The Dynamics of Thrombin Formation

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Abstract—The central event of the hemostatic process is the generation of thrombin through the tissue factor pathway. This is a highly regulated, dynamic process in which thrombin itself plays many roles, positively and negatively its production and destruction. The hemostatic process is essential to normal physiology and is also the Achilles heel of our aging population. The inappropriate generation of thrombin may lead to vascular occlusion with the consequence of myocardial infarction, stroke, pulmonary embolism, or venous thrombosis. In this review, we summarize our present views regarding the tissue factor pathway by which thrombin is generated and the roles played by extrinsic and intrinsic factor Xa generating complexes in hemostasis and the roles of the stoichiometric and dynamic inhibitors that regulate thrombin generation. (Arterioscler Thromb Vasc Biol. 2003;23:17-25.)

Key Words: coagulation ■ fibrinogen ■ aggregation ■ coagulation inhibitors

The inventory of molecular components and the presumed physiology of the hemostatic process and its regulation have been established on the basis of plasma abundance, hemorrhagic or thrombotic pathology, in vitro tests, and chemical signatures. Two in vitro plasma tests, the prothrombin time and the activated partial thromboplastin time, were prominent in the development of the inventory. The former test relies on the addition of an extrinsic tissue factor source (thromboplastin), whereas the latter is based on the introduction of a foreign surface to initiate coagulation using only this surface contact and the biological constituents intrinsic to plasma. Both tests rely on a fibrin formation (clotting) end point. These assays permitted identification of connectivity between the component activities identified as required for plasma coagulation and defined the concept of intrinsic and extrinsic coagulation pathways, which converge at the step of formation of the prothrombinase complex. However, the mechanisms established by in vitro tests are not always mirrored in human pathology associated with bleeding or thrombosis. The primary pathway leading to hemostatic and thrombotic pathologies is associated with the tissue factor–initiated extrinsic coagulation pathway, whereas components unique to the intrinsic or contact pathway (factor XI, factor XII, prekallikrein, HMW kininogen) may have accessory roles in the process. Therefore, in this review, we focus on the dynamics of the reactions associated with the introduction of tissue factor to blood, leading to the formation of thrombin.

An evaluation of the reactions involved in the formation of thrombin leads to the conclusion that the physiologically relevant hemostatic mechanism is composed of 3 procoagulant vitamin K–dependent enzyme complexes (which use the proteases factor IXα, factor Xa, and factor VIIα) and one anticoagulant vitamin K–dependent complex.1 Each complex involves a vitamin K–dependent serine protease and a cofactor protein with the protein-protein complex assembled on a membrane surface provided by activated or damaged cells. The same hemostatic process required for preventing leaks from the vasculature may also be life threatening when responsible for an intravascular occlusion. Thus, nature has elected a system for highly regulated, multiconstituent activity presentation that provides a process that will lead to the local arrest of hemorrhage. The plasma proteins involved in the process require activation to participate in the thrombin-generating process. In addition, platelet adhesion and activation are required to provide membrane binding sites explicitly at the region of vascular damage, because platelet adhesion and activation provides the discrete membrane sites on which all of the plasma-derived procoagulant complexes are assembled.2–7 Equally important are the stoichiometric and dynamic inhibitory systems, which block the presentation of thrombin. The sum of inhibitory functions is far in excess of the potential procoagulant response. These inhibitory processes act in synergy, providing minimal activation thresholds, which must be achieved before significant thrombin generation.8–10

The significance of the components involved in the procoagulant response and its regulation in a genetically homogeneous population is reflected in studies of function-deleted transgenic mice. In mice, elimination of tissue factor, factor VII, tissue factor pathway inhibitor (TFPI), factor X, factor V, prothrombin, and protein C is lethal, whereas deficiencies of factor VIII and factor IX contribute significantly to hemorrhagic risk.11–19 It is instructive to note that in the outbred human population, individuals with equally severe prothrombin, factor X, factor V, and factor VII deficiencies do exist,
and indeed most of these factors were discovered because of the presentation of a living propositus displaying hemorrhagic pathology.20–25 Thus, genetic and environmental events can significantly alter a potentially lethal outcome. It is also interesting to note that congenital fibrinogen deficiency both in mice and man is not lethal and frequently only mildly symptomatic. Therefore, fibrin formation does not seem to be essential for survival.26

Overview

The key initiating event in the generation of thrombin depends on the interaction of membrane-bound tissue factor and factor VIIa, the latter of which is preexistent in the plasma milieu at ~1% to 2% of the total factor VII concentration (10 nmol/L).27,28 The source and presentation of active tissue factor is controversial.29–33 However, its damage-related presentation is essential. The factor VII zymogen is cleaved at arginine 152 by a variety of proteases, including thrombin, factor IXa, factor Xa, and factor VIIa–tissue factor to produce the serine protease factor VIIa.34 However, although this “enzyme” seems to possess all of the appropriate catalytic machinery to display the active site of an effective serine protease, it does not express proteolytic activity unless it is bound to tissue factor. Thus, naked factor VIIa at natural biological concentrations has no significant activity toward either factor IX or factor X before its binding to tissue factor.35 The defective active site also makes factor VIIa impervious to the high concentration of antithrombin-III (AT-III) present in blood, which permits its continued existence.36 The factor VIIa–tissue factor protein–protein interaction increases the $k_{cat}$ of the enzyme for synthetic substrates by two orders of magnitude37,38 and increases the rate of factor X activation by four orders of magnitude.39,39 This latter increase is the result of the aforementioned improvement in catalytic efficiency and the membrane binding of the macromolecular substrates factor IX and factor X. Substrate–membrane binding leads to an effective reduction in $k_{m}$. Overall, the $k_{cat}$ increase and $k_{m}$ lowering leads to a $10^{4}$ increase in the expression rate of the enzyme toward its natural substrates.

The factor VIIa–tissue factor (extrinsic) complex (Figure 1) catalyzes the activation of both factor IX and factor X, the latter initially being the more efficient substrate. Thus, the initial product formed by the extrinsic factor Xase is factor IXa, which is completed with the second bond cleavage (arginine 145) to produce the intermediate factor IXa. Thrombin interacts with factor IXa on the activated platelet membrane surface, and this “prothrombinase” catalyst (Figure 1) converts prothrombin to thrombin. Prothrombinase is 300 000-fold more active than factor Xa in catalyzing prothrombin activation.

The coagulation system is under extraordinarily tight regulation by both stoichiometric and dynamic inhibition systems. The tissue factor concentration threshold for reaction initiation is steep, and the ultimate amount of thrombin produced is largely regulated by the stoichiometric inhibitors tissue factor pathway inhibitor (TFPI) and antithrombin III (AT-III) and by the dynamic anticoagulant process of protein C activation and functional expression (Figure 2).9,10,45–51

The principal influence of TFPI is to block the tissue factor-factor VIIa-factor Xa product complex, thus effectively neutralizing the extrinsic factor Xase and eliminating this catalyst’s generation of both factor Xa and factor IXa.46 TFPI is present in a low abundance (~2.5 nmol/L) in blood; it is also releasable from the vasculature by the action of heparin.52

The stoichiometric inhibitor AT-III is normally present in plasma at more than twice the concentration (3.4 µmol/L) of any potential target coagulation enzyme generated by the tissue factor pathway. AT-III is an effective neutralizer of all of the procoagulant serine proteases.45 The targets of AT-III are primarily the mature enzyme products of these reactions. AT-III is a weaker inhibitor of the factor VIIa–tissue factor complex; thus, the principal influence of this inhibitor is in quenching thrombin production and the thrombin produced.

![Figure 1](image-url)
Our laboratory has made use of 4 models in attempts to recapitulate the dynamic mechanism of the coagulation reaction system. These have included the following: (1) synthetic reaction mixtures prepared from purified procoagulants and anticoagulants and a natural or synthetic membrane plasma, in which whole blood exuding from a microvascular wound is sequentially sampled for relevant product formation.72–75 Each of the model systems used has specific benefits and limitations. The in vivo model, involving a “Simplate” wound, is the least flexible, involves the highest degree of discomfort for the volunteer, and is analytically difficult; however it is the most biologically relevant. The numerical model is the least expensive and the most rapid and convenient method of analysis. It provides insight into the regulation of reaction mechanisms occurring at concentrations of intermediates and products, which may be inferred but not measured with existing technology. It is (obviously) the least biologically relevant.

Models of the Blood Coagulation Reaction

Regardless of the model system chosen, the display of thrombin generation after tissue factor initiation of the hemostatic reaction is approximately the same.41,42,56,71,73 This behavior is illustrated in Figure 3, which shows the generation of the thrombin-AT-III complex (TAT) as a function of time using the paravivo model. From an operational perspective, thrombin generation may be described as occurring in two phases. Shortly after the addition of tissue factor, tiny amounts of thrombin (nanomolar) are produced in an interval, which we define as the initiation phase of the reaction. Subsequently, the major bolus (>96%) of thrombin is produced during the propagation phase of the reaction. During the initiation phase of the reaction, the factor VIIa-TF complex forms and generates small amounts (subpicomolar) of factor Xa and factor IXa.40,41,53 Factor Xa in collaboration with the membrane surface activates a small amount of prothrombin to thrombin, which serves to generate the platelet membrane and cofactor components required for the major generation of thrombin.

The early events associated with thrombin function during the initiation phase of the reaction are illustrated in Figure 4, which shows the inception points for the detection of thrombin products generated during the reaction measured in
paravivo experiments. Many of these products are required to provide the catalysts (Figure 1) that generate most of the thrombin produced during the propagation phase of the reaction.

Under normal circumstances, the rate-limiting component of most prothrombinase formation and the generation of thrombin activity is the concentration of factor Xa. Thus, under normal conditions, the activation of factor V and the activation of platelets (probably through thrombin-PAR-1 receptor interactions) occur rapidly to produce surplus factor Va and platelet membrane binding sites. However, under conditions of congenital deficiency, thrombocytopenia, platelet pathology, or pharmacologic intervention, the tissue factor-initiated reaction can become sensitive to factor V or platelets. In studies to assess the influence of preactivation of platelets, we used the thrombin receptor activation peptide to provide for the preexpression of complex binding sites on the platelet surface. Preactivation of the platelets did not change the rate of thrombin generation. However, pharmacologic interventions with agents such as PGE1, ReoPro (Abciximab), and Integrilin do influence the generation of thrombin in the reaction. Similarly, reductions in platelet counts (<10,000/mm³) elicit dependence on platelet concentration.

The end point used in evaluating hemostasis in most bioassays is the generation of a fibrin clot. As illustrated in Figure 4, the formation of a fibrin clot occurs at 10 to 30 nmol/L thrombin or ~3% of the total amount of thrombin produced during the reaction, which is provided by only ~7 pmol/L prothrombinase. Thus, most thrombin formation is ignored using present technology for evaluating clinical hemorrhagic risk or thrombosis.

Figure 4. The generation of thrombin activation products during the initiation phase of the reactions shown in Figure 3. Platelet activation (osteonectin release) is detected at <1 nmol/L thrombin (0.06% of the total thrombin ultimately produced). The clotting time, 4.7 minutes, is observed immediately after the inception of the propagation phase of thrombin generation. The dotted line (■) corresponds with the active thrombin present in the reaction mixture, as determined from fibrinopeptide release.


Reactant Evolution

Figures 5A and 5B represent the numerical model’s evaluation of product formation over the entire time course of the reaction illustrated in Figure 3. Panel 5A represents the first 100 seconds of the reaction and corresponds primarily to the initiation phase of the reaction. Note that the vertical axis is exponential. The initial burst of factor Xa (~10 fmol/L) (●), primarily generated by factor VIIa-TF, produces a small amount of thrombin (<10 fmol/L) (■), which activates some factor V and factor VIII to factor Va (●) and factor VIIIa (●) and leads to the initial formation of the intrinsic factor Xase (○) and prothrombinase (○) complexes. During this early phase of the reaction, thrombin generation is principally under the control of factor VIIa-tissue factor and factor Xa-membrane and the reaction is principally regulated by TFPI.

Figure 5B represents the entire reaction (20 minutes). Note that the formation of the prothrombinase complex (○) is identical to and ultimately limited by the concentration of factor Xa (●), because factor Va (●) is in excess. Also apparent is that the existence of factor VIIIa-factor IXa (the intrinsic factor Xase) (○) is dependent not only on the...
This corresponds to a tissue factor concentration of 10 pmol/L; at this concentration, robust generation of factor Xa by factor VIIa-factor IXa complex in clot end point assays. The relative factor Xa generation by the factor VIIa-tissue factor complex is illustrated in Figure 6.71 Initially, the concentration of the factor VIIa-factor IXa complex ($\approx 10^{-11}$ mol/L) is higher than the concentration of the factor VIIIa-factor IXa complex, which requires activation and assembly. As time progresses, however, the contribution of the latter, more active complex in factor X generation exceeds that of the extrinsic factor Xase. As a consequence, most of factor Xa (Figure 6 inset) is ultimately produced by the factor VIIIa-factor IXa complex in the tissue factor–initiated hemostatic processes. Clotting would have occurred at 250 to 300 seconds at this tissue factor concentration.

In the absence of factor VIII or factor IX, the intrinsic factor Xase cannot be assembled; thus no propagation phase occurs. This is the principal defect observed in hemophilia A and hemophilia B62,67 in all models, including paravivo blood studies from those affected with these hemorrhagic abnormalities.

Because the presentation of a clot depends only on the generation of 10 to 30 nmol/L thrombin, at high tissue factor concentration, robust generation of factor Xa by factor VIIa-tissue factor can completely mask the contribution of the factor VIIIa-factor IXa complex in clot end point assays. This is the case for the prothrombin time in which the concentrations of thromboplastin (tissue factor and phospholipid) are chosen to produce a clot time of 11 to 15 seconds. This corresponds to a tissue factor concentration of $>20$ nmol/L. For the illustrations of Figures 3 through 6, a concentration of 5 pmol/L TF was used, producing a clotting time of $\approx 5$ minutes. In hemophilia A and B, at these TF concentrations, although the clotting time is prolonged, the major defect is associated with the absence of a propagation phase.62,67

### Significance of Intrinsic Factor Xase

The relative factor Xa generation by the factor VIIa-tissue factor and the factor IXa-factor VIIIa complexes is illustrated in Figure 6.71 Initially, the concentration of the factor VIIa-factor VIIIa complex ($\approx 10^{-11}$ mol/L) is higher than the concentration of the factor VIIIa-factor IXa complex, which requires activation and assembly. As time progresses, however, the contribution of the latter, more active complex in factor X generation exceeds that of the extrinsic factor Xase. As a consequence, most of factor Xa (Figure 6 inset) is ultimately produced by the factor VIIIa-factor IXa complex in the tissue factor–initiated hemostatic processes. Clotting would have occurred at 250 to 300 seconds at this tissue factor concentration.

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### Attenuation of Thrombin Generation

The attenuation of the coagulation system is as important as the procoagulant process and involves both stoichiometric and dynamic regulators. TFPI and AT-III are the principal stoichiometric inhibitors of the process. TFPI is the principal regulator of the initiation phase of thrombin generation, whereas AT-III serves to attenuate thrombin activity and its generation.9 These two agents when combined provide a synergistic regulatory effect by inducing kinetic thresholds such that the initiating tissue factor stimulus must be of significant magnitude to propel thrombin generation. Tissue factor concentrations below the threshold concentration required are ineffective in promoting massive thrombin generation because of the cooperative influence of the inhibitors; concentrations in excess of the threshold yield robust and almost equivalent thrombin generation.9,71 In a similar fashion, TFPI and the PC-Tm-thrombin APC system cooperate to provide a similar, threshold-limited, synergistic inhibition of thrombin production.10 The dynamic activated protein C system is responsive only after thrombin has been generated, because this system depends on activation of the zymogen protein C by the thrombin produced in the procoagulant response. Thus, its influence is mostly associated with quenching the propagation phase of thrombin generation,10 although the two phases are significantly overlapped.

The activations of the cofactors factor V and factor VIII are multistep processes, and at high thrombomodulin concentrations, the protein C system is an effective neutralizer of the reaction. Factor V activation involves cleavages at arginines 709, 1018, and 1545. The cleavage at arginine 709 occurs first and produces the heavy chain (residues 1 through 709) of the molecule. Factor V activity, however, requires the cleavage at arginine 1545 to produce the light chain (residues 1546 through 2329) of factor Va.76–79 APC inactivates factor Va (and intermediates in the activation process) principally by cleavage at arginines 506 and 306. Each of these cleavages is in the heavy chain.53 Thus, the heavy chain can be inactivated before generation of the light chain of factor Va, eliminating its procoagulant activity.56

### Accessory Processes

The zymogen factor XI is a symmetrical two-chain serine protease precursor present in plasma and platelets that has been variably associated with hemorrhagic pathology.83–84 The significance of factor XI as an important procoagulant is established by the bleeding pathology associated with its qualitative or quantitative absence. This zymogen is also a substrate for thrombin and has been invoked in a revised pathway of coagulation.85 Paravivo studies of the clotting of natural hemophilia C blood and synthetic plasma experiments, which mimic factor XI deficiency, illustrate the importance of the feedback activation of factor XI but only at the lowest TF concentrations.62,86 At moderate concentrations of tissue factor (5 to 10 pmol/L), which produce clotting times in the range of 3 to 5 minutes, factor XI has little or no effect on thrombin generation or other procoagulant param-
etters. However, at lower levels of tissue factor (1 to 2 pmol/L), which produce clotting in the range of 12 to 15 minutes, the generation of thrombin and formation of fibrin are prolonged in factor XI deficiency.62 The variability of observations of pathology with factor XI deficiency is most likely a reflection of the dimension of the stimulus associated with the vascular lesion in factor XI–deficient individuals.

The platelet also provides a source of factor XI and factor V, both of which are secreted during the platelet activation process.87–91 In studies of synthetic systems with deletion of plasma factor XI and factor V in which the platelet is the sole contributor of factor V and factor XI, in vitro data would suggest that the contributions of factor V or factor XI from platelets are sufficient and at least equivalent to the contribution of factor V or factor XI from plasma.64 Conversely, plasma factor V and plasma factor XI seem to make the platelet contribution superfluous. These in vitro studies, however, are not entirely born out by in vivo observations of individuals who lack platelet factor V and have a bleeding diathesis.92 Pathology does not seem to be associated with factor XII, high molecular weight kininogen, or prekallikrein deficiency states. These zymogens are cleaved to their active species by thrombin and thus may participate in coagulation but not as primary initiators of the hemostatic response.

**Fibrin and Fibrinolysis**

In purified systems, the kinetics of fibrinogen cleavage by thrombin result in the sequential removal of fibrinopeptide A (FPA) followed by fibrinopeptide B (FPB). The removal of FPA permits end-to-end polymerization, whereas removal of FPB has been thought a requirement for side-to-side polymerization,93,94 with the latter required for covalent cross-linking, ie, the formation of isopeptides between ε-aminolysyl and γ-glutamyl residues of adjacent chains.95 Elegant x-ray crystallographic studies of fibrinogen fragments provide details of these associations at the atomic level.96 Although the processes have been well established in purified systems, fibrin formation during blood clotting per se has only recently been evaluated using the paravivo system.

During the tissue factor–induced coagulation of whole blood modified only by suppression of the contact pathway, the activation of factor XIII and the removal of FPA by thrombin occur simultaneously.63 Cross-linked products are observed in the solution phase of the blood before the observation of a clot. At the point of clotting, <50% of the Aα chains of fibrinogen are cleaved and there is virtually complete removal of all fibrinogen/fibrin from solution. Thus, one must conclude that the initial clot is a heteropolymer, most likely composed of fibrinogen, fibrin 1, and partially cleaved fibrin 1. Subsequent to clotting, one begins to observe FPB in solution. However, this process never goes to completion, and only ~40% of FPB is ever removed and is detectable in the fibrin clot as the Bβ chain of fibrin 1. In the initial clot, the products associated with γ-γ dimerization and α-chain cross-linked polymers are observed. The FPB, which is released into solution, is additionally processed by a carboxypeptidase B-like enzyme, which removes the −COOH terminal arginyl residue.65 A prospective candidate of this reaction is the thrombin activatable fibrinolysis inhibitor (TAFI), which is activated by thrombin-Tm to the product TAFIa.97,98 TAFIa cleaves terminal lysyl and arginyl residues from the partial plasmin digestion products of fibrin. The result is indirect stabilization of the clot, because clot lysis is accelerated by the relatively high affinity of plasmin for substrates terminating in lysine, thus accelerating plasminogen activation.99

**Pharmacological and Genetic Alterations**

Observations from fundamental kinetic studies can be translated back to considerations of the pathophysiology of hemorrhagic and thrombotic disease. Hemorrhagic syndromes are characterized by severe, moderate, or milder on the basis of the relative functional level of a coagulation factor. Severe hemophilia A is associated with factor VIII concentrations <1%.80 Similar observations have been made for other coagulation factor deficiencies and provide paradigms for management of replacement therapy. These subjective assessments derived from clinical experience are consequences of the effective plasma protein concentrations required to generate effective hemostasis. In contrast to expectations, which may be derived from steady-state kinetics, in the pre–steady-state processes relevant to biology, only tiny amounts of the procoagulants upstream from prothrombinase are consumed during the thrombin-generating reaction, and contributions to rate processes rather than stoichiometric considerations predominate.51,55,61,71

The pathophysiologic influence of qualitative and quantitative alterations to the hemostatic system reactants has primarily been explored at the extremes of population distribution curves. These extremes are associated with congenital hemophilia and thrombophilias. In most cases, diagnostic methods are based on evaluations of single analytes with respect to their anticipated influence on the generation of hemorrhagic or thrombotic pathology. Synthetic plasma, numerical, and paravivo analyses show that quantitative differences in coagulation factor levels within the normal range, when combined, can produce significant variability in response to a tissue factor stimulus.59,68 This sort of behavior, as illustrated for prothrombin concentrations in synthetic plasma and numerical models, is presented in Figure 7.71 This figure displays the active thrombin concentration evaluated using the synthetic plasma and numerical systems as a function of time for a variety of prothrombin concentrations ranging from 50% to 150% of the population mean value. It can be seen that the active thrombin concentration present at any point in time as well as the cumulative amount of thrombin produced is significantly different for values of prothrombin concentration, which remain within the normal range. The stimulus-response algorithm that is presented for individuals with different prothrombin concentrations may be significant for predicting hemorrhagic or thrombotic risk, especially when such states are combined with other excursions from mean values. In the future, it may be anticipated that algorithms that combine individual coagulation factor and inhibitor levels will be useful in diagnosis and selection of prophylactic regimens.

The paravivo and in vivo systems have been used to evaluate pharmacologic interventions used to treat hemor-
rhagic and thrombotic diseases, to evaluate congenital abnormalities, and to inspect the influence of common polymorphisms. Studies have been conducted on individuals with hemophilia A, B, and C, factor VII deficiency, the Pf4^1^, Pf4^2^ platelet receptor polymorphism, and the factor XIII Val34-Leu polymorphism. Pharmacological interventions have included ingestion of aspirin, Simvastatin, and Coumadin anticoagulation. Pharmacological agents and abnormalities with an adverse effect associated with hemorrhage risk or a positive influence with respect to protection from thrombosis slightly prolong the initiation phase of the reaction but have their most dramatic influences on the propagation phase, when most thrombin is generated. As a consequence, clot end point–based assays for evaluation of hemorrhage risk or antithrombotic effect frequently do not display prolongations of the fibrin clotting time with these agents.

**Summary**

Technical advances in molecular genetics, protein chemistry, bioinformatics, and physical biochemistry provide us with an impressive array of tools and information with respect to normal and pathologic processes leading to hemorrhagic or thrombotic disease. The challenge for the 21st century will be to merge these mechanism-based, quantitative data sets with epidemiologic studies and clinical experience associated with the tendency to bleed or thrombose and with the therapeutic management of individuals with thrombotic or hemorrhagic disease. Our knowledge of the biology of coagulation is incomplete without considerations of genetics, biochemistry, pathology, hematology, and vascular biology. Because the composite of biochemical models needs to become biologically relevant and biological approaches need to become quantitatively enabled, we need to distill and integrate mechanistic data with the vast amount of subjective clinical experience regarding the management of individuals with thrombotic and hemorrhagic disease. Algorithms must be developed that can combine the art of clinical management with the quantitative science available in this field to define the outcome of the challenge or the efficacy of an intervention. Ultimately, we must tailor diagnosis and pharmacologic intervention to the individual.

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**References**


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**Figure 7.** The concentration of active thrombin as a function of time in the presence of prothrombin at 150% (diamonds), 125% (triangles), 100% (squares), 75% (circles), and 50% (asterisk) of its mean physiological concentration. Represented are the empirical experiments of Butenas et al. and an in silico representation of the same experiment. Reprinted with permission from Hockin MF, Jones KC, Everse SJ, Mann KG. A model for the stoichiometric regulation of blood coagulation. *J Biol Chem*. 2002;277:18322–18333.


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