*Journal of Thrombosis and Haemostasis*, **1**: 1504–1514

REVIEW ARTICLE

# What is all that thrombin for?

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**To cite this article:** Mann KG, Brummel K, Butenas S. What is all that thrombin for? *J Thromb Haemost* 2003; **1**: 1504–14.

**Summary.** The hemostatic process initiated by the exposure of tissue factor to blood is a threshold limited reaction which occurs in two distinct phases. During an initiationphase, small amounts of factor (F)Xa, FIXa and thrombin are generated. The latter activates the procofactors FV and FVIII to the activated cofactors which together with their companion serine proteases form the intrinsic FX activator (FVIIIa-FIXa) and prothrombinase (FVa-FXa) which generate the bulk of FXa and thrombin during a propagation phase. The clotting process (fibrin formation) occurs at the inception of the propagation phase when only 5-10 nM thrombin has been produced. Consequently, the vast majority (greater than 95%) of thrombin is produced after clotting during the propagation phase of thrombin generation. The blood of individuals with either hemophilia A or hemophilia B has no ability to generate the intrinsic FXase, and hence is unable to support the propagation phase of the reaction. Since clot based assays conclude before the propagation phase they are not sensitive to hemophilia A and B. The inception and magnitude of the propagation phase of thrombin generation is influenced by genetic polymorphisms associated with thrombotic and hemorrhagic disease, by the natural abundance of pro- and anticoagulants in healthy individuals and by pharmacologic interventions which influence thrombotic pathology. Therefore, it is our suspicion that the performance of the entire process of thrombin generation from initiation through propagation and termination phases of the reaction are relevant with respect to both hemorrhagic and thrombotic pathology.

**Keywords**: fibrin, hemostasis, thrombosis, thrombin, tissue factor.

## Introduction

Blood loss through lack of hemorrhage control has captured the attention of individuals from all walks of life and throughout history. The significance of hemorrhage is discussed in the earliest known literature [1]. In the mid-19th century the clotting of fibrinogen by thrombin was accurately described by Schmitt [2], and since that time hemostasis, defined by *Stedman's Dictionary* [3] as the control of hemorrhage, has been synonymous with blood clotting.

The pursuit of knowledge of natural events can be divided into the categories of inventory, connectivity and dynamics. The development of plasma clot-based assays *in vitro*, in which the observation of fibrin strands is the endpoint has played a central role both in describing the inventory and in establishing the logical connectivity between the reactants and reactions relevant to the hemostatic process. Morowitz [4] formalized the relationship between the introduction of tissue components into plasma and the formation of a clot. This reaction was exploited quantitatively by Quick [5] in the development of the prothrombin time assay. In its current state, this series of reactions (represented in Fig. 1) includes additional components now known to be required to produce the fibrin endpoint effectively. Langdell and colleagues [6] subsequently explored the spontaneous clotting of recalcified citrate plasma and developed the partial thromboplastin time, thus expanding the inventory catalog and further defining the connectivity of the intrinsic pathway of coagulation [Fig. 1]. The connectivity of this expanded system was defined by the cascade/waterfall paradigms of MacFarlane [7], and Davie and Ratnoff [8]. Intersections connecting these pathways were extended by the work of Österud and Rapaport [9], and Galiani and Broze [10], who identified transactions that explicitly linked the two classical pathways leading to thrombin generation and a fibrin clot. The apparent absence of kinetic efficiency in the tissue factor (TF) activation of factor (F) VIIa was solved by Lawson *et al*. [11], who showed that FXa–membrane could cooperate in the FVIIa–TF activation of FIX.

The title selected by the organizers for this presentation is based upon activities championed by our laboratory and Hemker's, which have explored the dynamics of the total generation of thrombin and the significance of this process in maintaining hemorrhagic and antihemorrhagic qualities from the physiologic and pathologic perspectives [12,13]. *In vitro*, blood and plasma clot when only a tiny fraction of prothrombin is converted to thrombin, and a great deal of hemostatic and thrombotic physiology and pathology is not captured by the fibrin clotting endpoints used commonly to evaluate the hemostatic

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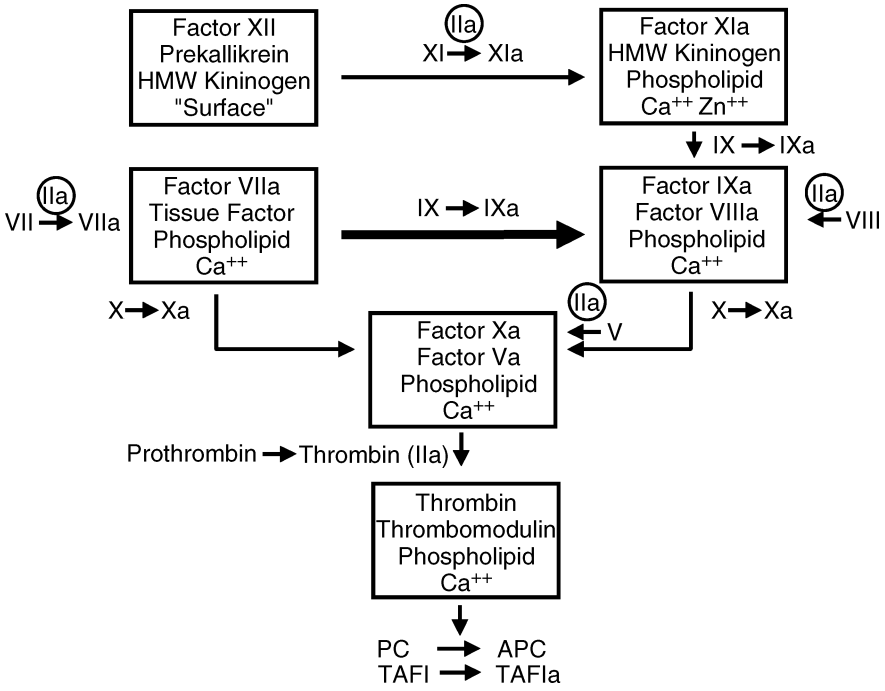


**Fig. 1.** Inventory and connectivity of the reaction complexes that lead to the generation of thrombin. Each complex catalyst is illustrated with its participating serine protease and cofactor protein. For the vitamin K-dependent catalysts, an anionic phospholipid-like membrane is required for assembly and expression of activity. Various thrombin substrates are illustrated as sidebars to the complexes.

process. In short, it appears that hemostasis is not synonymous with the endpoint of the fibrin-clotting reaction, and the latter is not a sufficient descriptor of the pathology associated with errors in the hemostatic process or the potential for development of a thrombotic occlusion.

We begin this review with a disclaimer with respect to the title of this manuscript: we do not know 'what all the thrombin is for', but we do believe that the hemostatic process cannot be adequately evaluated by a simple clotting endpoint. In this review, we provide: (i) a description of the dynamics of the TF pathway to thrombin from a mechanistic standpoint; (ii) a collection of our experiences dealing in thrombin generation in hemorrhagic and thrombotic situations; and (iii) some potential approaches to the anticipation of hemostatic and thrombotic responses by using an integrated approach to laboratory analysis.

## The central importance of thrombin

Thrombin is a multifaceted protein with functions extending from coagulation activator and inhibitor to cellular regulator. Its central importance in biology, physiology, and pathology is underscored by studies conducted in genetically homogenous transgenic mice made deficient in components of TF pathway that are essential to the thrombin generation process and its regulation: TF [14], FVII [15], TF pathway inhibitor (TFPI) [16], FX [17], FV [18], prothrombin [19], and protein C (PC) [20] $^{-/-}$ mouse constructs have been reported. In the mouse populations studied, deletion of these key components are lethal, indicating that when evaluated in a homogenous genetic background, thrombin formation/regulation is essential to life. FVIII$^{-/-}$ [21] and FIX $^{-/-}$ [22] mice incur hemorrhagic risk. In contrast, in the outbred human population, total deficiencies in coagulation components that are lethal in mice may yield consequences ranging from mild to extreme pathology. We hypothesize this is a consequence of human genetic heterogeneity that provides alternative pathways that can achieve survival in spite of potentially lethal abnormalities. In this hypothesis we assume that the majority of embryos congenitally deficient in the 'essential' components of the TF pathway do not survive.

Plasma clot-based assays have been the endpoints for the most commonly used tests of the hemostatic process. The prothrombin time (PT) and activated partial thromboplastin time (APTT) reflect, however, only the contributions of the diluted plasma component of the hemostatic system in adulterated form. Both tests terminate with endpoints that occur with less than 5% of the reaction complete. Fibrin clotting thus occurs when only minimal levels of prothrombin have been activated. It is also somewhat paradoxical that while we use the terms blood coagulation and hemostasis somewhat interchangeably, fibrinogen deficiency in mouse [23] is frequently only mildly symptomatic, in marked contrast to those deficiencies that negate thrombin formation. While clotting tests have been especially useful in identifying congenital abnormalities associated with hemophilia A, B and C, and in the evaluation of oral anticoagulant therapy, they overlook most thrombin formation, and their utility in evaluating many anticoagulant therapies [24] has been somewhat limited. In addition, prolongation of the endpoint in the APTT may reflect genetic deficiencies with little consequence for hemorrhagic pathology in the host.

## The tissue factor pathway

Most investigators believe that the generation of thrombin via the TF pathway is the biologically relevant process by which thrombin is elaborated and hemostasis is achieved. The reaction begins with the expression/exposure of TF, which is maintained in an inactive form either by compartmentalization or by some regulatory process that permits expression of this membrane-bound receptor protein [25–29]. The function of TF is expressed by binding pre-existent plasma FVIIa, which is present at approximately 1–2% of the total FVII zymogen concentration [30]. FVII competes with FVIIa for TF binding, thus serving as a negative regulator in the overall reaction [31]. When bound to TF, FVIIa function is efficiently expressed toward its macromolecular substrates, FIX and FX [9,32–34], with the latter being the more efficient and abundant substrate. The resultant initial FXa with a fraction of active membranes converts prothrombin to thrombin, albeit inefficiently. FXa also contributes to factor IX activation by cleaving the zymogen to the intermediate FIX [11]. The small amounts of thrombin produced by FXa–membrane initiates platelet activation [35], and activates minute amounts of plasma FV and FVIII to the cofactor forms FVa and FVIIIa [36]. These two cofactors ultimately form the receptor sites, both locating and activating FXa and FIXa, and forming the intrinsic FXase and prothrombinase complexes on the activated platelet surface [37]. Each membrane-bound, vitamin K-dependent enzyme complex [Fig. 1] is $10^4$–$10^6$ more active than the respective proteases towards their macromolecular substrates in solution [38].

In addition to the feedback activation by thrombin to activate FV and FVIII, other feedback steps accentuate the overall process of catalyst generation. FXa–membrane can also activate FV and FVIII to their respective cofactors [39], although the biological relevance of these processes is suspect considering the small amounts of FXa available and competitive substrates. Thrombin also activates FXI to FXIa [10], initiating an accessory pathway that enhances FIX activation [40].

The hemostatic reaction is under the control of both stoichiometric and dynamic inhibitory systems. TFPI is the principal inhibitor of the extrinsic FX activator [41,42]. TFPI is a high-affinity, low-abundance inhibitor present in plasma and secreted by vascular cells contributing to the local anticoagulant environment of the vascular wall. The major stoichiometric inhibitor of thrombin and its generation is antithrombin III [45]. FXa and IXa in complex display decreased reactivity with antithrombin III [43]. In contrast, the FVIIa–TF complex shows increased reactivity compared with FVIIa in plasma [44,45]. In plasma, FVIIa is almost impervious to inhibition by this ubiquitous serpin, and this lack of reactivity permits the existence of FVIIa in the hostile, inhibitor-rich blood environment. Antithrombin III is present at over twice the concentration (3.2 $\mu$mol $L^{-1}$) of the highest potential procoagulant enzyme concentration (thrombin) (1.4 $\mu$mol $L^{-1}$). This serpin effectively neutralizes all the serine proteases associated with the hemostatic process. Other serpins, including heparin cofactor II, may also contribute inhibitory capacity, suppressing the procoagulant proteases [46,47].

In addition to being an effective feedback procoagulant activator, thrombin is also an effective feedback anticoagulant. The enzyme binds to vascular cell-associated thrombomodulin [48]. The complexed thrombin effectively recognizes the vitamin K-dependent zymogen PC and ceases to be an activator for the procofactors and fibrinogen. The activated PC (APC) product interferes with the coagulation system by binding FVIIIa and FVa in competition with FXa and FIXa and proteolyzing the two cofactors, leading to their destruction [49,50]. The inactivated membrane-bound factor FVai and FVIIIai suppress APC function by continuing to effectively bind the enzyme, thus serving as product inhibitors.

The overall expression of thrombin function is a tightly regulated, highly intercalated system, the performance of which cannot be anticipated with the simple 'guesstimate' analyses that investigators typically apply to simple reactant systems. It is the composite of qualitative and quantitative features of the overall system that dictates the ultimate process, including the rates, extents of formation, and durabilities of catalysts and thrombin [51]. In addition, many products occur transiently during the course of the reaction, leading to increased complexity. Even alterations of plasma-protein concentrations in the 'normal range' observed in the human populations can have an extraordinary effect on the ultimate amount of procoagulant activity generated for a given level of TF stimulation of the system [52]. Even dilution of blood or plasma produces significant and often unexpected changes. 'Seat of the pants' speculation of how these reactions will be altered by changes in reactant concentrations, solvent conditions or temperature are inherently dangerous and have contributed to misinterpretations of the biological processes.

## Models of hemostasis

In attempting to examine how the TF pathway works in generating thrombin, our laboratory has evaluated four models to try to describe the dynamics of the thrombin generating system. These are: (i) synthetic plasma mixtures prepared using purified proteins and natural or synthetic membranes induced to react by the addition of lipid-reconstituted TF [52,53]; (ii) numerical models of the coagulation system based upon reaction-rate constants, concentrations and mechanisms [51,54,55]; (iii) *para vivo* studies involving whole blood induced to clot purely by a TF stimulus [11,56,57]; and (iv) *in vivo* studies in blood exuding from a microvascular wound [58–60].

Each model has its advantages and disadvantages. Models that involve human volunteers are constrained by both ethical and technical considerations. Synthetic and computer models are used to anticipate knowledge of the true biology of the process and aid in design of both *para vivo* and *in vivo* experiments. When the models converge with *in vivo* observations, the appropriateness of the approximations is assured.

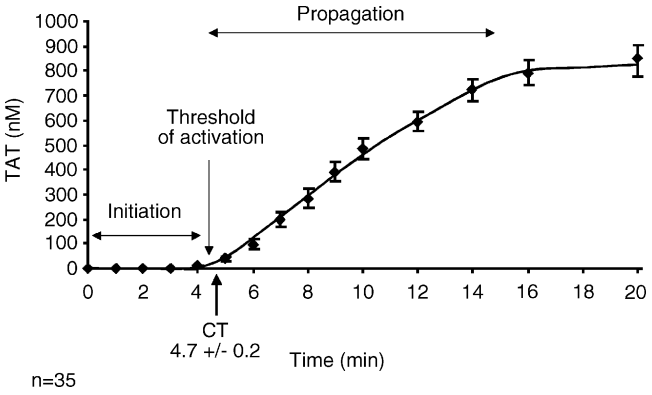


**Fig. 2.** Thrombin generation during *para vivo* whole-blood experiments. Thrombin–antithrombin III (TAT) complex formation is plotted as a function of time for sequential samples of human blood at 37 °C inhibited with respect to contact pathway activation and activated by the addition of 5 pmol $L^{-1}$ tissue factor, 10 pmol $L^{-1}$ phospholipid. The data (± SEM) are presented for 35 individual experiments with an average clot time of 4.7 ± 0.2 min. The operationally defined initiation and propagation phases correspond to the slow and fast kinetic expressions of thrombin formation. The propagation phase is invisible to clot-based assays. From Brummel *et al.* [58], by permission.

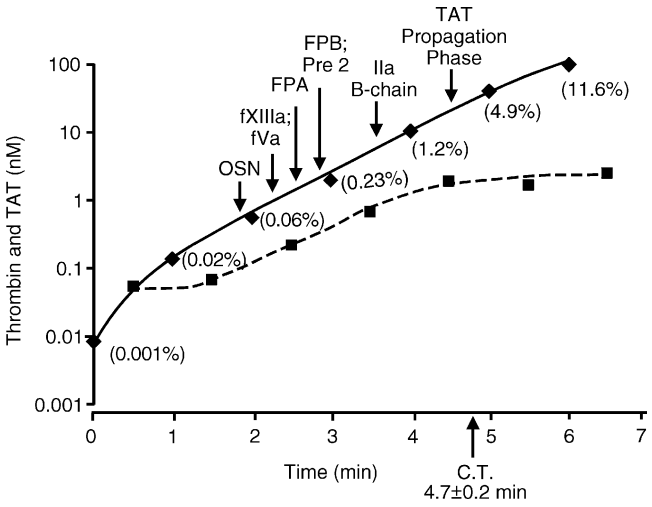


**Fig. 3.** The onset of detection of products from thrombin substrates during the initiation phase for the experiments presented in Fig. 2. Active thrombin and thrombin–antithrombin III (TAT) concentrations are plotted on the vertical axis on an exponential scale vs. time. The onset of product detection corresponding to platelet activation (OSN) and other well-established thrombin substrates are depicted. The inception of propagation phase corresponds to the point at which there is a transition from slow to rapid thrombin generation. From Brummel *et al.* [58], by permission.

## Thrombin generation

In all models, the TF-initiated display of thrombin generation is approximately the same. This behavior, illustrated in Fig. 2, may be operationally described as occurring in two phases. At first, tiny (nanomolar) amounts of thrombin are produced during an interval (the initiation phase). The major bolus (>96%) of thrombin is produced secondarily during a propagation phase. During the initiation phase, the FVIIa–TF complex forms, and generates sub-picomolar amounts of FXa and FIXa. FXa, 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. These autocatalytic processes lead to increased catalyst formation.

Signal events occurring during the initiation phase are illustrated in Fig. 3, which shows the inception points for the detection of thrombin products generated during the reaction measured in *para vivo* experiments [57]. These products provide the elements of the catalysts (Fig. 1) that generate the majority of the thrombin produced during the propagation phase of the reaction.

The cleavage of fibrinopeptide (FP) A and subsequent clot formation (Figs. 2 and 3) occur just prior to the propagation phase of the reaction. Under normal conditions, the activation of platelets and FV occurs rapidly to produce a surplus of FVa and platelet-membrane binding sites, leaving the rate-limiting reagent for prothrombinase formation as the concentration of FXa. However, with congenital deficiencies, thrombocytopenia, platelet pathology or pharmacologic interventions, the reaction can become sensitive to FV or platelets [61].

The endpoint utilized in evaluating hemostasis in most bioassays is the generation of a fibrin clot. As illustrated in Figs 2 and 3, in largely unadulterated whole blood at 37 °C, the formation of a visible fibrin clot occurs at 10–30 nmol $L^{-1}$ thrombin, or ~3–5% of the total amount of thrombin produced. This thrombin in turn is provided by only ~7 pmol $L^{-1}$ prothrombinase [12,57]. Thus, most catalyst and thrombin formation is undetected by current technology for evaluating clinical hemorrhagic risk or thrombosis.

Figure 4 illustrates the time course of removal of fibrinogen and fibrin products from the fluid phase of blood and the formation of products within the insoluble clot. This figure should be compared with the data of Figs 2 and 3 to register the formation of thrombin with the cleavage of fibrinogen and the formation of the fibrin clot. In Fig. 4A, at the point of visual clot formation (CT), virtually all fibrinogen (and some product already crosslinked) disappears from the fluid phase of the reaction [62]. At this point ~50% of the FPA has been cleaved, thus the 'clot' is a mixture composed of a mixture of fibrin 1 and fibrinogen. The insoluble material present in the fibrin clot (panel B) is virtually all cross-linked by FXIIIa, the activation of which is nearly simultaneous with FPA removal (Fig. 3). In purified systems it has been observed that FPB removal precedes the cross-linking reaction, however, as seen in the α-FPB immunoblot in Fig. 4C, the B peptide antigen epitope is still detectable associated with the Bβ chain.

## The significance of intrinsic factor Xase

During the transition between the initiation and propagation phases, increased concentrations of the FVIIIa–FIXa complex are generated, contributing an increasing concentration of FXa. The TFPI downregulation of FXa formation by the FVIIa–TF

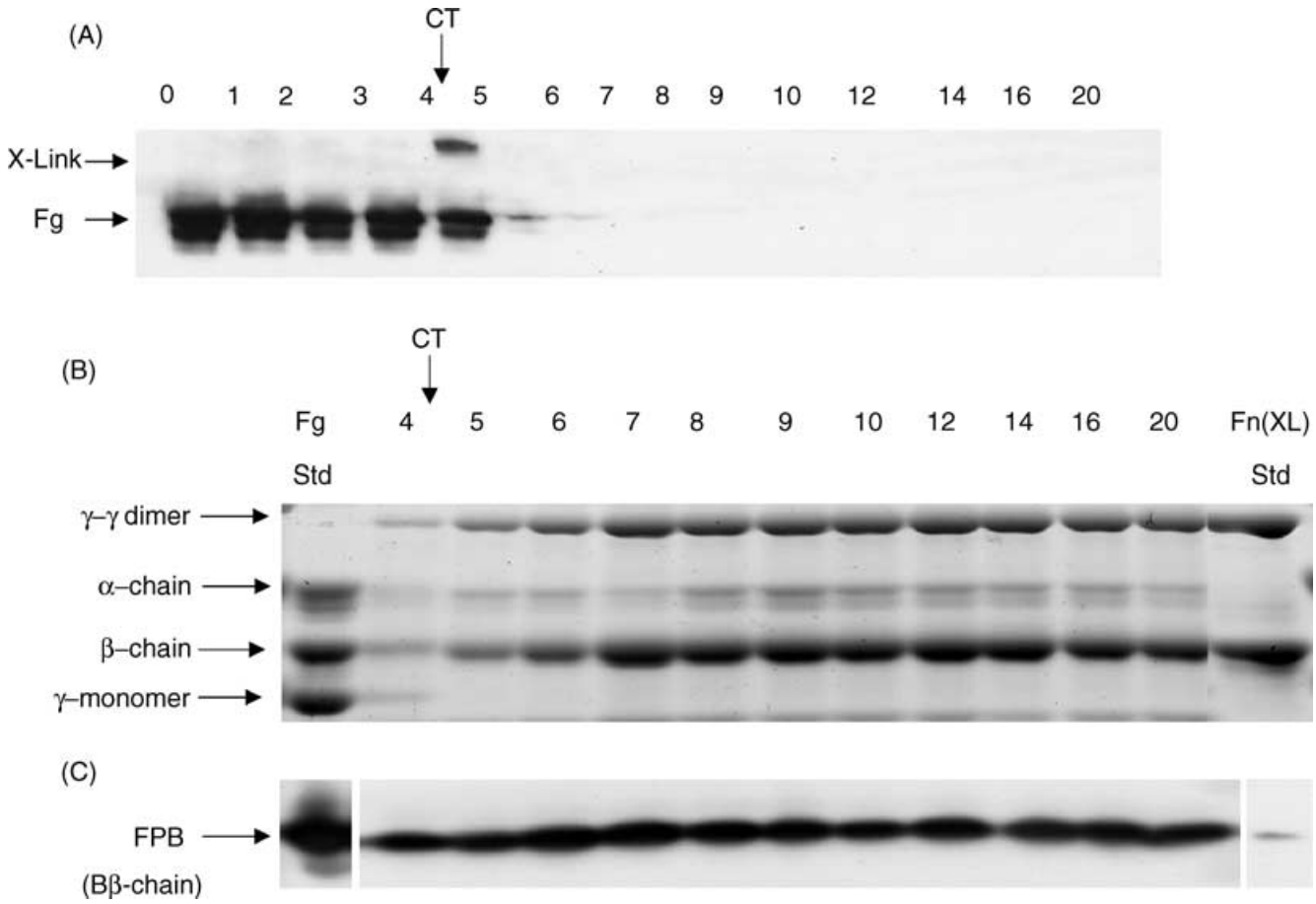


**Fig. 4.** Fibrinogen and fibrin products during the tissue factor-induced clotting of whole blood. Panel A reflects the immunoblot data for sequential aliquots taken from the solution phase of contact pathway inhibited whole blood induced to clot with 5 pmol $L^{-1}$ tissue factor–phospholipid. The label 'CT' corresponds to the clotting time observed visually for the experiment. Panel B illustrates a Coomassie Blue-stained gel of the reduced insoluble clotted material for the same experiment in Panel A. Panel C illustrates an immunoblot of the gel of panel B using an antibody (supplied by B. Kudryk) specific for the B peptide region of the Bβ chain. From Brummel *et al.* [63], by permission.

complex and the enhanced efficiency (~50-fold) of the FVIIIa–FIXa complex effectively switch the primary path of FXa production to the latter catalyst. Figure 5 illustrates a numerical analysis of the percentage formation of FXa by the two complexes during the progress of the reaction. Shortly before the propagation phase of thrombin generation is observed, the majority of FXa begins to be produced by the intrinsic FXase. Operationally, the onset of the propagation phase that signals enhanced thrombin generation is coincident with the intrinsic FXase being the principal generator of FXa.

From these observations one would conclude that in congenital hemophilia A and B a significant deficit in thrombin generation during the propagation phase would occur. This is in fact observed in all models, most significantly in *para vivo* studies of individuals with hemophilia A and B [Fig. 6]. The blood of these individuals displays a slight prolongation of the time to form a clot, but the major impairment is in thrombin generation during the propagation phase of the reaction. The deficit in hemophilia C, or FXI deficiency, is also observable as

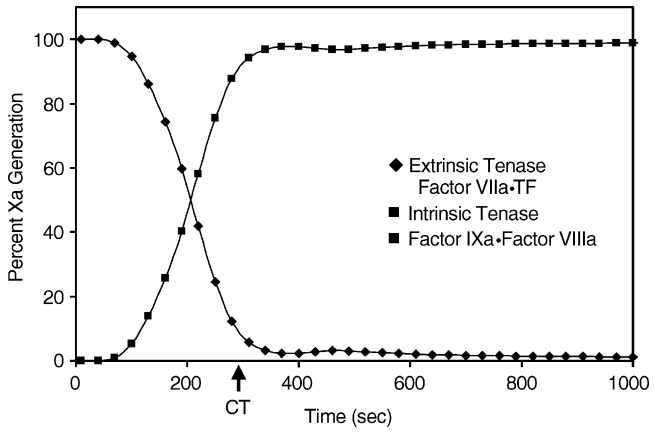


**Fig. 5.** A numerical estimation of the percentages of FXa produced by the intrinsic FXase and by the extrinsic FXase. Initially 100% of FXa is generated by FVIIa–tissue factor. However, as the reaction progresses, the major FXa production is contributed by the more efficient FIXa–FVIIIa catalyst. The arrow indicated the approximate clotting time. Modified from Hockin *et al.* [52], by permission.

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