Anniversary Issue Contribution

Models for reaction mechanisms in haemostasis – Contributions from the study of prothrombin activation

Craig M. Jackson
Hemosaga Diagnostics Corp, San Diego, California, USA

Summary
A brief history of the development of the contemporary model for the mechanism of prothrombin activation is presented. The focus is on the advances in understanding structure-function relationships in the molecules that comprise "prothrombinase" that occurred primarily during the 1970s. A link between the "classical theory" of hemostasis and the conceptual development of activation complexes as the activators of the precursors of coagulation proteases is developed. It is argued that advances occurred when new ideas arose that could be tested and new technologies enabled more definitive experiments to be performed.

Keywords
Prothrombinase, prothrombin, structure-function, coagulation models, models, history

Thromb Haemost 2007; 98: 24–35

Introduction

Studies of prothrombin activation occupy an important position in the quest to discover and understand reaction mechanisms in hemostasis. The classical theory of blood clotting propounded by Morawitz provided the framework into which postulated substances acting between thromboplastin (tissue factor) and fibrinogen could be placed (1). During the century following Morawitz new experiments were performed, interpretations were refined in the face of the new evidence, and in some cases abandoned. An assay procedure, the "prothrombin time" initially devised by Armand Quick (2, 3) and extended by Paul Owren (4–6) enabled further investigation of the classical theory[1]. The nagging question of the defects responsible for hemophilia and the new and distinct hemorrhagic diseases observed by Owren (4, 11), Duckert (7–9), Alexander (10, 12), Telfer (13) and Hougie (14) produced observations that challenged the classical theory. These observations provoked controversy only part of which was scientific[2].

Descriptions of the early history of prothrombin, thrombin and the search for and the nature of the agent(s) acting between the tissue factor and fibrinogen can be found in papers of Beck (15), Esnouf and Macfarlane (16,17), Milstone (1,18–21), Suttie and Jackson (22) Seegers (23–25), and from the perspective of a scientist/historian/philosopher by Marcum (26, 27).

A historical context – paradigms[3] and models[4]

Attempts to link injury, bleeding and cessation of bleeding to biochemical components requires conceptual models to facilitate discussion and promote laboratory investigation. Hemostasis is too complex to comprehend unless models are created to

1 These two assay procedures remain the methods for monitoring the "extrinsic pathway" of hemostasis, particularly, the effects of vitamin K antagonist drugs.

2 Controversy and disputes regarding priority of discovery or credit for it remain, perhaps even exacerbated today with the introduction of marketing and selling strategies into the "commerce of science".

3 Paradigm is used in the sense presented by Thomas Kuhn in his "The Structure of Scientific Revolutions" (30).

4 Citations in the text are primarily to review articles, but exceptions have been made to either call attention to commonly missed reports or reports significant in challenging or revising models that are simply no longer cited by many authors for reasons unknown. Monographs when published from meetings are also cited because they frequently indicate the state of the art at a particular time. As journals slowly make previously published papers available in online archives, access to these papers becomes simple and reading them a source of awe and respect for the insights of the earlier investigators.

Correspondence to:
Dr. Craig M. Jackson, PhD
Hemosaga Diagnostics Corp
5931 Seacrest View Rd
San Diego, CA 92121–4355, USA
Tel.: +1 858 638 0956
E-mail: cjackso2@san.rr.com

Received April 5, 2007
Accepted May 21, 2007

Prepublished online June 12, 2007
doi:10.1160/TH07–04–0253

reconcile biochemical and clinical observations[5]. In particular, patients with newly recognized bleeding disorders were key to forcing revisions to the classical theory as well as its successors. The inherent human desire for simple models that are useful for discussing, teaching and claiming scientific credit can foster resistance to new discoveries and challenges. Unfortunately, when models are too readily accepted they can impede progress because they impede thinking.

The focus of this essay is on biochemical investigations of prothrombin activation, primarily those during the period 1970–1980. Work from my laboratory will be summarized most extensively, but with grateful recognition that our work was built on foundations established previously by others and the model for prothrombin activation to which we contributed was derived from their work.

Setting the stage for subsequent discovery and revised models

Efforts to isolate and study the components postulated in the classical model for blood clotting occupied the attention of many groups during the period from the late 1940s until the 1970s[6]. These studies provided the knowledge base for the investigations of the 1970s that enabled a more detailed biochemical picture of prothrombin activation to be created. Several laboratories were pursuing the purification of prothrombin from bovine and human plasma, all with significant success, but limited by the technologies then available. Conventional practices involved Ba citrate[7] or $MgCO_3$ formed *in situ*; or adsorption to $Al(OH)_3$ or $BaSO_4$ when oxalated plasma was the starting material. Prothrombin was the predominant protein being sought, but as is now known the earliest preparations actually contained all vitamin K-dependent proteins. Although ion exchange resins were used, their resolving power was low, and efficient purification did not occur until ion exchange celluloses were introduced in 1958 (36).

Two competing hypotheses regarding prothrombin and the postulated "other components" involved in prothrombin activation complicated discussion and interpretation of experimental results. One was based on the view that prothrombin, the protease that converted it to thrombin and other clotting factors, could be derived from the prothrombin molecule itself by limited proteolysis (23–25). The other view was that preparations of "prothrombin" were actually mixtures of several vitamin K-dependent proteins and proteolysis artifacts (1, 18–21)[8]. New assay methods, better separation techniques and electrophoretic methods for assessing homogeneity ultimately resolved the conflicts. Renaming of the product the initial adsorption or precipitation step of prothrombin purification "prothrombin complex" also aided in more productive discussion among investigators.

Identification of the polypeptide products derived from prothrombin during its activation was the focus of many investigators during the 1950s and 1960s. Studies of the products of transformation of prothrombin into thrombin produced evidence that retrospectively was remarkably concordant. The activation process was shown to be accompanied by the formation of thrombin and two protein fragments, with very similar properties whether from bovine or human plasma. Work from the laboratories of Aronson (37–39), Lanchantin (40–43), Seegers (23–25, 44), Scheraga (45), Waugh (46–50) and Magnusson (51, 52) provided major insights into the origins, structures and properties of thrombin and the fragments of prothrombin. Molecular weights for prothrombin and thrombin, although not without confusion because of proteolytic degradation by thrombin also established prothrombin as approximately twice the mass of thrombin[9]. Amino acid sequencing by Magnusson (53) established the homology between thrombin and the pancreatic serine proteases; a relationship previously supported only by similarity in enzymatic activity (54)[10].

Studies by other investigators focused on the proteolytic enzyme most likely to directly cleave prothrombin and the functional roles played by phospholipids and factor V (accelerator

[5] The author's perspective is that chemical and biochemical investigations are performed to explain physiological function, more specifically a search for mechanisms that relate molecular structure to biological function. It has been his longstanding view, perhaps because of the influence the authors noted below had on him, that models for complex biological processes are hypotheses, even "straw men" to be challenged by experiments that test the predictions that the models provide. In that regard models are always tentative and require continued challenge as technology and concepts derived from other sciences make such challenges possible. Readings many years ago to which I attribute my perspective, correctly or erroneously, were: Medawar's "The Art of the Soluble", (28), Popper's "The Logic of Scientific Discovery" (29), Kuhn's "The Structure of Scientific Revolutions"(30), Clifford's "The Ethics of Belief " (31), Cervantes' "Commendatory Verses to The History of Don Quixote de la Mancha"(32), John Godfrey Saxe's poem, "The Blind Men and the Elephant " (33), based on a fable from India and most recently, Frankfort's "On Bullshit"(34). It has also been my opinion that the sharpest tool in science is "Occam's' Razor", the assertion that in choosing explanations (interpretations) simplest is best because it is the most easily attacked and falsifiable.

[6] Presentation is not chronological, but grouped into intervals of several years for convenience.

[7] Barium citrate became the most widely used precipitant. Although the process is often described as adsorption to Ba citrate, Ba citrate is soluble in water and thus the description as in-situ precipitation is probably more accurate, but to my knowledge has not been investigated.

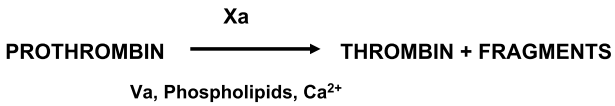
[8] The preparations of prothrombin subsequently called "prothrombin complex" were homogeneous by the criteria of that era. Unfortunately, homogeneity of proteins is a conclusion drawn from negative evidence, i.e. the absence of demonstrable contaminants. The author as a graduate student was fortunate to have stumbled upon a classical discussion of the problem of protein homogeneity; Pirie, NW. (35).

[9] This is specifically mentioned because the question; "what is the other half of prothrombin doing" was the underlying question for the studies carried out in my laboratory.

[10] Investigators focusing on human thrombin rather than bovine prothrombin used other methods very successfully. When Cohn fraction III was used as the starting material for producing human thrombin, Miller (55) and Fenton (56, 57), produced a preparation of human thrombin that served as the "gold standard" for the enzyme. For many years John Fenton generously supplied human thrombin to others who were investigating its functions. Clarification of the nature and origins of bovine thrombin proteolysis products were also being achieved (49, 50, 58).

globulin or proaccelerin[11]). Clear progress in verifying the direct participation of these components in prothrombin activation, in spite of different names given them, occurred when purification procedures devised in the laboratories of Aronson (37–39), Duckert (7, 9), Mann (58), Seegers (59), Esnouf (60–64), Hanahan (65–71), Milstone (18–21) and others (72, 73) provided materials that could be biochemically investigated. Although the purity of these materials was difficult to assess[12], primarily because of the very limited amounts that could be isolated and the insensitivity of most of the available analytical methods of this period, by the late 1960s the evidence that supported a general model for the components and their respective functions in prothrombin was quite concordant. Acceptance was not immediate; in fact the model for prothrombin activation that prevailed was generally that espoused by Milstone (18–21) and Seegers (23, 24) (without the complications associated with the molecular origins of factor X and Xa) that was proposed in the early 1960s and mid to late 1960s and strongly reinforced by work from the laboratories of Esnouf (60–64, 75, 76) and Hanahan (65–71, 74).

As already noted, during this same period, patients with bleeding disorders, as well as laboratory observations challenged the classical model for hemostasis with data that it could not explain and thus forced its expansion and revision. The "cascade" (77–79) with its proposed amplification properties[13] or "waterfall" (80–81) models replaced the classical model, but incorporated the elements of it that survived as the "extrinsic pathway". Relevant to prothrombin activation in the "cascade" model was the almost overwhelmingly appealing linear representation of this model which placed factors V and VIII in positions identified with proteases; a placement that by Occam's razor seemed entirely appropriate. Interpretation of the recognizably catalytic, but not necessarily proteolytic activity of factor V or Va was problematic[14]. Two completely different approaches to testing the assigned proteolytic function to factor V arose from the laboratories of Hanahan (74) and Esnouf (75) to resolve this issue. In the first laboratory, experiments showed that prothrombin could be converted to thrombin only when active factor Xa was present with factor V(Va). When DFP-inactivated Xa and factor V (Va) were employed, no activation occurred, even if the factor V had been previously incubated with active Xa[15]. In the second report, kinetic studies demonstrated that V(Va) was not directly activating prothrombin, conclusions entirely consistent with earlier work from both these laboratories as well as those of Milstone[16] and Seegers, i.e.



Studies carried out during the 1970s thus commenced with and built upon this model for prothrombin activation[17]. The model included five components: prothrombin (the substrate), factor Xa (the proteolytic enzyme), factor V(Va), a protein cofactor that increased the rate of thrombin formation (a non-protease catalyst), a phospholipid membrane surface and Ca^{2+}. The context for understanding proteolytic reactions that had been established guided the design and interpretation of experiments. Amino acid sequences and crystallographic structures for chymotrypsin and elastase and thrombin's homology with them (53) were known as were pathways for their production from their zymogen forms. Moreover, acrylamide gel electrophoresis, both without and with sodium dodecyl sulfate permitted quick and sensitive monitoring of the process of prothrombin cleavage. Although recognition of lipid participation in blood clotting preceded even the classical theory, its role was confusing in part because both proteins and lipids were included within the conceptual framework of colloids. Advances in chemistry and physical chemistry of phospholipids in aqueous dispersions, led by Bangham (82) who identified surface charge on phospholipid vesicles as a key variable in their ability to promote clotting had identified a key property of them. The extension of that work by Papahadjopoulos and Hanahan (65, 68, 69), Jobin and Esnouf (62, 64), Cole (72) and others enabled well characterized phospholipid bilayer vesicles to be used as surrogates for the cell membranes which provide such surfaces *in vivo*. Studies of platelet membrane lipids by Marcus (83) and Rouser (73) provided the link to the most physiologically relevant lipid source. Phospholipid vesicles were useful particularly for biochemical studies because they enabled investigations of individual properties that the complexity of cell membranes and the receptors on their surfaces preclude. They are not substitutes for biological, particularly platelet and cell, membranes with their associated receptors. However, their simplicity and ease of production in the laboratory made clear interpretation of experiments possible. Because of the work already

11 Even though a consensus on common designations for the clotting factors occurred in 1958, many groups continued to use the designations that the consensus was intended to eliminate. Multiple names are mentioned here because the adoption of new names, and the use of names that might be viewed as "proprietary", unfortunately can lead to much earlier contributions being overlooked or ignored.

12 Most techniques, including gel electrophoresis methods that depend on staining with the dyes used at the time would easily miss 1% contaminants, or in gels observed on slides, 5%.

13 A model for the linear cascade that is based on photomultiplier amplification, although requiring modifications in light of what we now know, still has substantial appeal for describing clotting, Esnouf and Macfarlane (16).

14 Factor V in its activated form was factor VI in the original systematic nomenclature for coagulation factors. The dependence on concentration of Xa and Va in prothrombin activation is in its essential features identical; both would show hyperbolic behavior with prothrombin concentration, but because of the subsequently demonstrated very large effect of Va on the rate of prothrombin activation, any contamination by Xa would be unobserved and would make misinterpretation of factor Va as an enzyme quite likely.

15 This letter to Nature (74), in which I was one of the authors took a year to get accepted and was only published after substantial prodding of the editor of that journal. Although interpretations of qualitative experiments frequently have more impact than quantitative ones, this paper appears to have had minimal impact being cited primarily only by Esnouf and its authors in later years.

16 Work by Milstone has been overlooked. Factor Xa in his papers was called thrombokinase, a name given in the classical theory by analogy with enterokinase, the protease that converts trypsinogen to trypsin.

17 A very useful summary of the "state of the field" in 1968 is the booklet provided at the 1968 Boerhaave Course held at the University of Leiden. Unfortunately this document may be difficult to acquire.

done addressing prothrombin activation, I, in my new academic position, selected it as a problem on which to work; and it seemed to meet the criteria of Medawar (28) for being a solvable problem. And, as under the influence of the essay of William Kingdon Clifford (31), I felt compelled to investigate.

Studies of prothrombin activation in a model system

Contributions to the understanding of prothrombin activation originating from my laboratory in the Department of Biological Chemistry at Washington University in St. Louis began by continuing my previous work on bovine factor X (84, 85). The large-scale purification and characterization of bovine factor X had been the subject of my own doctoral dissertation in the Department of Biochemistry at the University of Washington in Seattle in the mid 1960s under the direction of Donald Hanahan. A new, first year graduate student, Charles (Chuck) T. Esmon[18] joined the laboratory and became my first PhD student. Whyte G. Owen joined soon after as my first post-doctoral fellow. Chuck's PhD dissertation was directed to isolation of factor V, and Whyte took on prothrombin; I provided the factor Xa. Each of us had a protein to provide for the study of prothrombin activation. I, having been trained in a laboratory noted outside hemostasis for its contributions to the chemistry and metabolism of phospholipids, and a post-doctoral fellowship in England working on phospholipid monolayers provided me with the background to handle the fourth component needed for our planned studies, the phospholipids as well.

Our first intentions were to look at binding of the proteins to phospholipids, both vesicles and monolayers. Each individual would have his own protein and phospholipids, soon to be synthesized for us by an organic chemist hired once grant funds were available[19]. As frequently occurs in science, serendipity was responsible for the seminal experiment on phospholipid-protein interactions. Whyte Owen was looking at the enhancement of prothrombin conversion to thrombin by phospholipid vesicles and Ca^{2+} and as predicted from the work noted above, the rate of activation was increased by more than 100 times. But, on the next day, after storing the prothrombin in the refrigerator overnight, no acceleration was observed! Whyte ran a sodium dodecyl sulfate acrylamide electrophesis gel; the prothrombin had been transformed into two pieces, fragment 1 and intermediate 1 (now prethrombin 1). Sanford Gitel, a recent PhD from the Washington University Chemistry Department, was joining the group and, after confirming that the first observation could be repeated and verified using intentionally prepared fragment 1 and prethrombin 1, did the binding studies that resulted in the group's first paper in the Proceedings of the National Academy of Sciences (86)[20]. The products of prothrombin cleavage and the pathways finally established are illustrated in Figure 1.

Investigations of factor V and its activation were proceeding, but with difficulty in reproducibly obtaining factor V that could always be activated to the same extent by thrombin or the factor V activating enzyme of Russell's viper venom (87). Products with 10-fold activation relative to plasma, by a conventional clotting assay using aged, oxalated FV-deficient plasma, or three-fold were the norm – not always 10-fold which we assumed was the form most like that normally circulating in plasma. Alterations in the conditions employed in the purification steps, the addition of more protease inhibitors improved the quality of the product, but contaminants (proteolysis products?) could always be seen when electrophoresis gels were heavily loaded. Discussions of factor V frequently raised the question: "what does activation mean if the protein isn't an enzyme, even if it is a catalyst?" This led to a comparison of the binding to prothrombin-Sepharose of factor V in $BaSO_4$ -adsorbed plasma which had been activated using the Russell's viper enzyme with un-activated factor V from the same plasma. Activated factor V bound to the prothrombin-Sepharose; unactivated factor V did not. We concluded that activation, seen as an increase in specific activity and as fragmentation on sodium dodecyl sulfate acrylamide electrophesis gels resulted in exposure of binding sites for prothrombin (88). Similar studies were done several years later using factor Xa bound to Sepharose; the observations demonstrated that exposure of sites due to activation was required for binding to Xa as well as to prothrombin (89)[21].

A paper by Kurt Stenn (90) identifying a band in sodium dodecyl sulfate acrylamide electrophesis gels that corresponded to the expected behavior of the intact "propiece" of prothrombin and the confusion regarding precursor-product relationships among the "intermediates" observed led us to conclude that we needed to repeat almost all of our previously performed experiments. And, if we were to do so, we should examine every aspect that we could, chromatographic column elution patterns of the prothrombin proteolysis products from all appropriate combinations of activator components, molecular weights by sedimentation equilibrium in the ultracentrifuge and amino acid compositions. Similarly, we should present the results in a way

[18] As this was the time of the Vietnam War, my only verified success at prophecy was the letter that I wrote to Chuck's draft board to argue that deferring him was in the greater interest of science and the nation than his becoming cannon fodder. Fortunately, he came from a relatively small town in Illinois where such letters were actually read. He was deferred.

[19] The first grant application required an on-site visit that included two then well known scientists working on prothrombin activation, Gerald Lanchantin and Arnold Ware. My only recollection is of a question about Klotz plots of binding data from Lanchantin that I couldn't answer, In fact, I'd never heard of them and admitted so. Apparently ignorance was acceptable because the grant was funded.

[20] Initially there was substantial concern regarding this paper, principally because something that although called intermediate 1, but wasn't converted to the product as fast as its precursor, could not be a kinetic intermediate on the pathway from prothrombin to thrombin! The benefit of astute colleagues, George Drysdale, Carl Frieden and Roy Vagelos prevented a serious error in interpretation by those of us whose enthusiasm for the discovery got in the way of clear thinking.

[21] Regrettably, those studies, done by Marie-Claude Guillin and Annie Bezeaud during a visit to St Louis were never published except in an abstract (89).

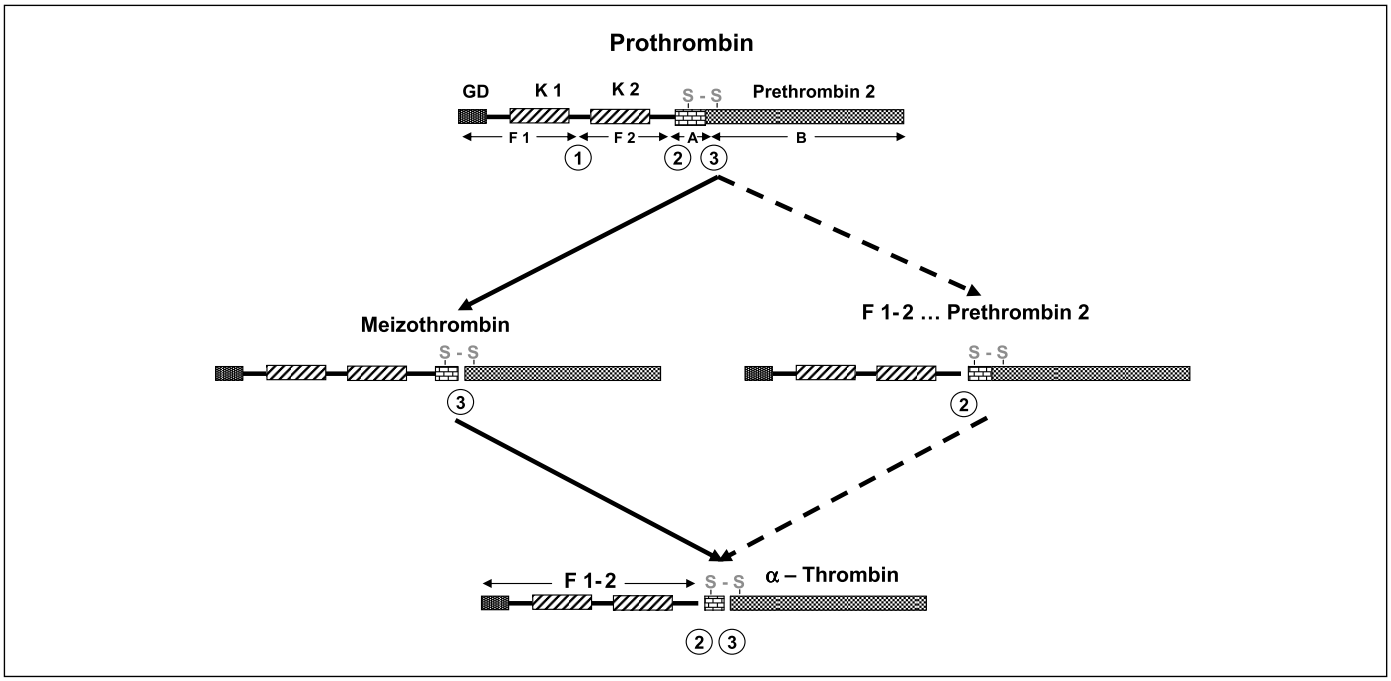


Figure 1: Prothrombin activation pathway and prothrombin proteolysis products. Prothrombin consists of two approximately equal size polypeptide regions, the fragment 1–2 (striped bars and dark crosshatched bar) and prethrombin 2 (crosshatched (B) and bricked (A) regions). The Gla domain (dark crosshatching) is the portion of prothrombin that contains 10 γ-carboxyglutamic acid residues; a region at the amino terminus of prothrombin. Factor Xa hydrolysis of the two peptide bonds marked by the encircled ② and ③ on the diagram for prothrombin produces two products, α-thrombin and fragment 1–2. Proteolytic action by thrombin on prothrombin at bond ① produces prethrombin 1 (not labeled on the diagram) and fragment 1 (F 1). Thrombin action on fragment 1–2 produces the separate polypeptides, fragment 1 (F 1) and fragment 2 (F 2). The pathway now accepted as predominant when prothrombinase catalyzes prothrombin activation occurs via initial cleavage of bond (3) to produce meizothrombin (solid arrows, left side of the figure). The fragment 1–2 region and the thrombin B chain region are held together by a disulfide bond between cysteine residues in the A and B chains of thrombin. In the absence of factor Va, i.e. activation using "partial activators", most of the prothrombin is converted to thrombin via initial cleavage of bond ② to produce fragment 1–2 and prethrombin 2 (dashed arrows, right side of figure). The final products of prothrombin activation are α-thrombin (includes both A and B chains) and fragment 1–2. Because of thrombin action, Fragments 1 and 2 are the predominant species at completion of prothrombin activation *in vitro*. Fragments 1 and 2 are not shown individually. Two homologous polypeptide sequences designated kringles (53), K 1 and K 2 are found in the fragments as marked on the diagram for prothrombin. The above description is for bovine prothrombin; human prothrombin is cleaved within the A chain of prethrombin 2 to produce a 13 residue longer fragment 2 and a shorter A chain in thrombin.

that would provide sufficient evidence to answer the questions surrounding prothrombin activation products as conclusively as we could. Although this was risky because others might make the observations regarding factor V in particular, we went ahead with the systematic approach that resulted in six papers published in the *Journal of Biological Chemistry* in 1974[22].

In **Paper I**, we attempted to ensure that all material derivable from prothrombin could be accounted for by recovery on chromatography, by mol wt analysis and by amino acid composition (93). This paper was devoted also to verifying the origins of each of the "phenomenological intermediates" and fragments, e.g. prothrombin 1 produces fragment 1 and prethrombin 1[23]; and prethrombin 1 produces prethrombin 2 and fragment 2. Although qualitatively adding very little to what was already known, this put the subsequent experiments on a firm basis and made drawing conclusions from them more straightforward (Fig. 1).

Paper II demonstrated that prethrombin 1 and fragment 1 were exclusively the products of thrombin catalyzed cleavage, whereas prethrombin 2 and fragment 2 were the products of Xa catalyzed cleavage (96). Using DFP to inhibit thrombin, the intact "propiece" of prothrombin (fragment 1–2) was formed and could be isolated and characterized. It was then shown that thrombin would produce the two, long known fragments, fragment 1 and fragment 2, but that Xa did not. These results confirmed the conclusion of Stenn (90) and enabled unambiguous placement of all the products of prothrombin proteolysis within the molecule. Two pathways were suggested to account for thrombin formation, one via prethrombin 2 and fragment 1–2; the other via an intermediate in which the first peptide bond

[22] A complete presentation of this work is available in the book published from the 1974 Boerhaave Course of the University of Leiden (91) and at a Cold Spring Harbor Conference (92). Publishing "complete" solutions to a defined problem has always been my preference, a practice that has distinct disadvantages but provides the greatest sense of personal accomplishment. Only a fool would recommend such a practice today!

[23] Designated intermediate 1; the name was changed to prethrombin 1; intermediate 2 to prethrombin 2 to be more informative with respect to their potential to produce thrombin (94).

cleavage produced a form of thrombin with the same mol wt as prothrombin, the species now called meizothrombin[24,25].

Paper III in this series addressed the question: "what are the products of prothrombin proteolysis by factor Xa in the absence of factor V or phospholipid", the other components of the physiological activation complex (97). Evidence that might support one or the other of the two putative pathways, via prethrombin 2 and fragment 2 or via meizothrombin was sought using ^{3}H-labeled prothrombin. Clear evidence for meizothrombin was not observed[26], and the bulk of the prothrombin was converted to thrombin via prethrombin 2. The necessary kinetic requirements were met by prethrombin 2, i.e. it was converted to thrombin more rapidly than prothrombin. Quoting from the discussion of this paper, "Although evidence apparently consistent with the existence of pathway III (*via meizothrombin, added here*) was obtained, this evidence is at best on the borderline of significance for the experimental procedures…. When these additional components are present, the pathway demonstrated to be responsible for prothrombin activation by [Xa, Ca^{2+}] may be altered and pathway III will need to be reconsidered in each particular case." Quantitative kinetic measurements performed in the mid 1980s by Thomas L. Carlisle (98), however, showed unambiguously by kinetic criteria that thrombin formed in the earliest phase of prethrombin 1 activation by Xa alone could not arise via prethrombin 2 and fragment 2. This was not published fully until 1990 (99) after Paul Bock's novel peptide chloromethyl ketone inhibitors[27] (100, 101) made it possible to observe the time course of meizothrombin 1 in gel electrophoresis[28].

In **Paper IV** the possibility that the fragment 2 region of prothrombin and prethrombin 1 was responsible for the ability of factor Va to enhance the rate of thrombin formation was investigated (102). Given the experience with the fragment 1 region and its function in binding to phospholipid vesicles, expectations were high, but science must be driven by evidence, not expectation. When the activator was [Xa, Va, Ca^{2+}], prethrombin 2 qualified as a kinetic intermediate in thrombin formation only if fragment 2, or fragment 1–2 were also present in a stoichiometric amount. However, for this to "make sense", interaction between these fragments and prethrombin 2 and/or with factor Va was required. Interaction between the fragments 2 and 1–2 and prethrombin 2 was sufficiently strong that complexes with the electrophoretic mobility of prethrombin 1 (fragment 2 plus prethrombin 2) or prothrombin (fragment 1–2 plus prethrombin 2) were readily seen in non-denaturing acrylamide gels. Once again, the intermediate comprising the fragment and prethrombin 2 met the necessary, although not sufficient criterion for its being a kinetic intermediate on the pathway.

In **Paper V**, we examined the products formed by Xa action on prothrombin in the presence of phospholipid vesicles and showed that the products and their formation were the same as in the absence of phospholipids (103). Phospholipid effects were therefore catalytic, and the pathway for Xa and prothrombin conversion to thrombin was indistinguishable from that occurring in solution. Based on what is known about binding of other substances to surfaces, the increase in activation rate in the presence of phospholipid was almost certainly accounted for by the increased concentrations of both the enzyme (Xa) and the substrate (prothrombin) on or adjacent to the surface. Another key observation that was made in this study was that fragment 1–2 and prethrombin 2 associated non-covalently such that it would be cleaved even after thrombin formation and by inference cleavage by thrombin. It was also demonstrated that only fragment 1–2 could support phospholipid vesicle surface catalysis, fragments 1 and 2, either alone or in mixture could not. Most importantly, these observations indicated collectively that the pathway involving prethrombin 2 and fragment 1–2 could be the kinetic pathway; but it was not demonstrated to be so because the kinetics of formation were not followed accurately enough to draw any unambiguous conclusions. Based on the criterion of Occam's razor, the pathway via prethrombin 2 was considered adequate, the simpler of the two and therefore preferred[29]. Quantitative studies of fragment 1 binding to vesicles of phosphatidyl choline and phosphatidyl glycerol by Frederick A. Dombrose (104) established that the binding constant was the same as the concentration of prothrombin in the circulating plasma; an observation that indicated that association with exposed phospholipids from injury and/or platelet factor 3 release would be a spontaneous process, and thus a chemical mechanism for localizing prothrombin activation to the injury site was proposed (104)[30].

The discovery of γ-carboxyglutamic acid by Johan Stenflo (105–109), which occurred following his identification that the difference between normal prothrombin and that found in individuals receiving vitamin K antagonists involved Ca^{2+} binding, led us to collaborate with John Suttie to experimentally challenge the conclusions derived from the comparisons of prethrombin 1 and prothrombin using true acarboxyprothrombin (110). Reassuringly, the acarboxyprothrombin neither bound to

[24] This intermediate could not be ignored because work by Morita (95) clearly demonstrated the formation of this entity when the activator was the protease from *Echis carninatus* venom.

[25] The name meizothrombin was created during an effort to rationalize the nomenclature of prothrombin proteolysis products. In this exercise parts of names from the all laboratories involved were adopted in an attempt to provide credit fairly; the name meizothrombin was suggested to me by Prof. Zola Packman of the Department of Classics at Washington University, St. Louis (94).

[26] It must be noted, and as was subsequently demonstrated, absence of evidence is not evidence for the absence of the pathway.

[27] Peptide chloromethyl ketone inhibitors, the active site titrant for trypsin-like proteases, nitrophenyl guanidobenzoate, among others were contributed by Elliot Shaw. Without these inhibitors to use as tools, neither isolation nor pathway elucidation would have been possible.

[28] Such demonstrations by analysis of the kinetics of the reaction, although definitive, are rarely compelling to persons not steeped in the mathematics that underpin enzyme kinetics. Moreover, as recognized by kineticists, kinetic data does not prove, but only disproves models for chemical and biochemical reactions.

[29] This is a relatively simple example of the danger and difficulty of drawing conclusions regarding kinetic mechanisms, particularly when two enzymes are involved and thrombin, because it is formed stoichiometrically from prothrombin, could be relatively inefficient, but dominate the observed product formation. Its concentration would be higher than Xa after less than 1 percent of the prothrombin had been activated.

[30] The molecular mechanism suggested was not compelling, in fact sufficient trepidation existed that its discussion was entirely separate from the discussion of the experimental results.

phospholipid vesicles, nor was its activation rate by [Xa, Ca^{2+}] increased in the presence of them. As predicted from the study with factor Va, acarboxyprothrombin and normal prothrombin were activated at the same rate by [Xa, Va, $Ca2^{+}$]. Similar conclusions were drawn by Guillin et al. (111).Takashi Morita[31] came to the laboratory during this period and developed an extremely useful derivative of factor X and Xa; Xa (-GD) by cleaving the Gla domain, residues 1–41 using chymotrypsin. Xa(-GD), behaved as predicted and thus exhibited no phospholipid vesicle dependent rate increase for thrombin formation from prothrombin, i.e. [Xa(-GD), PL, Ca^{2+}] behaved as if there were no phospholipid present in the reaction mixture (113).

The **final paper (VI)** in this series "A plausible mechanism for prothrombin activation by factor Xa, factor Va, phospholipid and calcium ions" (114) reported the results from the complete prothrombin activation complex, now called prothrombinase[32]. Magnitudes of the increases in the rate of prothrombin conversion to thrombin were estimated with an overall enhancement of >1 x 10^5 relative to [Xa, Ca^{2+}] alone[33]. It was notable, and a clue to conclusions regarding the preferred pathway in the prothrombinase catalyzed reaction, the rate of thrombin formation in the mixture of prethrombin 1 plus fragment 1–2 never exceeded the rate of prothrombin conversion to thrombin. As stated in this paper: "One specific feature of this mechanism, which is physiologically interesting, is the interaction of all of the components with phospholipid, and thus a mechanism exists by which the thrombin-forming process may be specifically localized at a site in which phospholipid surface is available. The release of platelet phospholipid upon aggregation of the platelets at the site of injury is one such source of the lipid."

Of course we were not the only ones working on prothrombin activation during this period; studies from the laboratories of Stenn (90), Esnouf (115–117), Iwanaga (118, 119), Seegers (120–123), Aronson (124–126), Mann (127–136) and Hanahan (137–139) were drawing similar conclusions regarding the pathways for thrombin formation. Our unique contribution was the identification of the functions of the domains of the propiece of prothrombin.

Prothrombin activation on platelets (human prothrombin activation)

Several inferences from these experiments were made that led to a subsequent series of investigations of prothrombin activation on platelets, experiments performed in collaboration with my colleague Philip Majerus. Platelets have long been considered to be the principal source of phospholipid (platelet factor 3) in hemostasis (83). With the information in hand from the above described experiments, and the desire to find out if the same behavior could be observed with human components and with platelets, studies of factor Xa binding and prothrombin activation on platelets were begun. Joseph P. Miletich came across the street from Phil Majerus' lab to begin these investigations. In the first set of experiments it was shown that Xa bound reversibly to platelets, but only after activation by thrombin formed either *in situ*, or activated using either exogenous thrombin or a Ca^{2+} ionophore. Inhibition of the release reaction with dibutryl cyclic AMP inhibited in parallel the binding of Xa and thrombin formation (140). A second study provided evidence that the platelet receptor was of very high affinity (~ 3 x 10^{10} M^{-1}). Identification of the receptor as factor Va was by inhibition of both binding and prothrombin activation using an antibody to factor V from a factor V deficient patient (141). In a third paper with William H. Kane and M. J. Lindhout[34] (142), it was shown that the Xa receptor was factor Va, not factor V, consistent with the earlier affinity chromatography experiments (88). It was observed also that it was factor V, not Va that was released from arachidonic acid or ionophore-stimulated platelets. Binding of Xa could occur to the external surface of unactivated platelets, but only to Va; apparently phospholipid "flip-flop" to expose phosphatidyl serine having occurred as the result of the platelet stimulating substances (143, 144). Bound Xa was shown also to be "protected" from inactivation by antithrombin, confirming earlier in observations by Ewa Marciniak (145).

Similar work on prothrombin activation on platelets and other cells was being reported by Tracy (147–151), and from Hemker's group in Maastricht (152, 153). The original work in St. Louis was extended independently by Majerus (146, 154, 155). A more current picture of coagulation reactions on platelets can be found in the review by Walsh (156).

Concluding remarks

The structural basis for the functions of the components of prothrombin activation became clearly defined and convincingly demonstrated during the 1960s to 1980s. Postulated components were replaced by molecules that have as one or more of their properties the expression of a particular function in the activation process. A molecular mechanism (a more detailed model) was thus provided for localized, rapid thrombin formation, a model involving an organized activation complex of [Xa, Va, PL, Ca^{2+}]; the entity envisioned in the earliest revisions of the classical theory of coagulation. With the benefit of hindsight, the process of prothrombin activation by a complex activator is in its key features the model championed by J. H. Milstone and Walter H. Seegers. Unfortunately, the tools available to them did not permit the simple demonstrations we were able to make, and their choice of names for the components probably hindered appreci-

[31] Takashi Morita also identified the structural basis for bovine factor X separating into two variants on anion exchange chromatography (112). A single tyrosyl residue was sulfated in approximately 1/3 of the factor X of the molecules, a single charge difference thus enabling the two species to be resolved.

[32] Although firmly entrenched in the literature beginning in 1968, the term prothrombinase has been avoided by the author as it was unacceptable according to the international nomenclature rules.

[33] What is most dramatic is that this difference in rate is comparable in time to the same amount of thrombin that would require 6 months to form with Xa alone would be formed in one minute with the complete "prothrombinase".

[34] Theo Lindhout, from Coen Hemker's laboratory spent a year learning to isolate factor V; Bill Kane was an MD, PhD student with Phil Majerus.

ation of their work[35]. Other groups of investigators since the 1970s, particularly those of Mann, Nesheim and Krishnaswamy (157–161), Hemker and Rosing (172–178) and Dahlback (179-181) greatly expanded and extended the investigations of prothrombin and refined the model for it. The pathway argued in 1974 to be sufficient for [Xa, Va, PL, Ca^{2+}] by kinetic criteria has been replaced; evidence for factor Va altering the pathway to the route via meizothrombin is compelling, and very interestingly regulated (182–184). Today a model for prothrombinase based on a "synthesis" of crystallographic and homology modeling exists for prothrombinase that can guide the design of structure-function experiments (181) and will undoubtedly lead to even further understanding of prothrombin activation.

Our discovery of the functions of the two fragments derived from "propiece" of prothrombin enabled structure-function studies to expand from the protease domain of the proenzyme to the entire molecule. In that the amino terminal domains serve similar functions in all of the coagulation proenzymes, it might be argued that these functions identified in prothrombin and factor Xa served to initiate a paradigm shift (30) in the understanding of the functions of the homologous regions of all of the proenzymes of the hemostatic process.

I would like to believe that the progress in developing the current model of the entire coagulation process owes much to the identification, isolation and characterization of the proteins that participate in prothrombin activation. Acceptance of this "activation complex model" is in part due to the fact that appreciation for such discoveries can be almost universal because tangible evidence, e.g. gel photographs, amino acid or cDNA sequences and three-dimensional structures could be acquired. Such evidence is **positive** and seems to be "real". Interpretation is usually evident, simple and straightforward, although opportunity for misinterpretation existed when the evidence came in "bits and pieces". From my perspective structural evidence is but the starting point; the more biologically and medically interesting insights are built on structure and how structure produces function.

The work on prothrombin activation is most significant because it provided insight into a mechanism by which all of the components and reactions of hemostasis could be localized to an injury site. Chemical mechanisms for binding, for dramatic rate increases and the implied small amounts of active enzyme required to achieve hemostatic response are general and not limited to prothrombin activation. The model for the coagulation "cascade" today still consists of a series of reactions, but at each stage it is system of complex activators [enzyme, substrate, protein cofactor (in an "activated" form) and a surface/receptor]. With the work on prothrombin activation on the platelet surface, and on monocytes and other cells the model has advanced from its simplified form to a more biologically relevant one.

It is now generally recognized that interactions between and among the components of reaction complexes regulate their function. Even at the stage of fibrinogen conversion to fibrin interactions between thrombin and fibrin are not limited to enzyme-substrate interactions, but are rather enzyme effector interactions. A functionally important Na^{+}-binding site and exosites on thrombin and the other proteases (185–189) provide the structural features or attributes that are responsible for these interactions and the regulation of their specificity. Similar interactions are characteristic of the fibrinolytic system. Was prothrombin the model system for a paradigm change in our model of the clotting cascade? I would like think so (190).

Future models for regulation of hemostasis will be quantitative and involve binding and kinetic measurements. Conceptually these models will be more complex and will appear to be esoteric. In contrast to structures, reaction rate increases and changes in reaction mechanisms, changes in values of kinetic parameters are less easy to interpret. And usually they offer few intuitive insights to those whose backgrounds are not steeped in such lore. Moreover, rate constants, even to those who "love them" are nothing without the model to which they are related and by which they are interpreted. Nonetheless, it is worth reminding ourselves that the most common measurement made in assessing the function of the clotting system is a clotting time, an "inverse", but intuitive measure of the rate of the reaction. It is intuitively clear that the longer a reaction takes to complete, the slower it is. The simple indicator of inadequacy of the system function is that if the clotting time is too long hemorrhagic risk is implied or if it is too short the implication is thrombotic risk. Although this is clearly oversimplified, the tools now provided by inexpensive, but powerful computers will enable the next generation of investigators to refine and perhaps disprove the models that today are dear to our claims of discovery and PowerPoint™ slides. Work is already underway (191–200), but appears to be inadequately appreciated and supported. Perhaps the lack of appreciation is understandable, kinetic studies provide **negative** evidence with respect to a model and eliminates alternative mechanisms, but cannot prove them. Popper's falsification approach to hypothesis testing (29) is clearly relevant, informative and powerful. Models and cartoons should not to be confused with evidence, only data provide evidence; models are just vehicles that permit data to be interpreted and hypotheses discussed.

Although the descriptive models I've described and quantitative kinetic models I predict will supplant them will be refined primarily from investigations *in vitro;* "The Art of the Soluble" (28) will guide our choice. The future, however, is *in vivo*. The vessel wall with its normally exposed endothelium, the subendothelium exposed upon injury and the surfaces of atheromatous plaque exposed as a consequence of rupture provide a new and more exciting frontier. It may be important to consider the surfaces presented to flowing blood *in vivo* as active. The in-vitro observations and models described above will have served the purpose of creating hypotheses that can be tested for their adequacy to explain and predict behavior in the living organism.

Personal rewards from the studies from my own laboratory that I've described above cannot be better described than already done by Peter Medawar (28). "Scientific discovery is a private event, and the delight that accompanies it, or the despair of find-

[35] An acknowledgement of the priority of conceptualization by J. H. Milstone was made in a talk in London and published as Jackson, C. M. The Biochemistry of Prothrombin Activation. In: Heparin Chemistry and Clinical Usage (Kakkar, V. V., Thomas, D. P., Eds.), Academic Press, London, 1976, pp. 61–99. The thanks returned by J. H. Milstone's widow personally to me after a seminar some years later at Yale University must stand as refutation of the cynic's aphorism "No good deed goes unpunished".

ing it illusory, does not travel. One scientist may get great satisfaction from another's work and admire it deeply; it may give him great intellectual pleasure; but it gives him no sense of participation in the discovery, it does not carry him away, and his appreciation of it does not depend on his being carried away. If it were otherwise the inspirational origin of scientific discovery would never have been in doubt." But discovery is never the exclusive property of one individual or group, as noted above it is built on the work that went before it. Again, as noted by Medawar, "Simultaneous discovery is utterly commonplace, and it was only the rarity of scientists, not the inherent improbability of the phenomenon, that made it remarkable in the past. Scientists on the same road may be expected to arrive at the same destination, often not far apart." On a final note, it seems appropriate to recall Cervantes (32), "Win the attention of the wise, give the thinker food for thought, whoso indites (writes) frivolities will but by simpletons be sought".

Acknowledgements

The author thanks M. Peter Esnouf for his invaluable comments, particularly those solicited to limit the inaccuracy and bias of the author's recollections. Of course, all errors are the responsibility of the author.

References

1. Milstone, JH. On the evolution of blood clotting theory. Medicine 1952; 31: 411–447.
2. Quick AJ, Stanley-Brown M, Bancroft FW. A study of the coagulation defect in hemophilia and in jaundice. Am J Med Sci 1935; 190: 501.
3. Quick AJ. The coagulation defect in sweet clover disease and in the hemorrhagic chick disease of dietary origin. Am J Physiol 1937; 118: 260–271.
4. Owren PA. The fifth coagulation factor ('Factor V'). Preparation and properties. Biochem J 1948; 43: 136–139.
5. Owren PA. Prothrombin and accessory factors; clinical significance. Am J Med 1953; 14: 201–215.
6. Owren PA. Thrombotest. A new method for controlling anticoagulant therapy. Lancet 1959; 2: 754–758.
7. Duckert F, Koller F, Matter M. Purification and physiological properties of factor VII from plasma and serum; separation from prothrombin. Proc Soc Exp Biol Med 1953; 82: 259–261.
8. Duckert F, Fluckiger P, Koller F. The role of factor X in the formation of blood thromboplastin. Rev Hematol 1954; 9: 489–492.
9. Duckert F, Fluckiger P, Matter M, et al. Clotting factor X; physiologic and physico-chemical properties. Proc Soc Exp Biol Med 1955; 90: 17–22.
10. Alexander B, De Vries A, Addelson E. Human prothrombin: Quantitative studies on the plasma labile factor and the restorative effects of normal, hypofibrinogenemic, and hemophilic plasma on the prothrombin of stored plasma. J Clin Invest 1949; 28: 24–31.
11. Gonyea LM, Hjort P, Owren PA. A study of the relationship of concentrations of prothrombin, proconvertin, and proaccelerin to three methods for measuring prothrombin time. J Lab Clin Med 1956; 48: 624–633.
12. Pechet L, Alexander B, Tishkoff GH. Separation, interaction and properties of clotting components essential for prothrombin activation with special reference to Stuart factor. Thromb Diath Haemorrh 1960; 4 (Suppl): 47–57.
13. Telfer TP, Denson KW, Wright DR. A new coagulation defect. Br J Haematol 1956; 2: 308–316.
14. Hougie C, Barrow EM, Graham JB. The Stuart factor: a hitherto unrecognized blood coagulation factor. Bibl Haematol 1958; 7: 336–340.
15. Beck EA. Historical development of the prothrombin concept. In: Prothrombin and Related Coagulation Factors. Leiden: Leiden University Press 1975: 15–24.
16. Esnouf MP, Macfarlane RG. Enzymology and the blood clotting mechanism. Adv Enzymol Related Areas Mol Biol 1968; 30: 255–315.
17. Esnouf MP. The nature of prothrombinase. Boerhaave Course, 1968; University of Leiden
18. Milstone J. Fractionation of plasma globulin for prothrombin, thrombokinase, and accessory thromboplastin. J Gen Physiol 1951; 35: 67–87.
19. Milstone JH. Protein thrombokinase and lipoid thromboplastin as distinct factors with complementary functions. Yale J Biol Med 1952; 25: 19–33.
20. Milstone JH. Thrombokinase as prime activator of prothrombin: Historical perspectives and present status. Fed Proc 1964; 23: 742–748.
21. Milstone J. Fractionation of plasma globulin for prothrombin, thrombokinase, and accessory thromboplastin. J Gen Physiol 1951; 35: 67–87.
22. Suttie JW, Jackson CM. Prothrombin structure, activation and biosynthesis. Physiol Rev 1977; 57: 1–70.
23. Seegers WH. A personal perspective on hemostasis and thrombosis (1937–1981). Semin Thromb Hemost 1981; 7: 177–307.
24. Seegers WH. Prothrombinase: Recognition and developments. Adv Exp Med Biol 1990; 281: 93–96.
25. Seegers WH. Solving the Riddle of Blood Clotting. In: Thrombosis: Mechanisms and Control. Stuttgart – New York: F. K. Schattauer Verlag 1973: pp. 9–30.
26. Marcum, J. Constructing a scientific paper: Howell's prothrombin laboratory notebook and paper. Int Studies Philos Sci 2001; 15: 293–310.
27. Marcum JA. Defending the priority of 'remarkable researches': the discovery of fibrin ferment. Hist Philos Life Sci 1998; 20: 51–76.
28. Medawar P. Pluto's Republic, Incorporating The Art of the Soluble and Induction and Intuition in Scientific Thought. Oxford: Oxford University Press, 1984.
29. Popper K. The Logic of Scientific Discovery. London: Routledge Classics, 1952.
30. Kuhn TS. The Structure of Scientific Revolutions, 2nd Ed. University of Chicago Press, 1962. pp. 1–223.
31. Clifford WK. The Ethics of Belief. In: Gateway to the Great Books, Philosophical Essays. Chicago: Encyclopaedia Britannica, Inc. 1963: pp. 14–36.
32. Cervantes M. Commendatory Verses. The History of Don Quixote de la Mancha. Britannica Great Books of the Western World, Vol. 29. Chicago: Encyclopaedia Britannica, Inc. 1952: xiv-xvi.
33. Saxe, John Godfrey, The Blind Men and the Elephant. Available at http://www.wordinfo.info/words/index/info/view_unit/1/?letter=B&spage=3, Accessed 4-April-2007, American Poet, 1816–1887
34. Frankfurt H. On Bullshit. Princeton, Princeton University Press, 2005: 1–67.
35. Pirie NW. Purification and Crystallization of Proteins. Biol. Rev. Cambridge Philos. Soc. 1940; 15: 377 (Reprinted in Ann NY Acad Sci 1979; 325: 21–36.)
36. Sober HA, Peterson EA. Protein chromatography on ion exchange cellulose. Fed Proc 1958; 17: 1116–1126.
37. Aronson DL. N-Terminal amino-acids formed during the activation of prothrombin. Nature 1962; 194: 475–476.
38. Aronson DL, Preiss JW. Molecular weights of human prothrombin and thrombin by electron irradiation. Radiat Res 1962; 16: 138–143.
39. Aronson DL, Menache D. Chromatographic analysis of the activation of human prothrombin with human thrombokinase. Biochemistry 1966; 5: 2635–2640.
40. Ware AG, Lanchantin GF. Purification of fibrinogen, prothrombin and thrombin. J Biol Chem 1954; 34: 714–721.
41. Lanchantin GF, Friedmann JA, Hart DW. The Conversion of Human Prothrombin to Thrombin by Sodium Citrate: Analysis of the Activation Mixture. J Biol Chem 1965; 240: 3276–3282.
42. Lanchantin GF, Hart DW, Friedmann JA, et al. Amino acid composition of human plasma prothrombin. J Biol Chem 1968; 243: 5479–5485.
43. Lanchantin GF, Friedmann JA, Hart DW. Interaction of soybean trypsin inhibitor with thrombin and its effect on prothrombin activation. J Biol Chem 1969; 244: 865–875.
44. Seegers WH, Marciniak E, Kipfer RK, et al. Isolation and Some Properties of Prethrombin and Autoprothrombin III. Arch Biochem Biophys 1967; 121: 372–383.
45. Ingwall JS, Scheraga HA. Purification and properties of bovine prothrombin. Biochemistry 1969; 8: 1860–1869.
46. Lamy F, Waugh DF. Certain physical properties of bovine prothrombin. J Biol Chem 1953; 203: 489–499.
47. Lamy F, Waugh DF. Transformation of prothrombin into thrombin. Physiol Rev 1954; 34: 722–729.
48. Shapiro SS, Waugh DF. The purification of human prothrombin. Thromb Diath Haemorrh 1966; 16: 468–490.
49. Rosenberg RD, Waugh DF. Multiple bovine thrombin components. J Biol Chem 1970; 245: 5049–5056.
50. Waugh DF, Rosenberg RD. Specific activities of bovine thrombin and thrombin components. Thromb Diath Haemorrh 1972; 27: 183–195.
51. Magnusson S. Preparation and carbohydrate analysis of bovine prothrombin. Arkiv Kemi 1964; 23: 285–298.
52. Magnusson S. Preparation of highly purified bovine thrombin (E.C.3.4.4.13) and determination of its N-terminal amino acid residues. Arkiv Kemi 1965; 24: 349–358.
53. Magnusson S, Sottrup-Jensen L, Petersen TE, et al. The primary structure of prothrombin, the role of vitamin K in blood coagulation and a thrombin-catalyzed 'negative feedback' control mechanism for limiting the activation of prothrombin. In: Prothrombin and Related Coagulation Factors. Leiden: Leiden University Press 1975: pp. 25–46.
54. Sherry S, Troll W, Glueck H. Thrombin as a proteolytic enzyme. Physiol Rev 1954; 34: 736–741.
55. Miller KD, Copeland WH. Human thrombin: isolation and stability. Exp Mol Pathol 1965; 26: 431–437.

56. Fenton JW, Fasco MJ, Stackrow AB. Human thrombins. Production, evaluation, and properties of alpha-thrombin. J Biol Chem 1977; 252: 3587–3598.
57. Fenton JW, Campbell WP, Harrington JC, et al. Large-scale preparation and preliminary characterization of human thrombin. Biochim Biophys Acta 1971; 229: 26–32.
58. Mann KG, Batt CW. The molecular weights of bovine thrombin and its primary autolysis products. J Biol Chem 1969; 244: 6555–6557.
59. Seegers WH, Murano G, McCoy L. Structural changes in prothrombin during activation: A theory. Thromb Diath Haemorrh 1970; 23: 26–36.
60. Williams WJ, Esnouf MP. The fractionation of Russell's-viper (Vipera russellii) venom with special reference to the coagulant protein. Biochem J 1962; 84: 52–61.
61. Esnouf MP, Williams WJ. The isolation and purification of a bovine-plasma protein which is a substrate for the coagulant fraction of Russell's viper venom. Biochem J 1962; 84: 62–71.
62. Esnouf MP, Jobin F. Lipids in prothrombin conversion. Thromb Diath Haemorrh 1965; 17: 103–111.
63. Esnouf MP, Jobin F. The isolation of factor V from bovine plasma. Biochem J 1967; 102: 660–665.
64. Jobin F, Esnouf MP. Studies on the formation of the prothrombin-converting complex. Biochem J 1967; 102: 666–674.
65. Papahadjopoulos D, Hougie C, Hanahan D. Influence of surface charge of phospholipids on their clot- promoting activity. Proc Soc Exp Biol Med 1962; 111: 412–415.
66. Papahadjopoulos D, Hougie C, Hanahan DJ. Purification and properties of bovine factor V: A change of molecular size during blood coagulation. Biochemistry 1964; 3: 264–270.
67. Papahadjopoulos D, Yin ET, Hanahan DJ. Purification and properties of bovine factor X: Molecular changes during activation. Biochemistry 1964; 3: 1931–1939.
68. Papahadjopoulos D, Hanahan DJ. Observations on the interaction of phospholipids and certain clotting factors in prothrombin activator formation. Biochim Biophys Acta 1964; 90: 436–439.
69. Hanahan DJ, Papahadjopoulos D. Interactions of phospholipids with coagulation factors. Thromb Diath Haemorrh 1965; 17: 71–84.
70. Barton PG, Hanahan DJ. The Preparation and Properties of a Stable Factor V from Bovine Plasma. Biochim Biophys Acta 1967; 133: 506–518.
71. Barton P, Hanahan D. Some lipid-protein interactions involved in prothrombin activation. Biochim Biophys Acta 1969; 187: 319–327.
72. Cole ER, Koppel JL, Olwin JH. Phospholipid-protein interactions in the formation of prothrombin activator. Thromb Diath Haemorrh 1965; 14: 431–444.
73. Rouser G, Schloredt D. Phospholipid structure and thromboplastic activity II. The fatty acid composition of the active phosphatidyl ethanolamines. Biochim Biophys Acta 1958; 28: 81–87.
74. Barton P, Jackson CM, Hanahan D. Relationship between factor V and activated factor X in the generation of prothrombinase. Nature 1967; 214: 923–924.
75. Hemker HC, Esnouf MP, Hemker PW, et al. Formation of prothrombin converting activity. Nature 1967; 215: 248–251.
76. Esnouf MP. Biochemical aspects of blood coagulation. Proc R Soc Lond B Biol Sci 1969; 173: 269–275.
77. Macfarlane RG. An enzyme cascade in the blood clotting mechanism and its function as a biochemical amplifier. Nature 1964; 202: 498–499.
78. Macfarlane RG. A clotting scheme for 1964. Thromb Diath Haemorrh 1965; Suppl: 45–52.
79. Macfarlane RG. The basis of the cascade hypothesis of blood clotting. Thromb Diath Haemorrh 1965; 15: 591–602.
80. Davie EW, Ratnoff OD. Waterfall sequence for intrinsic blood clotting. Science 1964; 145: 1310–1311.
81. Davie EW, Fujikawa K. Basic mechanisms in blood coagulation. Annu Rev Biochem 1975; 44: 799–829.
82. Bangham AD. A correlation between surface charge and coagulant action of phospholipids. Nature 1961; 192: 1197–1198.
83. Marcus A. The role of lipids in blood coagulation. Adv Lip Res 1966; 4: 1–33.
84. Jackson CM, Hanahan DJ. Studies on bovine Factor X: I. Large scale purification of the bovine plasma protein possessing factor X activity. Biochemistry 1968; 7: 4492–4505.
85. Jackson CM, Hanahan D. Studies on bovine factor X: II. Characterization of purified factor X observations on some alterations in zone electrophoretic and chromatographic behavior occurring during purification. Biochemistry 1968; 7: 4506–4517.
86. Gitel SN, Owen WG, Esmon CT, et al. A polypeptide region of bovine prothrombin specific for binding to phospholipids. Proc Natl Acad Sci USA 1973; 70: 1344–1348.
87. Jackson CM, Gordon JG, Hanahan DJ. Separation of the tosyl arginine esterase activity from the factor X activating enzyme of Russell's viper venom. Biochim Biophys Acta 1971; 252: 255–261.
88. Esmon CT, Owen WG, Duiguid D, et al. The action of thrombin on blood clotting factor V: Conversion of factor V to a prothrombin-binding protein. Biochim Biophys Acta 1973; 310: 289–294.
89. Freeman JP, Guillin M-C, Bezeaud A, et al. Activation of bovine blood coagulation factor V. A prerequisite for it to bind both pothrombin and factor Xa. Fed Proc 1977; 36: 675.
90. Stenn K, Blout E. Mechanism of bovine prothrombin activation by an insoluble preparation of bovine factor Xa (thrombokinase). Biochemistry 1972; 11: 4502–4515.
91. Jackson CM, Esmon CT, Gitel SN, et al. The conversion of prothrombin to thrombin: The function of the propiece of prothrombin. In: Prothrombin and related coagulation factors. Leiden: Leiden University Press 1974: pp. 59–88.
92. Jackson CM, Esmon CT, Owen WG. The activation of bovine prothrombin. In: Cold Spring Harbor Conferences on Cell Proliferation 2: Proteases and Biological Control. New York: Cold Sprint Harbor Laboratory 1975: pp. 95–109.
93. Owen WG, Esmon CT, Jackson CM. The conversion of prothrombin to thrombin: I. Characterization of the reaction products formed during the activation of bovine prothrombin. J Biol Chem 1974; 249: 594–605.
94. Jackson CM. Report of the Task Force on Blood Clotting Zymogens and Zymogen Intermediates. Thromb Haemost 1978; 38: 567–577.
95. Morita T, Iwanaga S, Suzuki T. The mechanism of activation of bovine prothrombin by an activator isolated from Echis carinatus venom and characterization of the new active intermediates. J Biochem 1976; 79: 1089–1108.
96. Esmon CT, Owen WG, Jackson CM. The conversion of prothrombin to thrombin II. Differentiation between thrombin and factor Xa catalyzed proteolyses. J Biol Chem 1974; 249: 606–611.
97. Esmon CT, Jackson CM. The conversion of prothrombin to thrombin. III. The factor Xa catalyzed activation of prothrombin. J Biol Chem 1974; 249: 7782–7790.
98. Carlisle TL, Jackson CM. The two peptide bonds in bovine prethrombin 1 are cleaved at equal rates by bovine Factor Xa. Thromb Haemost 1985; 54: 46.
99. Carlisle TL, Bock PE, Jackson CM. Kinetic intermediates in prothrombin activation: Bovine prethrombin 1 conversion to thrombin by factor X. J Biol Chem 1990; 265: 22044–22055.
100. Bock PE. Active site-selective labeling of thrombin with fluorescence probes using thioester derivatives of peptide-chloromethyl ketones. Thromb Haemost 1987; 58: 506.
101. Bock PE. Active-site-selective labeling of blood coagulation proteinases with fluorescence probes by the use of thioester peptide chloromethyl ketones: II. Properties of thrombin derivatives as reporters of prothrombin fragment 2 binding and specificity of the labeling approach for other proteinases. J Biol Chem 1992; 267: 14974–14981.
102. Esmon CT, Jackson CM. The conversion of prothrombin to thrombin. IV. The function of the fragment 2 region during activation in the presence of factor V. J Biol Chem 1974; 249: 7791–7797.
103. Esmon CT, Owen WG, Jackson CM. The conversion of prothrombin to thrombin V: The activation of prothrombin by factor Xa in the presence of phospholipid. J Biol Chem 1974; 249: 7798–7807.
104. Dombrose FA, Gitel SN, Zawalich K, et al. The association of bovine prothrombin fragment 1 with phospholipid quantitative characterization of the Ca2+ ion-mediated binding of prothrombin fragment 1 to phospholipid vesicles and a molecular model for its association with phospholipids. J Biol Chem 1979; 254: 5027–5040.
105. Stenflo J, Ganrot PO. Vitamin K and the biosynthesis of prothrombin. I. Identification and purification of a dicoumarol-induced abnormal prothrombin from bovine plasma. J Biol Chem 1972; 247: 8160–8166.
106. Stenflo J, Ganrot P. Binding of Ca2+ to normal and dicoumarol-induced prothrombin. Biochem Biophys Res Comm 1972; 50: 98–104.
107. Stenflo J. Structural comparison of normal and dicoumarol-induced prothrombin. In: Prothrombin and Related Coagulation Factors. Leiden: Leiden University Press 1975: pp. 152–158.
108. Stenflo J, Fernlund P, Roepstorff P. Structure of a vitamin K-dependent portion of prothrombin. In: Proteases and Biological Control. Cold Spring Harbor: Cold Spring Harbor Conference on Cell Proliferation 1975: pp. 111–122.
109. Stenflo J, Suttie JW. Vitamin K-dependent formation of γ-carboxyglutamic acid. Annu Rev Biochem 1977; 46: 157–172.
110. Esmon CT, Suttie JW, Jackson CM. The functional significance of vitamin K action. Difference in phospholipid binding between normal and abnormal prothrombin. J Biol Chem 1975; 250: 4095–4099.
111. Guillin MC, Aronson DL, Bezeaud A, et al. The purification of human acarboxy prothrombin. Characterization of its derivatives after thrombin cleavage. Thromb Res 1977; 10: 223–233.
112. Morita T, Jackson CM. Localization of the structural difference between bovine blood coagulation Factors X1 and X2 to tyrosine 18 in the activation peptide. J Biol Chem 1986; 261: 4008–4015.
113. Morita T, Jackson CM. Preparation and properties of derivatives of bovine factor X and factor Xa from which the γ-carboxyglutamic acid containing domain has been removed. J Biol Chem 1986; 261: 4015–4023.
114. Esmon CT, Owen WG, Jackson CM. A plausible mechanism for prothrombin activation by factor Xa, factor Va, phospholipid, and calcium ions. J Biol Chem 1974; 249: 8045–8047.
115. Esnouf MP. The blood clotting mechanism. Prog Clin Biol Res 1976; 5: 69–84.
116. Esnouf MP. Biochemistry of blood coagulation. Br Med Bull 1977; 33: 213–218.

117. Jesty J, Esnouf MP. The preparation of activated factor X and its action on prothrombin. Biochem J 1973; 131: 791–799.
118. Morita T, Iwanaga S, Suzuki T, et al. Characterization of amino-terminal fragment liberated from bovine prothrombin by activated factor X. FEBS Lett 1973; 36: 313–317.
119. Morita T, Nishibe H, Iwanaga S, et al. Studies on the activation of bovine prothrombin isolation and characterization of the fragments released from the prothrombin by activated factor X. J Biochem 1974; 76: 1031–1048.
120. Seegers WH, Sakuragawa N, McCoy LE, et al. Prothrombin activation: Ac-globulin, lipid, platelet membrane, and autoprothrombin C (factor Xa) requirements. Thromb Res 1972; 1: 293–310.
121. Seegers WH, McCoy LE, Sakuragawa N. Mechanisms of thrombin formation. In: Physiology and Biochemistry of Prothrombin Conversion. Stuttgart: F. K. Schattauer Verlag 1974: pp. 255–272.
122. Seegers WH, McCoy LE, Walz DA. Properties of Bovine Prothrombin. In: Physiology and Biochemistry of Prothrombin Converstion. Stuttgart – New York: F. K. Schattauer Verlag 1974: pp. 1–5.
123. Seegers WH, Novoa E, Walz DA, et al. Effects of prothrombin fragments on thrombin, on thrombin formation, and separation from Ac-globulin (factor V). Thromb Res 1976; 8: 83–97.
124. Ball AP, Fenton J, Aronson DL, et al. Proceedings: Purification and characterization of activation fragments F1 and F2 from human prothrombin. Thromb Diath Haemorrh 1975; 34: 591.
125. Aronson DL, Stevan L, Ball AP, et al. Generation of the combined prothrombin activation peptide (F1–2) during the clotting of blood and plasma. J Clin Invest 1977; 60: 1410–1418.
126. Franza BR, Jr., Aronson DL, Finlayson JS. Activation of human prothrombin by a procoagulant fraction from the venom of Echis carinatus. Identification of a high molecular weight intermediate with thrombin activity. J Biol Chem 1975; 250: 7057–7068.
127. Heldebrant CM, Mann KG. The activation of prothrombin. I. Isolation and preliminary characterization of intermediates. J Biol Chem 1973; 248: 3642–3652.
128. Mann KG, Heldebrant CM, Fass DN. Multiple active forms of thrombin. II. Mechanism of production from prothrombin. J Biol Chem 1971; 246: 6106–6114.
129. Fass DN, Mann KG. Activation of fluorescein-labeled prothrombin. J Biol Chem 1973; 248: 3280–3287.
130. Heldebrant CM, Butkowski RJ, Bajaj SP, et al. The activation of prothrombin. II. Partial reactions, physical and chemical characterization of the intermediates of activation. J Biol Chem 1973; 248: 7149–7163.
131. Heldebrant CM, Noyes C, Kingdon HS, et al. The activation of prothrombin. III. The partial amino acid sequences at the amino terminal of prothrombin and the intermediates of activation. Biochem Biophys Res Commun 1973; 54: 155–160.
132. Butkowski RJ, Bajaj SP, Mann KG. The preparation and activation of (sialyl-3H)prothrombin. J Biol Chem 1974; 249: 6562–6569.
133. Bajaj SP, Butkowski RJ, Mann KG. Prothrombin fragments. Ca2+ binding and activation kinetics. J Biol Chem 1975; 250: 2150–2156.
134. Downing MR, Butkowski RJ, Clark MM, et al. Human prothrombin activation. J Biol Chem 1975; 250: 8897–8906.
135. Myrmel KH, Lundblad RL, Mann KG. Characteristics of the association between prothrombin fragment 2 and alpha-thrombin. Biochemistry 1976; 15: 1767–1773.
136. Butkowski RJ, Elion J, Downing MR, et al. Primary structure of human prethrombin 2 and alpha-thrombin. J Biol Chem 1977; 252: 4942–4957.
137. Kisiel W, Hanahan DJ. Purification and characterization of human factor II. Biochim Biophys Acta 1973; 304: 103–113.
138. Kisiel W, Hanahan DJ. The action of factor Xa, thrombin and trypsin on human factor II. Biochim Biophys Acta 1973; 329: 221–232.
139. Kisiel W, Hanahan DJ. Proteolysis of human factor II by factor Xa in the presence of hirudin. Biochem Biophys Res Comm 1974; 59: 570–577.
140. Miletich JP, Jackson CM, Majerus PW. Interaction of coagulation factor Xa with human platelets. Proc Natl Acad Sci USA 1977; 74: 4033–4036.
141. Miletich JP, Jackson CM, Majerus PW. Properties of the factor Xa binding site on human platelets. J Biol Chem 1978; 253: 6908–6916.
142. Kane W, Lindhout M, Jackson CM, et al. Factor Va-dependent binding of factor Xa to human platelets. J Biol Chem 1978; 255: 1170–1174.
143. Zwaal RFA, Comfurius P, van Deenen LLM. Membrane asymmetry and blood coagulation. Nature 1977; 268: 358–360.
144. Bevers EM, Comfuriue P, Zwaal RFA. Changes in membrane phospholipid distribution during platelet activation. Biochim Biophys Acta 1983; 736: 57–66.
145. Marciniak E. Factor-Xa inactivation by antithrombin III: Evidence for biological stabilization of factor Xa by factor V-phospholipid complex. Br J Haematol 1973; 24: 391–400.
146. Miletich J, Kane W, Hofmann S. Deficiency of factor Xa – factor Va binding sites on the platelets of a patient with a bleeding disorder. Blood 1979; 54: 1015–1022.
147. Tracy PB, Peterson JM, Nesheim ME, et al. Interaction of coagulation factor V and factor Va with platelets. J Biol Chem 1979; 254: 10354–10361.
148. Tracy PB, Nesheim ME, Mann KG. Coordinate binding of factor Va and factor Xa to the unstimulated platelet. J Biol Chem 1981; 256: 743–751.
149. Tracy PB, Rohrbach MS, Mann KG. Functional prothrombinase complex assembly on isolated monocytes and lymphocytes. J Biol Chem 1983; 258: 7264–7267.
150. Tracy PB, Nesheim ME, Mann KG. Platelet factor Xa receptor. Methods Enzymol 1992; 215: 329–360.
151. Swords NA, Mann KG. The assembly of the prothrombinase complex on adherent platelets. Arterioscler Thromb 1993; 13: 1602–1612.
152. Bevers EM, Comfurius P, van Rijn JLML, et al. Generation of prothrombin-converting activity and the exposure of phosphatidylserine at the outer surface of platelets. Eur J Biochem 1982; 122: 429–436.
153. Bevers EM, Tilly RHJ, Senden JMG, et al. Exposure of endogenous phosphatidylserine at the outer surface of stimulated platelets is reversed by restoration of aminophospholipid translocase activity. Biochemistry 1989; 28: 2382–2387.
154. Miletich J, Majerus D, Majerus P. Patients with congenital factor V deficiency have decreased factor Xa binding sites on their platelets. J Clin Invest 1978; 62: 824–831.
155. Majerus PW, Miletich JP. Relationships between platelets and coagulation factors in hemostasis. Ann Rev Med 1978; 29: 41–49.
156. Walsh PN. Platelet coagulation-protein interactions. Semin Thromb Hemost 2004; 30: 461–471.
157. Nesheim ME, Prendergast FG, Mann KG. Interactions of a fluorescent active-site-directed inhibitor of thrombin: Dansylarginine n-(3-ethyl-1,5-pentanediyl) amide. Biochemistry 1979; 18: 990–1003.
158. Nesheim MW, Tasswell JB, Mann KG. The contribution of bovine factor V and factor Va to the activity of prothrombinase. J Biol Chem 1979; 254: 10952–10962.
159. Nesheim ME, Kettner C, Shaw E, et al. Cofactor dependence of factor Xa incorporation into the prothrombinase complex. J Biol Chem 1981; 256: 6537–6540.
160. Mann KG, Nesheim ME, Tracy PB, et al. Assembly of the prothrombinase complex. Biophys J 1982; 37: 106–107.
161. Nesheim ME, Mann KG. The kinetics and cofactor dependence of the two cleavages involved in prothrombin activation. J Biol Chem 1983; 258: 5386–5391.
162. Nesheim ME, Foster WB, Hewick R, et al. Characterization of factor V activation intermediates. J Biol Chem 1984; 259: 3187–3196.
163. Krishnaswamy S, Mann KG, Nesheim ME. The prothrombinase-catalyzed activation of prothrombin proceeds through the intermediate meizothrombin in an ordered, sequential reaction. J Biol Chem 1986; 261: 8977–8984.
164. Krishnaswamy S, Church WR, Nesheim ME, et al. Activation of human prothrombin by human prothrombinase. Influence of factor Va on the reaction mechanism. J Biol Chem 1987; 262: 3291–3299.
165. Mann KG. Biochemistry and physiology of blood coagulation. Thromb Haemost 1999; 82: 165–174.
166. Kalafatis M, Swords NA, Rand MD, et al. Membrane-dependent reactions in blood coagulation: role of the vitamin K-dependent enzyme complexes. Biochim Biophys Acta 1994; 1227: 113–129.
167. Bovill EG, Tracy RP, Hayes TE, et al. Evidence that meizothrombin is an intermediate product in the clotting of whole blood. Arterioscler Thromb Vasc Biol 1995; 15: 754–758.
168. Krishnaswamy S, Nesheim ME, Pryzdial EL, et al. Assembly of prothrombinase complex. Methods Enzymol 1993; 222: 260–280.
169. Baugh RJ, Dickinson CD, Ruf W, et al. Exosite interactions determine the affinity of factor X for the extrinsic Xase complex. J Biol Chem 2000; 275: 28826–28833.
170. Doyle MF, Mann KG. Multiple active forms of thrombin. IV. Relative activities of meizothrombins. J Biol Chem 1990; 265: 10693–10701.
171. Mann KG, Nesheim ME, Church WR, et al. Surface-dependent reactions of the vitamin K-dependent enzyme complexes. Blood 1990; 76: 1–16.
172. Rosing J, Tans G, Govers-Riemslag JWP, et al. The role of phospholipids and factor Va in the prothrombinase complex. J Biol Chem 1980; 255: 274–283.
173. Lindhout T, Govers-Riemslag JWP, van de Waart P, et al. Factor Va-factor Xa interaction. Effects of phospholipid vesicles of varying composition. Biochemistry 1982; 21: 5494–5502.
174. Rosing J, van Rijn JLML, Bevers EM, et al. The role of activated human platelets in prothrombin and factor X activation. Blood 1985; 65: 319–331.
175. Rosing J, Zwaal RFA, Tans G. Formation of meizothrombin as intermediate in factor Xa-catalyzed prothrombin activation. J Biol Chem 1986; 261: 4224–4228.
176. Rosing J, Tans G. Meizothrombin, a major product of factor Xa-catalyzed prothrombin activation. Thromb Haemost 1988; 60: 355–360.
177. Tans G, Janssen-Claessen T, Hemker HC, et al. Meizothrombin formation during factor Xa-catalyzed prothrombin activation. Formation in a purified system and in plasma. J Biol Chem 1991; 266: 21864–21873.
178. van Rijn JLML, Govers-Riemslag JWP, Zwaal RFA, et al. Kinetic studies of prothrombin activation: Effect of factor Va and phospholipids on the formation of the enzyme-substrate complex. Biochemistry 1984; 23: 4557–4564.
179. Dahlback B, Stenflo J. The activation of prothrombin by platelet-bound factor Xa. Eur J Biochem 1980; 104: 549–557.

180. Suzuki K, Dahlback B, Stenflo J. Thrombin-catalyzed activation of human coagulation factor V. J Biol Chem 1982; 257: 6556–6564.
181. Autin L, Stehen M, Dahlback B, et al. Proposed structural models of the prothrombinase (FXa-FVa) complex. Proteins 2006; 63: 440–450.
182. Brufatto N, Nesheim ME. Analysis of the kinetics of prothrombin activation and evidence that two equilibrating forms of prothrombinase are involved in the process. J Biol Chem 2003; 278: 6755–6764.
183. Bukys MA, Blum MA, Kim PY, et al. Incorporation of factor Va into prothrombinase is required for coordinated cleavage of prothrombin by factor Xa. J Biol Chem 2005; 280: 27393–27401.
184. Bukys MA, Kim PY, Nesheim ME, et al. A control switch for prothrombinase: characterization of a hirudin-like pentapeptide from the COOH terminus of factor Va heavy chain that regulates the rate and pathway for prothrombin activation. J Biol Chem 2006; 281: 39194–39204.
185. Di Cera, Guinto ER, Vindigni A et al. The Na+ binding site of thrombin. J Biol Chem 1995; 270: 22089–22092.
186. Di Cera, Dang QD, Ayala YM. Molecular mechanisms of thrombin function. Cell Mol Life Sci 1997; 53: 701–730.
187. Camire RM. Prothrombinase assembly and S1 site occupation restore the catalytic activity of FXa impaired by mutation at the sodium-binding site. J Biol Chem 2002; 277: 37863–37870.
188. Di Cera. A structural perspective on enzymes activated by monovalent cations. J Biol Chem 2006; 281: 1305–1308.
189. Bianchini EP, Pike RN, Le Bonniec BF. The elusive role of the potential factor X cation-binding exosite-1 in substrate and inhibitor interactions. J Biol Chem 2004; 279: 3671–3679.
190. Jackson CM, Nemerson Y. Blood coagulation. Annu Rev Biochem 1980; 49: 765–781.
191. Nesheim ME, Tracy RP, Mann KG. "Clotspeed" a mathematical simulation of the functional properties of prothrombinase. J Biol Chem 1984; 259: 1447–1453.
192. Nesheim ME, Tracy RP, Tracy PB, et al. Mathematical simulation of prothrombinase. Methods Enzymol 1992; 215: 316–328.
193. Jesty J, Beltrami E, Willems G. Mathematical analysis of a proteolytic positive-feedback loop: dependence of lag time and enzyme yields on the initial conditions and kinetic parameters. Biochemistry 1993; 32: 6266–6274.
194. Beltrami E, Jesty J. Mathematical analysis of activation thresholds in enzyme-catalyzed positive feedbacks: application to the feedbacks of blood coagulation. Proc Natl Acad Sci USA 1995; 92: 8744–8748.
195. Gir S, Slack SM, Turitto VT. A numerical analysis of factor X activation in the presence of tissue factor--factor VIIa complex in a flow reactor. Ann Biomed Eng 1996; 24: 394–399.
196. Beltrami E, Jesty J. The role of membrane patch size and flow in regulating a proteolytic feedback threshold on a membrane: possible application in blood coagulation. Math Biosci 2001; 172: 1–13.
197. Jesty J, Beltrami E. Positive feedbacks of coagulation: their role in threshold regulation. Arterioscler Thromb Vasc Biol 2005; 25: 2463–2469.
198. Obraztsov IF, Kardakov DV, Kogan AE, et al. A mathematical model of the background state of the blood coagulation system. Dokl Biochem Biophys 2001; 376: 10–12.
199. Ataullakhanov FI, Panteleev MA. Mathematical modeling and computer simulation in blood coagulation. Pathophysiol Haemost Thromb 2005; 34: 60–70.
200. Orfeo T, Mann KG. Mathematical and biological models of blood coagulation. J Thromb Haemost 2005; 3: 2397–2398.