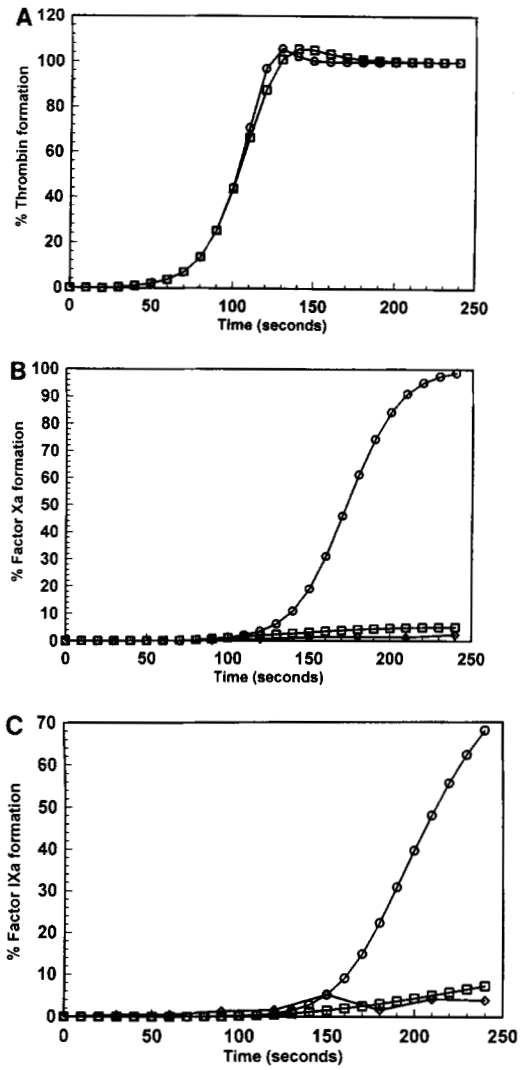

FIG. 2. **The effect of stable factor VIII-factor IXa.** The percent thrombin, factor Xa, or factor IXa is plotted *versus* time. The basic model (□) is simulated as described under "Methods." The alternative model (○) does not include the degradation of the active factor VIIIa-factor IXa complex. In *A*, the relative specific activity for αIIa = 1 and mIIa = 1.2. 100% formation corresponds to the complete formation of αIIa (1.4 μM). (Note: meizothrombin is also known to posess proteolytic activity similar to thrombin. In this paper, the kinetics of meizothrombin activity are assumed to be equivalent to thrombin activity.) In *B*, the relative specific activity for Xa = Va·Xa = Va·Xa-II = 1. The *diamond symbols* represent the empirically determined level of factor Xa reported in Lawson *et al.* (1). 100% formation corresponds to the complete formation of Xa (170 nM). In *C*, the relative specific activity for IXa = VIIIa·IXa = VIIIa·IXa – X = 1. 100% formation corresponds to the complete formation of IXa (90 nM). The *diamond symbols* represent the empirically determined levels of factor IXa.

levels of factor IXa and factor Xa are crucial processes, the complete activation of factor X and IX are not critical events in achieving the maximal rate of thrombin generation.

Influence of the Concentration of Factor VIIa-Tissue Factor Complex—The importance of the initial activation of factor IX and factor X can be modeled by varying the initial concentration of the tissue factor-factor VIIa complex. At the initial (plasma) concentrations of factor IX and factor X, both are significant substrates for the tissue factor-factor VIIa enzyme complex, and this complex plays an essential role in the initial activation of both factors (16, 17). Lawson *et al.* (1) note that changing the concentration of tissue factor-factor VIIa has only minimal effects on the maximal rate of thrombin formation observed during the propagation phase of the reaction, while

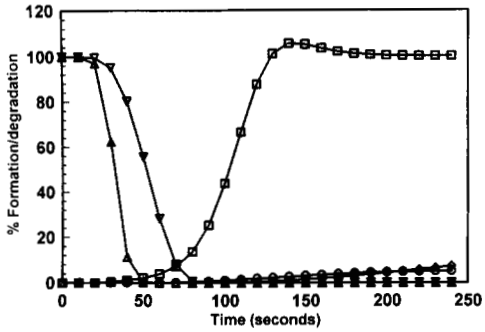

FIG. 3. **The reaction progress for each protein.** The basic model is simulated as described under "Methods" using the rate constants in Table I. The formation of thrombin (□) is modeled using relative specific activity of αIIa = 1 and mIIa = 1.2. 100% formation corresponds to the complete formation of IIa (1.4 μM). The formation of Xa (○) is modeled using relative specific activity: Xa = Va·Xa = Va·Xa – II = 1. 100% formation corresponds to the complete formation of Xa (170 nM). The formation of IXa (◇) is modeled using the relative specific activity for IXa = VIIIa·IXa = VIIIa·IXa – X = 1. 100% formation corresponds to the complete formation of IXa (90 nM). The cleavage of factor V (△) is modeled using the relative specific activity for V = 1. 100% corresponds to initial conditions [V] = 20 nM. The cleavage of factor VIII (▽) is modeled using the relative specific activity for VIII = 1. 100% corresponds to initial conditions [VIII] = 0.7 nM.

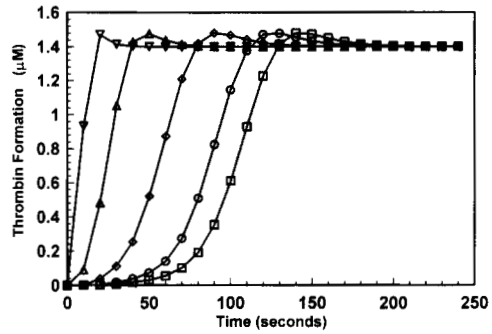

FIG. 4. **The effect of tissue factor-factor VIIa concentration.** The percent thrombin formed is plotted as a function of time. The model (□) is simulated as described under "Methods" using the rate constants in Table I and protein concentrations as described in Table I of Ref. 1 (with 5 pM tissue factor-VIIa). The relative specific activity of αIIa = 1 and mIIa = 1.2. Each of the other results was simulated using the same rate constants but increased concentrations of TF·VIIa. ○, [TF·VIIa] = 10 pM; ◇, [TF·VIIa] = 50 pM; △, [TF·VIIa] = 500 pM; ▽, [TF·VIIa] = 5 nM.

having the greatest influence on the initiation phase of the reaction which precedes the phase of maximal thrombin formation. Fig. 4 presents the predicted progress curves for thrombin generation when the reaction is initiated at different tissue factor-factor VIIa concentrations. The data of Fig. 4 illustrate the same form of dependence on the initiating tissue factor-factor VIIa concentration observed for the empirical results of Lawson *et al.* (1). The empirical studies using concentrations of the activator complex which varied over a 1000-fold range produced only a 5-fold change in the observed propagation phase rate of thrombin generation; the major effect observed was a prolongation of the "lag" or initiation phase of the reaction. Virtually the same observations are made for the computer model over a 1000-fold range of activator complex concentration.

The Effect of Factor VIII on the Reaction Progress—It is known that factor VIII plays a critical role in the initiation of coagulation, and that, in the absence of factor VIII, the activated partial thromboplastin time (21) is extended. In contrast, factor VIII deficiency does not result in prolongation of the prothrombin time (22). While the tissue factor-factor VIIa complex is important in forming the initial concentrations of factor Xa required for thrombin generation to proceed, the contribution of the factor VIIIa-factor IXa complex can be shown in the model by setting the initial concentration of factor VIII to 0. Fig. 5*A* shows that when low tissue factor-factor VIIa concentrations are used, the thrombin formation rate is depressed as a consequence of the much slower rate of factor Xa formation. This prediction by the model is virtually identical with the observation made in the empirical studies of Lawson *et al.* (1) which show that at low (picomolar) tissue factor-factor VIIa concentrations, the propagation phase of thrombin generation is significantly depressed when factor VIII is left out of the initial reaction mixture.

Similarly, both the model and the data of Lawson *et al.* (1) show that at high tissue factor-factor VIIa concentrations, omitting factor VIII from the reaction mixture has a minimal effect on the time course or form of the α-thrombin generation curve. Fig. 5*B* shows the predicted α-thrombin generation rate at 1 nM tissue factor-factor VIIa. The rates of thrombin generation are identical whether factor VIII is present or not. Thus, the presence of factor VIII is not critical to achieve rapid maximal formation of thrombin when high levels of factor VIIa-tissue factor are available. This is the result of the tissue factor-factor VIIa complex forming the threshold levels of factor Xa required to achieve explosive prothrombin activation without the need for the factor VIIIa-factor IXa complex.

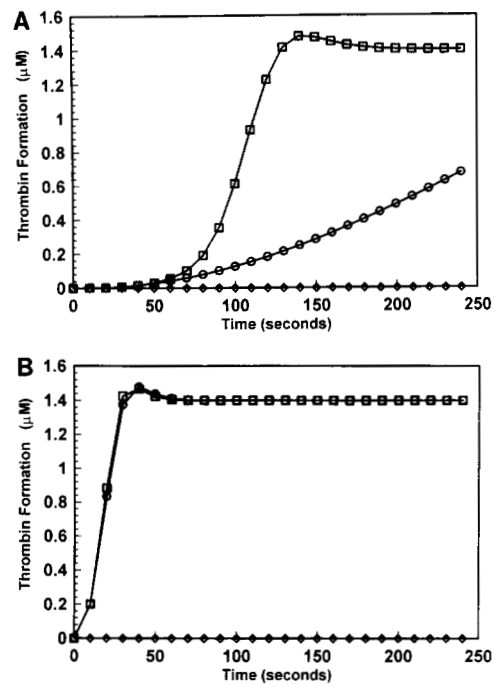

FIG. 5. *A*, the effect of pro-cofactors on thrombin formation. The percent thrombin formed is plotted as a function of time. The model (□) is simulated as described under "Methods" using the rate constants in Table I and protein concentrations as described in Table I of Ref. 1. The relative specific activity of αIIa = 1 and mIIa = 1.2. The simulation of IIa formation in the absence of VIII (○) was modeled using the same rate constants but starting with the concentration of factor VIII = 0. The simulation of IIa formation in the absence of factor V (◇) was modeled using the same rate constants but starting with the concentration of factor V = 0. *B*, the effect of pro-cofactors on thrombin formation. The percent thrombin formed is plotted as a function of time. The model (□) is simulated as described under "Methods" using the rate constants in Table I and plasma protein concentrations as described by Lawson *et al.* (1) in Table I except that the concentration of TF·VIIa = 1 nM. The relative specific activity of αIIa = 1 and mIIa = 1.2. The simulation of IIa formation in the absence of VIII (○) was modeled using the same rate constants but starting with the concentration of factor VIII = 0. The simulation of IIa formation in the absence of factor V (◇) was modeled using the same rate constants but starting with the concentration of factor V = 0.

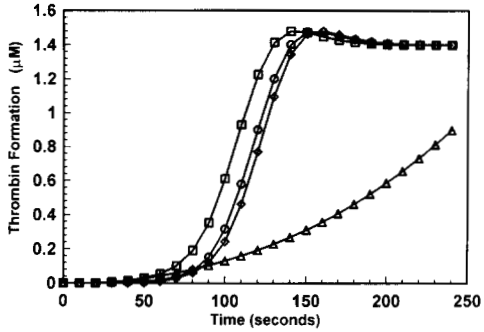

FIG. 6. **The contributions of factor IIa and factor Xa to factor V and factor VIII activation.** The percent thrombin formed is plotted as a function of time. The model (□) is simulated as described under "Methods" using the rate constants in Table I and plasma protein concentrations as described in Table I of Ref. 1. The relative specific activity of αIIa = 1 and mIIa = 1.2. The decreasing rate of thrombin formation is modeled by changing one of the rate constants. k_2 = 0 (◇), thrombin activation of factor V = 0; k_1 = 0 (○), factor Xa of factor V activation = 0; k_4 = 0 (△), thrombin activation of factor VIII = 0.

The observations of both the empirical data (1) and the mathematical model are consistent with the observations of Biggs and Nossel (26), who reported that the sensitivity of the prothrombin time to factor VIII or factor IX deficiency could be enhanced by dilution of the thromboplastin reagent used to initiate the clotting reaction.

Nature of the Activator of Factor V—Both thrombin and factor Xa are known to activate factor V to factor Va. The mathematical model can use the simulation approach to explore the relative contribution of each of these enzymes to the activation of factor V. In the mathematical simulation, the relative rates of factor V activation by factor Xa and by thrombin can be individually manipulated to exclude either enzyme from participating in this reaction process. This selection experiment can *only* be conducted by the computer since in the real world this selective reaction manipulation would be impossible. Fig. 6 shows the results of such an experimental simulation. The simulated reaction profile predicts that the elimination of either thrombin or factor Xa as a catalyst for the activation of factor V to factor Va would result in a substantial prolongation of the initiation phase of the reaction. The empirical data are thus best modeled by a synthesis in which both factor Xa and thrombin participate as activators of factor V during the initiation phase of the reaction.

A simulation was also performed in which the selective deletions of thrombin or factor Xa as the activator of factor VIII were explored. The result of this computer experiment (Fig. 6) suggest that thrombin plays the greater role in the activation of factor VIIIa. When factor VIII is not activated to factor VIIIa by thrombin, the reaction proceeds in a manner appropriate to those simulations in which factor VIII is deleted altogether from the initial reaction conditions.

The Relevant Pathway to the Activation of Factor Xa—One advantage of this computer model is the ability to determine the pathway by which the most significant proportion of factor X is activated. By assigning a rate of 0 to the factor X activating activity of factor VIIIa-factor IXa, that pathway can be eliminated in the model. The results of simulations in which the activation of factor X by factor VIIIa-tissue factor alone or in conjunction with factor VIIIa-factor IXa indicate that the initial activation of factor Xa is not affected by deleting the factor VIIIa-factor IXa pathway, but after 40 s the importance of this catalyst's activity to generating factor Xa at low tissue factor-factor VIIa concentrations becomes clear.

CONCLUSIONS

A difficulty inherent in many kinetic studies is the reliance upon steady state kinetics for the determination of individual rate constants. In the process of the activation of prothrombin to α-thrombin under pseudophysiological conditions of procoagulants, the concentrations of each zymogen, enzyme, procofactor, cofactor, and substrate vary widely and change significantly. For this reason, it is not obvious how the full ensemble of reactions will progress, nor is it obvious how the various forward and feedback reactions quantitatively influence the generation of the intermediate products. The ability to model the entire procoagulation reaction using specific individual rate constants allows a quantitative approximation of the proteolytic and catalytic events that lead to α-thrombin formation. The analytical model also provides the ability of the investigator to ascertain the relative significance of the quantitative levels of any species at any point in the reaction time course.

The results of this paper do *not* suggest new knowledge about previously unknown reactions or kinetic constants. Rather, the prediction data comply reasonably in form and magnitude to the empirical progress curves for the reaction. This result is a testimonial to both the mathematical model and the quality and the appropriateness of the empirical kinetic data published for each separate reaction. One must also specify caution since there may be other sets of rate constants which will match the experimental results by combining changes in any of the following: (*a*) factor VIIIa-factor IXa on-rates; (*b*) factor VIII activation by factor Xa or IIa; (*c*) factor Va-factor Xa on-rates; (*d*) factor V activation by factor Xa or IIa; (*e*) factor VIIIa degradation; (*f*) Michaelis-Menten complex on-rates.

The rate constants used in the model for this paper do show that no additional kinetic events must be invoked to explain the empirical data for α-thrombin generation. The model also provides insights into the kinetic events that precede and initiate the rapid formation of thrombin. The ability of the model to reflect empirical results also suggests that product inhibition may not be a necessary consideration to fully explain the progress of events in α-thrombin formation.

At low tissue factor-factor VIIa concentration, during the first 20–40 s of the reaction, an almost steady state rate of activation of factors IX and X occurs. Factor Xa begins to act on factors V, VIII, IX, and II. Two of the most kinetically significant reactions are the activations of the profactors factor V and factor VIII to the active species factor Va and factor VIIIa. The results of the model suggest that both factor Xa and thrombin play essential roles in the activation of factor V to factor Va, a result consistent with the empirical data of Lawson *et al.* (1). After activation, factor Va complexes with factor Xa to form *prothrombinase*, resulting in the initial generation of both meizothrombin and α-thrombin. While both thrombin and factor Xa are effective activators of factor VIII resulting in factor VIIIa, the model suggests that in the system utilized here, thrombin appears principally responsible for factor VIII activation.

At the point in the reaction when the activation of factor V and factor VIII are essentially complete, thrombin formation accelerates dramatically. However, at this point, which signals the propagation phase of thrombin generation, the concentrations of enzyme factor Xa and factor IXa do not reflect complete activation of their proenzymes. This observation raises the question of apparent reaction inefficiencies in having the "extra" proenzyme. Most likely, the concentrations of factor X and factor IX specified by the natural physiology of coagulation are selected not in terms of the ultimate enzyme concentrations required, but rather relative to their utilization as substrates. In this consideration, the satisfaction of the reaction K_m for

tissue factor-factor VIIa and for factor VIIIa-factor IXa complex becomes the determining basis of the physiologically necessary concentrations of factor IX and factor X.

The results of the empirical data of Lawson *et al.* (1), which have been incorporated into the model, indicate that something is causing the factor VIIIa-factor IXa complex-related activity in the expression of factor Xa to be diminished after the initial, essential formation of some factor VIIIa-factor IXa complex. One plausible contributor to this loss of activity is the dissociation of the subunits of factor VIIIa (13) or the proteolytic inactivation of factor VIIIa by factor IXa (14). Another possible explanation is proteolytic inactivation of either factor VIIIa or factor IXa under the conditions in which a vast excess of thrombin concentration occurs. For the purposes of the model, a mathematical relationship was introduced which results in a decay of the factor VIIIa-factor IXa complex, but only after the 3 pM complex is formed and a steady state level of 3 pM is maintained. The actual level of active complex can be approximated from empirical results of the rate of formation of factor Xa. In the experimental results reported by Lawson *et al.* (1), the results suggest that there is never more than a 5 pM active factor VIIIa-factor IXa complex. However, from the standpoint of either the model system or the empirical data, the high levels of factor Xa, which would occur if a stable factor VIII-factor IXa complex was present, would not substantially influence the ultimate maximum rate of thrombin generation seen during the propagation phase of the reaction. This lack of contribution would occur because the increase in factor Xa levels, which would occur with a stable factor VIIIa-factor IXa complex, would be formed subsequent to the maximum rate of thrombin generation; *i.e.* the propagation phase.

Reviewing the model results suggests the following sequence of events in the formation of thrombin. 1) The tissue factor-factor VIIa complex (5 pM) causes the activation of factor IX and factor X at the rates of 0.5 pM s^{-1} and 1 pM s^{-1}, respectively. 2) The factor Xa formed is then able to initiate the conversion of factor V to factor Va. 3) The factor Va formed then complexes with factor Xa to form the *prothrombinase* complex which begins the conversion of prothrombin to thrombin (via the meizothrombin intermediate). 4) The resulting thrombin formed accelerates the rate of factor V activation and together with factor Xa results in the conversion of factor VIII to factor VIIIa. 5) With factor VIIIa present, the enzymatic activity of factor IXa is enhanced resulting in a greater rate of formation of factor Xa. It is at this point that the initiation phase of the reaction is complete. 6) From the experimental results, at 30 s, the concentration of factor IXa is still less than 20 pM; the concentration of factor Xa is also less than 100 pM, but beginning to increase because of the additional conversion of factor X by the small but significant concentration of the factor VIIIa-factor IXa complex. The concentration of thrombin is also low at this time, but increasing because of the newly forming *prothrombinase* complex. By 60 s, the concentration of factors Va and VIIIa have reached levels easily saturating their respective apoenzymes (factor Xa and factor IXa, respectively), ultimately leading to maximal rates of factor Xa and thrombin formation.

An analysis of the simulation model shows that the observations of activation of individual factors and cofactors are consistent with earlier work on the protein activation pathway. At the start of the propagation phase of thrombin formation, the amount of *prothrombinase* necessary to generate the thrombin (Fig. 1) is equal to the concentration of factor Xa formed. Factor Va, which is probably formed by both factor Xa and thrombin, appears at a rate which saturates factor Xa at the end of the initiation phase.

The most important conclusion from these experiments is that there is no need for any enhanced rate of activation of any of the components of the extrinsic pathway. The formation rate of factor IXa observed empirically is sufficient to generate a significant concentration of the factor VIIIa-factor IXa complex, and the resulting formation of factor Xa allows for enough *prothrombinase* to rapidly convert prothrombin to thrombin. We anticipate that the computer models which are identified in this paper will have utility in exploring the influence of alterations in the concentrations of any species in the coagulation reaction and also how alterations in their concentrations, as a consequence of the presence of either natural or synthetic inhibitors of the coagulation reaction system, will influence outcome. The efficiency of the computer explorations in terms of time spent, materials required, and personnel cost makes it a valuable tool for the exploration of research concepts, the identification of appropriate experimental conditions for empirical exploration, and for development of model-generated hypotheses.

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