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City University of New York PH.D. 1986

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A UNIQUE ROLE FOR ACIDIC PHOSPHOLIPIDS IN THE TISSUE FACTOR PATHWAY OF BLOOD COAGULATION

by

STEVEN DAVID FORMAN

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1986

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The City University of New York

Abstract

A UNIQUE ROLE FOR ACIDIC PHOSPHOLIPIDS IN THE TISSUE FACTOR PATHWAY OF BLOOD COAGULATION

by

Steven David Forman

Adviser: Professor Yale Nemerson

Negatively-charged phospholipids enhance the activity of several procoagulant reactions, including the activation of factor X by the membrane-localized complex of tissue factor-factor VIIa; a suggested mechanism is that binding of the gammacarboxyglutamic acid-containing zymogens to negatively-charged membranes increases their effective concentration as substrates. Prothrombin fragment 1, the 156 residue amino-terminal peptide from prothrombin, and factor X compete for binding sites on negatively-charged phospholipid surfaces. This phenomenon was exploited to determine how phosphatidylserine modulates the tissue factor pathway. In a tissue factor system containing 30% phosphatidylserine, prothrombin fragment 1 displaced phospholipid-bound factor X, increasing the fluid-phase factor X concentration and the rate of factor X activation nearly 4-fold. The increased reaction velocities correlated completely with the increases in the fluid-phase factor X concentration; therefore, fluid-phase factor X regulates the

tissue factor pathway. In the phosphatidylserine system the Km (calculated using the fluid-phase factor X concentration) was 41 nM and 63 nM in the presence and absence of prothrombin fragment 1, respectively. In contrast, the Km in a neutral phosphatidylcholine system (to which neither factor X nor prothrombin fragment 1 binds) was 877 nM and 791 nM in the presence and absence of prothrombin fragment 1, respectively. Thus, phosphatidylserine accelerates the tissue factor pathway not by concentrating substrate but by a direct effect on the catalytic complex, increasing the association of fluid-phase substrate. Binding of factor X to the phospholipid surface creates new pathways to encounter the catalytic complex: binding to the membrane surface with subsequent surface diffusion. Simulations indicate that surface diffusion of membrane-bound substrate should dominate the rate of substrate-enzyme encounters, yet membrane-bound substrate did not influence reaction rates. This finding combined with the fact that negatively-charged phospholipids inhibit factor X activation by three other proteases (which all attack the same peptide bond as that attacked by tissue factor-factor VIIa) leads to the conclusion that phospholipid-binding protects factor X from activation. Thus, contrary to the prevailing view in which binding of substrates to phospholipids enhances the rates of procoagulant reactions; phospholipid-binding of factor X inhibits its activation.

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My parents, who sacrificed more than I will probably ever know to give me the opportunities which I have had;

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CHAPTER 1

PERSPECTIVES AND OVERVIEW

Blood clots when monomeric fibrin polymerizes to form a fibrin meshwork. Monomeric fibrin is formed by proteolysis of a precursor protein, fibrinogen. This proteolytic event is the last of a series of proteolytic reactions involving interactions among at least 17 protein components, calcium ions and lipid. I will describe experiments aimed at understanding the effects of one component, negatively-charged phospholipids, on one coagulation reaction, the activation of factor X by a membrane-bound catalytic complex of tissue factor and factor VIIa. Although a literature review is included, I believe it will be helpful to summarize the nomenclature of the coagulation factors and current concepts regarding the overall structure of the coagulation process. This information will be useful not only in keeping track of the various clotting factors but also in gaining a perspective of where this work fits into the study of blood coagulation.

Although the details of the coagulation system are complex, there are common, unifying motifs. Protein coagulation factors are of two types. First, there are the serine proteases. These proteases circulate in precursor (zymogen) forms which are, by and large, catalytically inactive. During the coagulation process these zymogens are activated by limited proteolysis at specific peptide

bonds. However, even after activation certain of these "activated proteases" are essentially incapable of procoagulant activity except in complex with specific protein cofactors, which are the second type of protein coagulation factor. In addition to the protein components, phospholipid and calcium ions also potentiate procoagulant activity.

Precursor forms of blood coagulation factors (zymogens and inactive cofactors) are designated by Roman numerals (I-XIII). Activated forms are denoted by the appropriate Roman numeral followed by the suffix "a". Unfortunately, the order of enumeration does not relate to the order in which the factors interact but instead was derived from the order in which the factors were discovered. (Calcium is factor IV, but is no longer referred to by Roman numeral designation.) Table 1.1a is a glossary of the coagulation factors. Table 1.1b is a list of abbreviations used in this dissertation.

Table 1.1a Glossary of Coagulation Factors

Name (Roman Numeral)	Characteristics
Fibrinogen (I)	Circulates; Converted to fibrin by thrombin; Fibrin polymers form clot
Prothrombin (II)	Circulates; Vitamin K-dependent
Tissue Factor (III) (also, thromboplastin)	Membrane protein; Cofactor for factor VII(a)
Calcium (IV)	Divalent cation required for clotting activity and lipid binding
Factor V	Circulates and also released from platelets; Binds to acidic phospholipid; Cofactor for factor Xa
Factor VII	Circulates; Vitamin K-dependent; Activates factors IX & X
Factor VIII	Circulates; Association with von Willebrand's factor; Cofactor for factor IXa; Deficiency causes Hemophilia A
Factor IX	Circulates; Vitamin K-dependent; Deficiency causes Hemophilia B
Factor X	Circulates; Vitamin K-dependent; Factor X activation is central common pathway of coagulation
Factor XI	Circulates; In activated form can activate factor IX

Table 1.1a - Continuation

Factor XII	Circulates; Initiator of Contact System
Factor XIII	Circulates; Stabilizes fresh clots by crosslinking the fibrin
Prekallikrein	Kallikrein activates XII and HMWK
High-molecular weight kininogen	Bradykinin is released during activation.
Thrombomodulin	Membrane Protein; Modulates thrombin activity so that it preferentially activates Protein C, rather than factor X or platelets
Protein C	Activated by thrombin-thrombomodulin; Anti-coagulant; Proteolytically degrades factors Va and VIIIa
Protein S	Cofactor for Protein C
Plasminogen	Activated form dissolves newly formed fibrin clot

Most of the information and the general form of this table were taken from ref. [1].

Table 1.1b List of Abbreviations

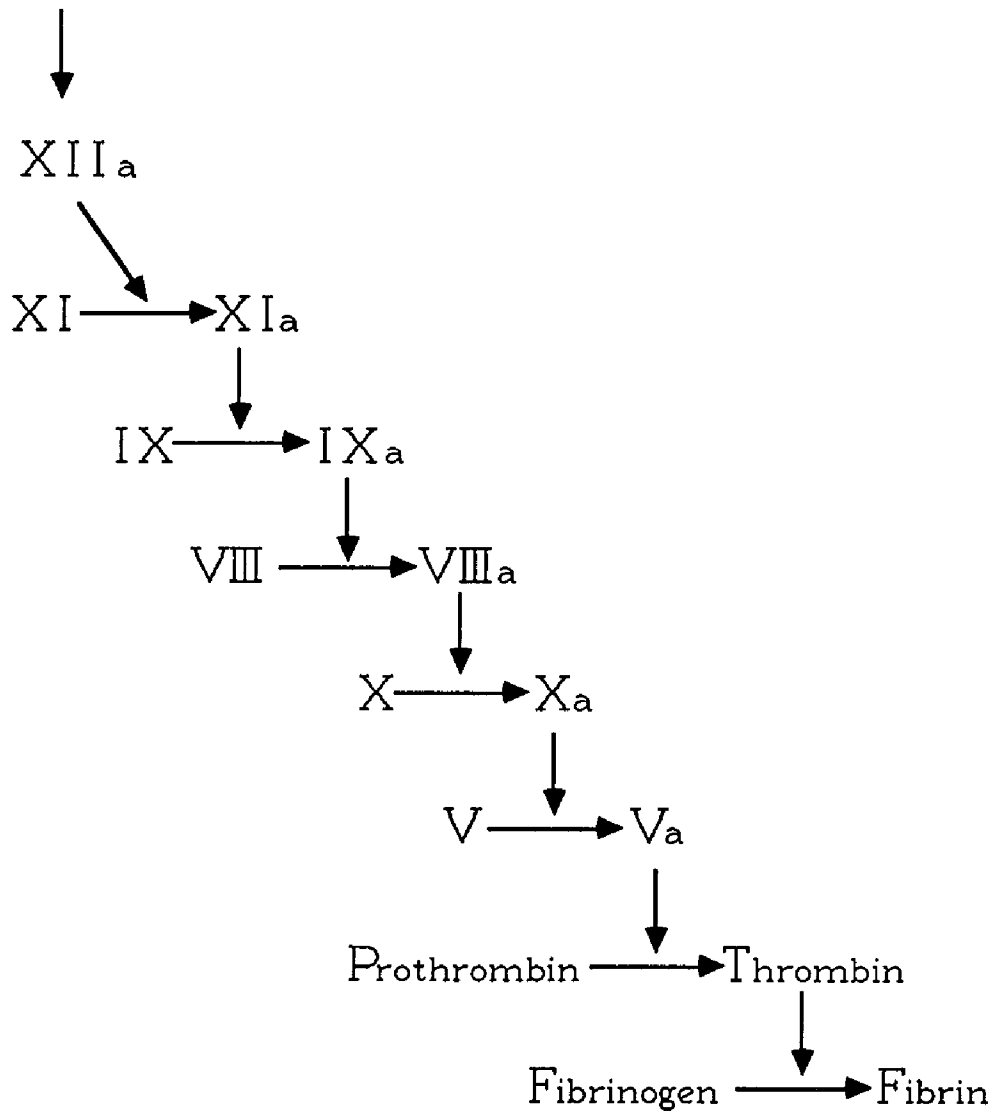
PS, phosphatidylserine; PC, phosphatidylcholine; PA, phosphatidic acid; PE, phosphatidylethanolamine; Gla, gamma-carboxyglutamic acid; DFP, diisopropylfluorophosphate; RVV-CP, factor X coagulant protein from Russell's viper venom; Hepes, N-2-hydroxyethyl piperazine-N'-2 ethane sulfonic acid

---

Although coagulation had already been studied for over 100 years, a watershed was reached in 1964 when Davie and Ratnoff and Macfarlane [2,3] independently proposed the "waterfall" or "cascade" hypotheses for blood coagulation (see Figure 1.1a). In these hypotheses, coagulation is initiated by contact of factor XII with a 'foreign' surface and proceeds via a linear sequence of zymogen activations in which each activated factor is a protease which catalyzes the subsequent activation reaction. The main concept of an amplifying series of reactions was astute, elegant and has influenced the thinking of a generation of biochemists; however, the original "cascade" models have required considerable modification.

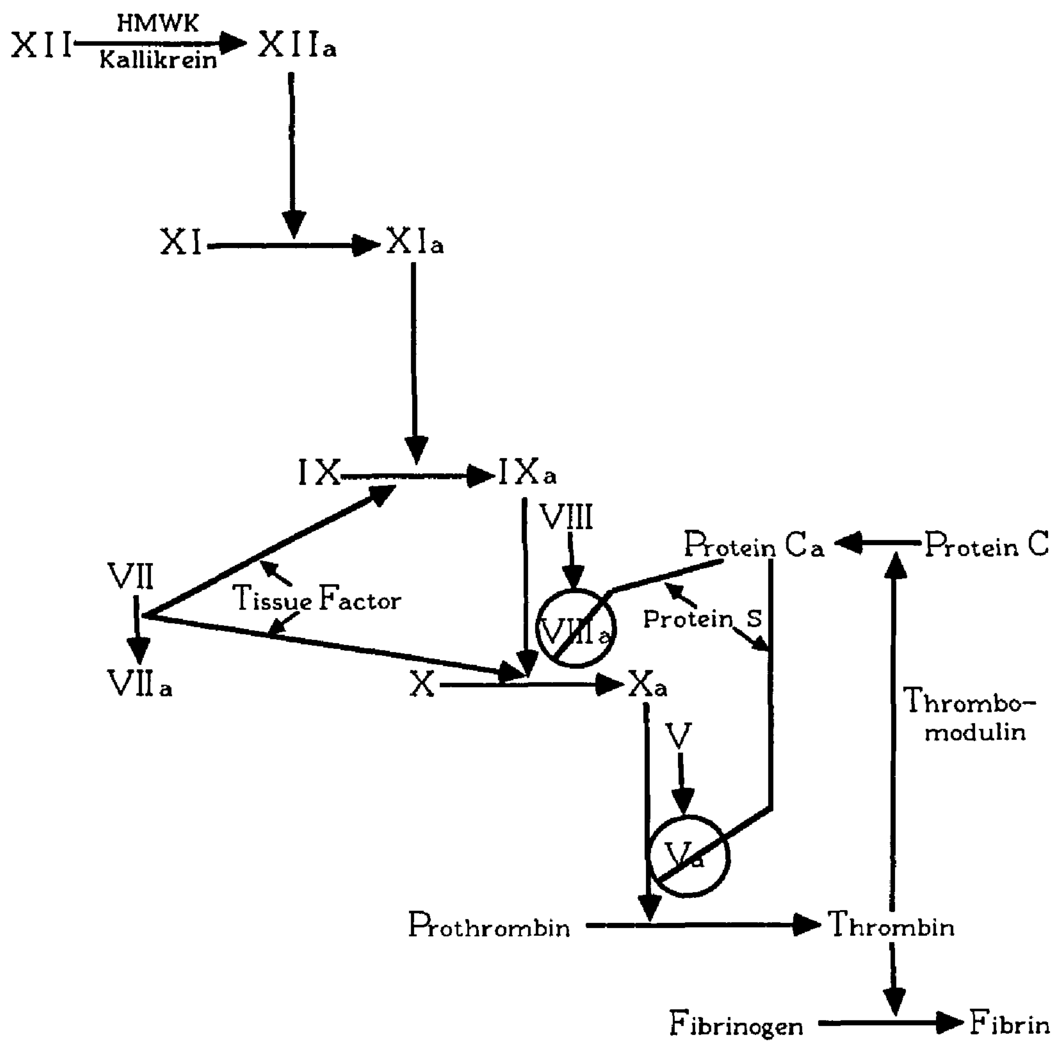
Originally, all the blood coagulation factors were considered to be proteases which were each successively activated in a simple, linear sequence. Recognizing the

Figure 1.1a



existence of the protein cofactors was the first major modification of the hypotheses. This recognition was more important than it first appears. The original scheme was a pure linear amplifying system with little provision for control once the process was initiated. The final output of fibrin depended only on the initial input of activated factor XII. Because the cofactors are not on the direct linear pathway, separate reaction stages can develop which can be independently regulated. Downstream events can influence upstream processes, especially by modifying cofactor activity. Figure 1.1b illustrates a simplified version of current concepts of the coagulation system; it is based on a diagram by Jackson and Nemerson [1], but incorporates the thrombomodulin-Protein C system [4]. Control is provided at two levels. First, several negative feedback reactions have been identified; some work by modifying or destroying cofactor activity (Protein C system - as shown in Fig 1.1b), others work by reacting with proteinase active sites (antithrombin III) or by degrading newly formed fibrin polymers (plasmin). (For clarity, these latter two types of negative feedback loops are omitted from Fig 1.1b). A second level of control is potentially supplied by positive feedback loops in which two of the cofactors (factors VIII and V) are activated by exposure to thrombin [5-7]. A balance between positive and negative control regulates clot formation.

Figure 1.1b



This leads into the second major modification of the original cascade: the identification of the initiating event. Tissue factor had a relatively minor role in the original schemes. Tissue factor-dependent reactions were called the Extrinsic Pathway because tissue factor does not circulate (i.e. it was *extrinsic* to the blood). Factor XII activation was the chief candidate for the initiating event of coagulation. In retrospect this position for factor XII activation is somewhat surprising because even in 1964 it was known that clot formation initiated by factor XII required minutes in vitro, while addition of tissue accelerated the process to about 12 seconds. Moreover, the absence of a hemorraghic disorder in patients exhibiting deficiency of factor XII casts additional doubt on its role in the initiation of coagulation. In contrast, inherited deficiency of tissue factor has not been described. Although this may be because tissue factor (and its deficiency) is physiologically inconsequential; alternatively tissue factor deficiency may be lethal with tissue factor having an essential role in coagulation. Later, I will present additional reasons why vascular damage leading to tissue factor exposure is the leading candidate for the initiating event of physiological coagulation.

The three cofactor-dependent procoagulant reactions are: tissue factor-mediated activity of factor VII(a) against factors IX and X; factor VIIIa-mediated activity of factor IXa against factor X; and factor Va-mediated activity of

factor Xa against prothrombin. How the cofactor-protease interactions modulate procoagulant activity is a much-studied subject (for Review see [8]). All three reactions above are enhanced by the presence of negatively-charged lipid surfaces. This thesis examines the contribution of negatively-charged phospholipids to the tissue factor pathway. Kinetic studies of tissue factor-mediated reactions have a major advantage over the more widely studied factor Va or VIIIa reactions. Factors Va and VIIIa reversibly adsorb to negatively-charged lipid surfaces. A general model for a cofactor-dependent coagulation reaction must include the equilibrium between the cofactor and the lipid surface. However, because tissue factor is an integral membrane protein, there is no adsorption equilibrium. Kinetic analysis is correspondingly simplified. Nonetheless, one can hope that our experimental protocols may be useful in studying the other cofactor-dependent coagulation reactions.

The main theme of the present work has been the development of techniques to distinguish lipid-dependent effects on substrate from lipid-dependent effects on the catalytic complex. Chapter 1 details historical development of the tissue factor pathway and the role of lipid in coagulation. Chapter 2 details the demonstration that fluid-phase substrate regulates the reaction rate, leading to the conclusion that negatively-charged lipids enhance factor X activation in the tissue factor pathway by direct

effects on the catalytic complex. The majority of this chapter is taken from a paper by Yale Nemerson and myself published in July 1986 in the Proceedings of the National Academy of Sciences USA. Chapter 3 provides a mathematical treatment describing the major contribution of nonspecific binding pathways (i.e. factor X binding to lipid with subsequent lateral diffusion to the enzyme) to the rate of encounters between substrate and enzyme. This information coupled with the experimental results leads to the conclusion that phospholipid binding protects factor X from proteolytic activation.

REVIEW OF LITERATURE

Identification of the Tissue Components of the Extrinsic Pathway

Damage to tissue initiates many processes including blood coagulation. In 1886 Wooldridge [9] produced extensive intravascular coagulation and death in dogs, cats and rabbits by injection of extracts from various tissues of calves. Wooldridge considered the extract to be "a compound of lecithin and proteid [protein]" because activity was abolished by either treatment with pepsin (which digests protein) or extraction with alcohol and ether (which removes lipid). In 1912 Howell [10] extracted two fractions from tissue both of which potentiated coagulation: 1) an aqueous extract and 2) a lipid fraction. The aqueous extract was similar to Wooldridge's extract and the activity was probably due to tissue factor. The lipid fraction, which Howell considered the more important, probably accelerated coagulation reactions mediated by soluble protein cofactors, factors VIII and V.

In the 1940s Chargaff and coworkers isolated an active fraction from lung which contained only lipid and protein. The fraction consisted "of a large percentage of almost perfect spheres with a diameter of 80 to 120 mμ", which were sedimented at 31,000 × g [11]. After exhaustive extraction of lipid with alcohol-ether mixtures, the protein portion had no clotting activity [12]. If the original

lipoprotein fraction (Chargaff's description) was mixed with sodium deoxycholate, centrifuged at 31,000 × g, and then both the pellet and the supernatant dialyzed, "about two-thirds of the protein [was recovered] in a non-sedimentable form retaining full thromboplastic activity" [13]. Chargaff had probably succeeded in solubilizing the tissue factor apoprotein with the detergent (solubilized apoprotein being non-sedimentable) and then reconstituting an "active thromboplastin" from the apoprotein and residual lipid upon removal of the detergent by dialysis. In this same paper Chargaff also demonstrated that tissue factor functioned in a "catalytic" fashion; i. e. tissue factor was not "consumed" during the activation of 80 mg of prothrombin. Chargaff's work had reestablished the importance of both the protein and the phospholipid moieties for procoagulant activity of tissue factor. More importantly, although the fact was not utilized for some years, he had established that the protein could be solubilized by detergent and then recovered in an active form. Detergent solubilization is still used in purifying the tissue factor apoprotein.

The specific phospholipid requirements for tissue factor activity were explored by Nemerson [14] who used acetone extraction followed by repeated butanol extractions to remove 95% of the lipid from a bovine brain homogenate. The extracted material was recombined in butanol with either purified phospholipids or mixed brain lipids and the solvent removed by evaporation. The activities of the

reconstituted preparations were determined by a two stage clotting assay. In the first stage, factors VII and X (purified from serum) were mixed with tissue factor and calcium and factor X activation proceeded for 1 minute. After the reaction was stopped, the extent of factor X activation (which was proportional to the tissue factor activity) was measured by adding an aliquot of this mixture to a plasma sample containing mixed phospholipids optimal for prothrombin activation. By performing the assay in this way, the final clotting time was related to the amount (or activity) of the tissue factor added and not to some enhancing effect of the phospholipids on some later stage of the reaction.

Of the purified lipids recombined with lipid-extracted tissue factor, phosphatidylethanolamine (PE) was the most active, followed by phosphatidylcholine (PC). Phosphatidylserine (PS) and phosphatidic acid (PA) were inactive. Tissue factor activity of the PE preparations had approximately twice the specific activity of the PC or mixed brain lipid preparations. Equal amounts of PE, PC and PS were associated with the protein (tissue factor) moiety. Therefore, the differences in activity were real effects of the differences in lipid composition and not just due to more or less lipid. The observation that neutral PC was capable of sustaining significant activity was contrary to the prevailing view which stated that negatively-charged phospholipids were required for coagulant activity [15].

One suggestion is that the PC is either contaminated with or has degraded to phosphatidic acid [16]. However, numerous tests by thin layer chromatography over a period of several years have never revealed evidence of contamination [17]. [In a real sense, the activity of PC in the tissue factor pathway was the seminal observation which, 18 years later, resulted in the present work. I will return to this observation later.]

At that time, the tissue extract was relatively crude and contained enough residual lipid that one could not state with assurance whether the tissue "activity" was due to a single protein or to residual lipid or, if a protein was reponsible, whether it had an absolute lipid requirement for activity. In 1969, Nemerson [18] used deoxycholate to solubilize tissue extracts from lung and brain (previously pioneered by Chargaff [13] and Hvatum, M., and Prydz, H. [19]). Stepwise precipitation with ammonium sulfate produced a "soluble" protein fraction which contained $< 1\%$ residual lipid and had no intrinsic coagulant activity. Upon relipidation with purified lipids or mixed brain lipids 5-10 fold increases in activity were obtained compared to the activity of the initial acetone extract. This active protein fraction (prior to relipidation) from both lung and brain extracts had eluted on Sephadex G-200 as a single peak with an apparent molecular weight of 425,000. [N. B.: Molecular weight determinations in this size range are fairly imprecise using G-200.] The lung and brain activities

both eluted as single peaks from TEAE-cellulose although at differing ionic strengths. The interpretation was that the "tissue activity" was due to a single, specific protein component, tissue factor, which may differ in different tissues. In this work, PE, PC and PS maintained their relative positions in ability to restore procoagulant activity; however, in contrast to the previous work, mixed lipids (containing up to 50% negatively-charged phospholipids) were the most active in restoring activity. As we now know that bovine tissue factor apoprotein has a molecular weight of 43,000 and is insoluble in aqueous buffers, the "soluble" fraction was probably a multimeric aggregate.

The phospholipid requirements of porcine tissue factor were described by Wijngaards et al. [16]. In contrast to the previously described results, they found that reconstitution with pure phosphatidylcholine resulted in inactive material and phosphatidylethanolamine reconstitution produced minimally active tissue factor. Binary mixtures of anionic and neutral lipids were quite active however. One possible reason for the discrepancy regarding PC and PE may relate to the assay used to determine tissue factor activity. Their assay has two stages: first, the tissue factor sample is incubated with factor VII and calcium; second, this mixture is added to VII-deficient plasma (containing a lipid extract from brain at a concentration optimum for prothrombin activation) and the clotting time measured. The problem may have been the formation of factor VIIa in the first

stage. Nemerson and Repke [20] have reported that tisssue factor enhances the rate of factor VII activation by factor Xa. Appreciable activation occurred within five seconds and the rate of activation was lipid-composition dependent. If Wijngaards et al. used factor VII preparations containing even a small amount of contaminating factor Xa, then the two-stage assay they used may actually have measured the phospholipid requirements of tissue factor-mediated activation of factor VII by factor Xa, rather than the rate of factor X activation by tissue factor-factor VII. This was probably not a problem in Nemerson's earlier work on phospholipid requirements [14] because the "factor VII" used had been purified from serum and was almost certainly already activated to factor VIIa [21].

The nature of the reconstituted tissue factor-lipid complex was explored by Pitlick and Nemerson [22] who showed that tissue factor is an intrinsic membrane protein. Using isopynic centrifugation in a sucrose gradient, active tissue factor complex came to equilibrium at a density between its constituent phospholipid and protein components. It did not dissociate thus demonstrating that tissue factor is an integral component of the membrane.

Over this entire period, steady progress was made in the purification of bovine tissue factor, culminating in its purification to homogeneity by Bach et al. in 1981 [23]. The purified bovine tissue factor apoprotein is an integral membrane glycoprotein with a molecular weight of

43,000 [23]. Tissue factor requires reconstitution into lipid vesicles for optimal activity (although, in detergent, tissue factor retains the capacity to bind to factor VIIa and to act as a cofactor in factor X activation [24]). Unlike factors V and VIII, tissue factor requires no proteolysis for full activity and inherited deficiency of tissue factor has not been described. Upon reconstitution into large unilammelar vesicles (LUV), tissue factor is randomly oriented [25]. Using the information that tissue factor and factor VIIa retain a calcium-dependent binding capacity in the presence of detergent, human tissue factor has recently been purified in several laboratories [24,26].

Factor VII

In 1957 Hjort [27] postulated calcium-dependent formation of a tissue factor-factor VII complex during coagulation. It was known that tissue extracts added to plasma produce factor X activation [28-31]. In 1966 Williams [32] and Nemerson [33] first showed that the complex of tissue factor and factor VII is necessary to activate factor X.

Two important observations were made by Nemerson [33]. First, the intermediate complex could be inactivated by diisopropylfluorophosphate (DFP) or soybean trypsin inhibitor (SBTI). This indicated that the complex might activate factor X in a proteolytic fashion. Second, after mixing factor VII and the tissue factor extract in the

presence of calcium, the active complex sedimented with the tissue pellet and could not be recovered in the supernatant. Although at this time it was not known whether factor VII or tissue factor was the enzymatic species, active fractions required an intact complex of tissue factor and factor VII; a dissociated active species was not detectable.

The observation that factor VII in plasma is sensitive to inactivation by DFP, while tissue factor is unaffected, suggested that factor VII is the enzyme species [34]. Factor VII circulates as a single-chain zymogen with a molecular weight of 53,000 [21,35]. Single-chain factor VII is rapidly hydrolyzed by factor Xa to yield a more active two-chain form, factor VIIa [21,35]. Unless purification procedures are performed entirely in the presence of protease inhibitors, two-chain factor VIIa is probably isolated [21]. Thus, most experiments performed prior to 1975, probably involved factor VIIa rather than factor VII. As factor VII was the only coagulation zymogen which is inactivated by DFP at relatively rapid rates [34] the idea arose that perhaps it possesses procoagulant capability (after complexation with tissue factor) even before activation to its two-chain form. It took several years to resolve the issue of whether the procoagulant activity of purified single-chain factor VII (in combination with tissue factor) was intrinsic or the result of minor contamination with factor VIIa. However, in an elegant paper, Zur et al [36]

demonstrated that zymogen factor VII has 0.8% of the proteolytic activity of factor VIIa. This experiment resolved a conundrum present in the "cascade-like", sequential zymogen activation models for coagulation. Namely, if zymogens have essentially no proteolytic activity, how does the cascade get started? How does the first zymogen get activated? However, factor VII requires no proteolysis for procoagulant activity, only complexation with tissue factor (which is not exposed on an unperturbed endothelial surface). Thus, endothelial stimulation or rupture exposing tissue factor is an attractive candidate for the initiating event of physiological coagulation.

Radiometric Assays

In 1974, Jesty et al. [37] showed that the tissue factor-factor VII complex hydrolizes the same peptide bond in factor X as is hydrolized by the factor X coagulant protein from Russell's viper venom (RVV-CP) [38]. This bond cleavage results in the release of a small peptide (9500 daltons) from the NH_2-terminus of the factor X heavy chain. Silverberg et al. [39] used this information to devise the first direct assay for the activation of factor X. The assay measured the release of the activation peptide (radiolabeled by reductive tritiation of its sialic acid residues) into 5% trichloroacetic acid (TCA). The zymogen, factor X, and the enzyme, factor Xa, precipitates in 5% TCA and hence can be separated from the activation peptide.

Previous assays were all based on elaborate variations of coagulation tests which do not allow isolation of single reaction pathways. The radioassay was a major advance in allowing the study of isolated reaction pathways in systems containing defined components.

A major development occurred in 1977 when Østerud and Rapaport described the activation of factor IX by tissue factor-factor VII complex [40]. This observation allows for explanation of the two major hemorraghic states (hemophilias A & B) within the context of tissue factor-initiated coagulation (see Fig 1.1b). [Previously, the tissue factor pathway had been thought to bypass factors VIII and IX. As these factors are clearly essential for physiological coagulation, the relevance of tissue factor was questioned.] Shortly thereafter, Zur and Nemerson used the radioassay technology to study the kinetics of factor IX activation [41]. The radioassay is an effective tool for measuring the activation of factors X and IX by tissue factor-factor VIIa complex because during both activations a small TCA-soluble peptide is released (See Fig. 1.2). Selective precipitation by TCA unfortunately cannot be utilized to measure the activation of factor VII to VIIa or factor IX to IXaα, because no peptide is released during these reactions.

Figure 1.2

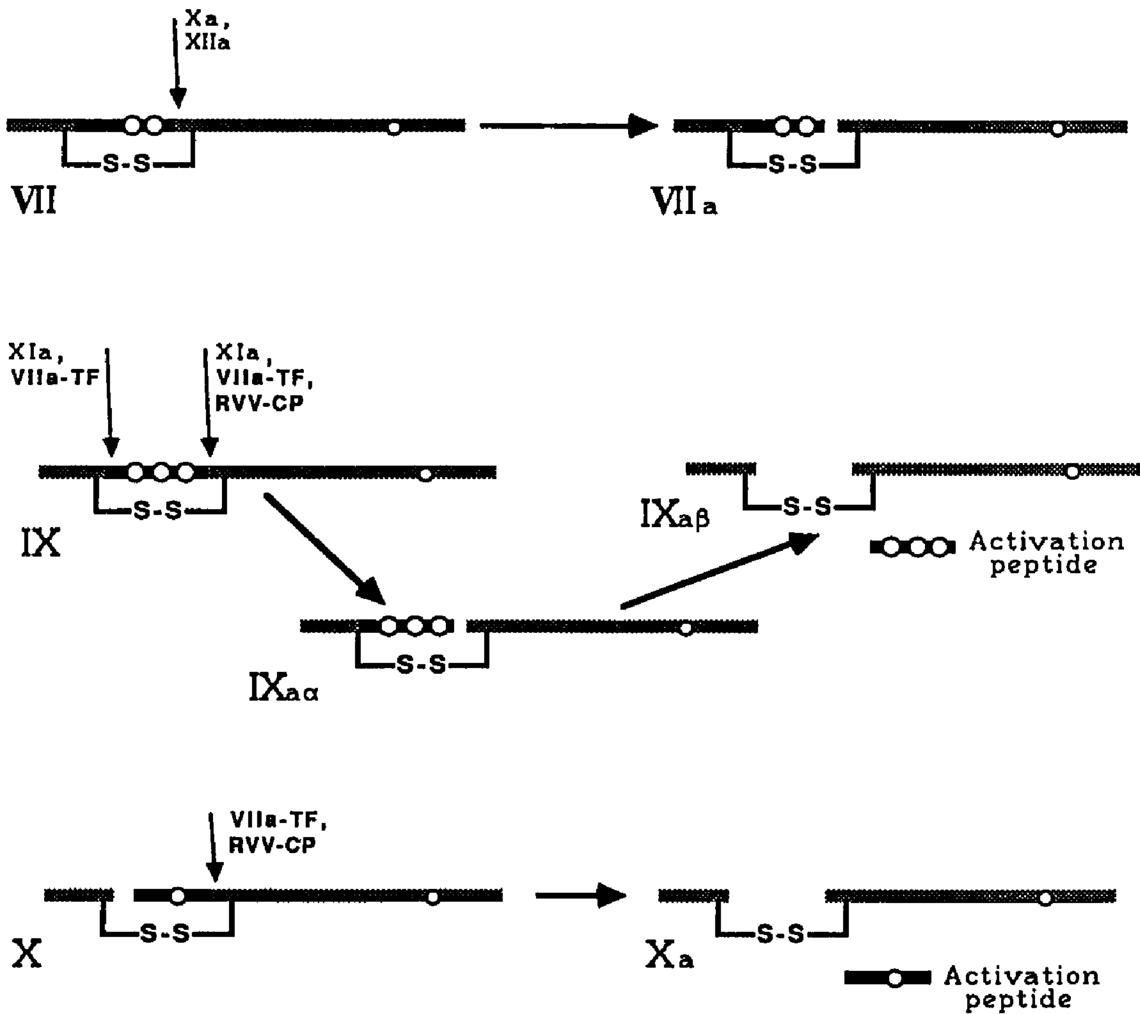


Figure after M. Zur and Y. Nemerson "Tissue factor pathways of blood coagulation" in Haemostasis and Thrombosis. (Churchill Livingstone, New York) pp. 130-131. Arrows denote cleavage sites; circles mark carbohydrate; figure is not drawn to scale.

A kinetic model for factor X activation by factor VIIa has recently been described by Nemerson and Gentry [42]. The formation of the ternary complex of tissue factor-factor VIIa-factor X is via an ordered addition of factor VIIa onto tissue factor followed by the addition of factor X.

The activity of tissue factor after reconstitution into neutral PC vesicles has proven extremely useful. Because neither factor X nor factor VIIa binds to PC vesicles, use of this phospholipid simplifies analysis of tissue factor pathway kinetics. There are simply fewer adsorption equilibria involved. The experimental reaction velocity data upon which the Nemerson-Gentry model is based were all obtained using tissue factor reconstituted into neutral PC vesicles. As mentioned above, PC was the second most potent of the pure phospholipids in reconstitutions of tissue factor activity. In other coagulation systems PC is generally considered inactive.

The Role of Phospholipids in Coagulation Reactions Other Than The Extrinsic Pathway

In 1958, Milstone found that prothrombin activation by factors Xa, V, and Ca^{2+} was accelerated by phospholipid [43]. Rouser et al. found neutral lipids inactive and PE from platelets most active [44]. In contrast Marcus and Spaet [45] found that PS was the most active phospholipid fraction. By the end of the 1950s there was a consensus that phospholipids accelerate prothrombin activation but

which phospholipids were active and the mechanism of the acceleration remained unknown.

In 1961 Bangham [15] showed that negative surface charge rather than specific phospholipid type was the determining factor in accelerating clotting. He correlated the electrophoretic mobility of varying dicetyl phosphoric acid:PC or phosphatidylethanolamine:PC mixtures with the clotting time of plasma after addition of Russell's Viper Venom (which contains a protease which activates factor X). Over the range of surface potentials studied, the relationship of clotting activity to potential was linear and increased with increasing negative charge. Papahadjopoulos et al. [46] confirmed Bangham's observations and found that clotting activity was optimal with phospholipid mixtures having an electrophoretic mobility of -4.5×10^{-4} $cm^2sec^{-1}volt^{-1}$; clotting activity decreased as the surfaces became more or less negative than this value. Why this level of negative surface charge provides for optimal activity is still unknown.

The connection between negative surface charge and enhanced rates of prothrombin activation has survived more than two decades. Recently however, Jones et al. [47] have measured the relative abilities of various phospholipid species "to enhance the prothrombinase-catalyzed conversion of prothrombin to thrombin." They found the following order for relative ability of phospholipids to enhance prothrombin activation: platelet-

derived membranes ≅ phosphatidylserine:phosphatidyl-choline (PSPC) ≅ phosphatidic acid:phosphatidylcholine (PAPC) >> monomethyl or monoethyl PAPC >> phosphatidyl-inositol:phosphatidylcholine (PIPC) or phosphatidylglycer-ol:phosphatidylcholine (PGPC). The latter lipid has an "appropriately", negatively-charged surface yet does not seem to enhance prothrombin activation. Clearly, factors other than simple charge are involved.

Nonetheless, negatively-charged phospholipids have also been found to accelerate the activation of factor X by factors IXa and VIIIa [48]. Thus, the activities of the three major protein cofactor-enzyme procoagulant complexes (tissue factor-factor VII, factor VIIIa-factor IXa and factor Va-factor Xa) are accelerated by the presence of acidic phospholipids. However, the question of mechanism has not been resolved and is one of the major reasons for the present study.

During the late 1960s it also became known that the Vitamin K-dependent coagulation factors (prothrombin, factors VII, IX and X) bind to negatively-charged phos-pholipid surfaces in the presence of Ca^{2+} [49-53]. This binding requires the presence of gammacarboxyglutamic acid (Gla) residues which are clustered in domains at the amino terminal portions of the above coagulation factors [54]. The Gla residues are produced by post-translational modification of glutamic acids by a reaction requiring Vitamin K [55]. The Gla residues are capable of binding di-

and tri-valent cations [56]. Upon the addition of calcium, the Glá-containing proteins undergo a decrease of intrinsic fluorescence which has been interpreted as indicating a conformational change [57]. There are two main theories for the mechanism of association with negatively-charged membranes: 1) the calcium ions bound to the protein and to the negatively-charged lipid surface serve to "bridge" [58,59] the two components; 2) the conformational change exposes hydrophobic residues which interact with the core of the membrane bilayer [60-63]. Because the second theory cannot conveniently explain the contribution of negatively-charged lipids to protein-lipid association, the first theory is generally more popular. For a given mixed lipid combination, there is a good correlation between negative charge and binding affinity (i.e. as you increase the PS content of a PSPC mixture both factor X and prothrombin bind more tightly [64]). However, the correlation between charge and affinity disappears between mixtures of different lipids. The affinity of prothrombin or factor X for 30:70 PSPC is nearly an order of magnitude greater than for an equivalent mixture in which phosphatidylglycerol (PG) was substituted for PS, even though the surface charge is nominally identical [64]. Moreover, the addition of a few percent PS to a 35:65 PGPC mixture provides an increase in affinity equivalent using a 35:65 PSPC mixture [64]. Thus, the exact role of negative surface charge in the binding process is controversial.

Macfarlane suggested that lipid surfaces might act as "catalysts absorbing specific proteins which then react together" [65]. Subsequently, several investigators have suggested that negatively-charged phospholipids accelerate coagulation by localizing and concentrating the substrates by adsorption at the membrane surface [50,66].

Adsorption of substrate to the negatively-charged membrane surface creates a new pathway for substrate to encounter enzyme: sliding in the plane of the membrane. Depending on the geometry of the system and the actual diffusion parameters, the influence of a surface diffusion pathway can theoretically range from inconsequential to total dominance (see Figure 3.3a-c). In the latter case, negatively-charged phospholipids should produce a decrease in the apparent Km for substrate activation. Rosing et al. [67] found that the apparent Km for prothrombin activation by factor Va-factor Xa complex decreases in the presence of negatively-charged phospholipids. They attributed the decrease in the apparent Km to phospholipid-substrate binding but the effect could as easily be caused by direct influence of the charged milieu on the catalytic complex favoring association. Consequently, there is current dispute whether bound substrate or fluid-phase substrate controls the rate of prothrombin activation [67-70].

SUMMARY OF THE PROBLEM AND APPROACH

The presence of negatively-charged phospholipids enhances the activity of several procoagulant reaction pathways, including the activation of factor X by tissue factor-factor VIIa. Because the presence of negatively-charged phospholipids also results in binding of factor X to the membrane surface, the prevailing view has been that the kinetic enhancements are related to, and indeed caused by, the binding process. That is, nonspecific binding of substrate to the phospholipid surface creates new pathways for substrate to encounter enzyme: binding to the membrane surface with subsequent lateral diffusion. Potentially, these additional pathways could increase the rates of substrate-enzyme encounters. Does the reaction rate enhancement in the presence of negatively-charged phospholipids result from increased substrate-enzyme encounters from surface diffusion or has the charged environment altered the catalytic complex directly?

Because both hypothetical mechanisms for producing the reaction rate enhancement depend on the presence of negatively-charged phospholipids, one cannot distinguish between the two possibilities by adjusting the variable of lipid composition. My approach was to obtain a ligand which would compete for phospholipid binding sites with substrate, but which would not interfere with specific substrate-catalytic complex recognition. By using such a reagent to displace surface-bound substrate, one could

determine the contribution of surface binding to the rate of substrate activation without varying the negatively-charged environment.

The Gla-containing, amino-terminal domain can be selectively cleaved from bovine prothrombin using bovine thrombin [71,72]. The isolated 156-amino acid residue peptide, prothrombin fragment 1, retains the capacity to bind to negatively-charged phospholipid vesicles in the presence of Ca^{2+} [73]. Prothrombin fragment 1 is known to competively inhibit prothrombin activation in the presence of negatively-charged phospholipids [74]. This inhibition was attributed to competition with prothrombin for phospholipid binding sites. Unfortunately, because the fragment is derived from the natural substrate, one cannot rule out interference with specific enzyme-substrate recognition. In the tissue factor pathway, prothrombin fragment 1 is potentially a "cleaner" reagent because it is not derived from a natural substrate of tissue factor-factor VIIa.

If tissue factor exposure initiates coagulation in vivo then understanding the contribution of the phospholipid environment is important. Moreover, the system may provide a model for studying the contribution of surface diffusion pathways to ligand-receptor (or substrate-enzyme) association.

In this thesis I will try to establish that fluid-phase factor X is the "true" substrate for activation by tissue factor-factor VIIa; that negatively-charged phospholipids accelerate the activation of factor X by a direct effect on the cofactor-enzyme complex leading to a 90% decrease in the "intrinsic" Km; and that, contrary to the prevailing view in which binding of substrates to phospholipids enhances the rates of procoagulant reactions, in fact, phospholipid-binding of factor X inhibits its activation by tissue factor-factor VIIa complex as well as several snake venoms. The latter assertion is based on numerical simulations of bimolecular association using models of Berg [75] and Wiegel and DeLisi [76].

CHAPTER 2

INTRODUCTION

The specific binding of ligands to receptor molecules embedded in cell membranes mediates many biological functions. In coagulation the binding of zymogens (factors IX, X and prothrombin) to catalytic complexes localized on membranes regulates the rate of several reactions (for review see ref. [1]). Analysis of these reactions is complicated because the zymogens also bind non-specifically to membrane surfaces, thus introducing uncertainty about the effective substrate concentration. The substrates can reach the enzymatic complexes either by fluid-phase diffusion or by binding to the membrane surface and then "sliding" in the plane of the membrane. Although these mechanisms are not mutually exclusive, the dominant process will govern the substrate-enzyme association rate. While the theory of both processes has been extensively developed [77,78], there have been few attempts to distinguish experimentally which mechanism regulates specific systems.

I studied the activation of bovine coagulation factor X by tissue factor-factor VIIa complex. This complex is analogous to a receptor in several respects. Tissue factor is an integral membrane protein which requires insertion into phospholipid vesicles for biological activity [14,79]. The formation of a 1:1 complex of the enzyme, factor VIIa, with

tissue factor is essential for the activation of factor X [42]. Factor X, upon binding to the enzyme-activator ("receptor") complex, is hydrolytically converted into factor Xa and an activation peptide. Because Factor X binds to negatively-charged phospholipids [49,64], two pools of substrate exist: one free in solution and one membrane-bound. To interpret kinetic data, one must know the concentration of substrate in the pool being hydrolyzed. If fluid-phase diffusion dominates, then the concentration of free factor X will control the association rate of factor X with enzyme. If surface diffusion dominates, then the concentration of membrane-bound factor X will govern the rate of association with enzyme. Thus, the rate of factor X activation should directly depend on the concentration of one of the two factor X pools. These concentrations, however, are not independent; the concentration of free factor X determines the concentration of surface-bound factor X. In this study, I "uncoupled" the two pools by adding a reagent, prothrombin fragment 1, which competes with factor X for membrane binding sites, but which I show does not interfere with the specific association of factor X with enzyme. Because prothrombin fragment 1 simultaneously decreases the concentration of surface-bound factor X and increases the concentration of free factor X, I was able to correlate reaction velocity with differing concentrations of each pool. I show that free factor X dominates the rate of factor X activation. Thus, the

binding of substrate to membrane does not enhance substrate-enzyme association and free factor X is the "true" substrate in the tissue factor pathway of coagulation.

MATERIALS & METHODS

Reagents

DEAE-Sephadex, Sepharose CL-2B and Sephadex G-100 are products of Pharmacia. Affigel-10 is the product of Bio-Rad. Phosphatidylcholine (PC) from egg and phosphatidylserine (PS) from bovine brain were obtained from Supelco (Bellefonte, PA). Dansyl-L-glutamyl-L-glycyl-L-arginine chloromethyl ketone and n-octyl-β-D-glucopyranoside were obtained from Calbiochem-Behring; Hepes was from Research Organics (Cleveland, OH). Formula 963 scintillation cocktail (New England Nuclear-Dupont) was used. All other reagents were of reagent grade or better and were obtained from standard sources.

Buffers

Tris: 50 mM Trizma base, 100 mM NaCl, adjusted to pH 7.5 with HCl. Hepes: 10 mM Hepes, 150 mM NaCl, adjusted to pH 7.4 with NaOH. Where indicated bovine serum albumin at 1 mg/ml was added.

Proteins

Protein concentrations were determined from absorbance at 280 nm using values for $A^{1\%}_{1cm}$ of 12.9, 10.1, 9.6 and 14.4 for bovine factor VII [80], prothrombin fragment 1 [72], factor X [81], and prothrombin [72], respectively. Proteins were stored at -80°C. Coagulation factors VII, X [82], and prothrombin were prepared from a barium citrate eluate of bovine plasma by chromatography on DEAE-Sephadex [83]. Factor VII, which was purified to

homogeneity by chromatography on an anti-factor VII immunoaffinity column [84], was converted to its more active 2-chain derivative, factor VIIa, by factor Xa [83]. Purified factor X coagulant protein from Russell's viper venom (RVV-CP) was a generous gift of Doris Repke.

Prothrombin fragment 1 was prepared from prothrombin by incubating 200 mg of prothrombin with 250 units of bovine thrombin (Calbiochem-Behring, 2,000 units/mg) in 125 ml of Tris buffer at 37°C for 40 hours. The reaction was stopped by adding diisopropylfluorophosphate to 1.5 mM. After 30 minutes, solid sodium citrate was added to 40 mM. The solution was cooled to 4°C and 20 ml of 1M barium chloride was added dropwise. After 15 min, the mixture was centrifuged for 40 min at 17,000 $\times$ g at 4°C. The pellet was washed twice with 50 ml of 5 mM barium chloride and then dissolved in a minimum amount of 0.2 M EDTA, pH 7.5. After dialysis vs. 2 liters of Tris buffer, trace amounts of factor VII were removed by passage over a 50 ml anti-factor VII immunoaffinity column. The sample was concentrated to <5 ml on an Amicon PM-10 membrane and then gel filtered on a Sephadex G-100 column (2.5$\times$100 cm) using 500 ml of Hepes buffer. The final material was homogeneous by polyacrylamide gel electrophoresis [85] in sodium dodecyl sulfate under non-reducing conditions; after reduction two bands of similar molecular weight were present indicating probable loss of a small terminal peptide [86].

Purified bovine tissue factor [87] was reconstituted with PSPC or PC using n-octyl-β-D-glucopyranoside [88] to solubilize the lipid and protein. The final tissue factor:phospholipid molar ratio was 1:100,000. Identically prepared vesicles (without tissue factor) were added to obtain the final phospholipid concentrations. Tissue factor is randomly oriented when inserted into phospholipid vesicles [25], thus tissue factor concentrations used for all calculations are 0.5×[total tissue factor].

Radioactive labeling

The carbohydrate moieties of factor X were reductively labeled with sodium [^{3}H]borohydride (New England Nuclear-Dupont, 5-15 Ci/mmol) using the method of Van Lenten and Ashwell [89], as described [90]. The specific radioactivity of the [^{3}H]-factor X was 150,000 cpm/ug. This value was obtained by measuring the radioactivity of 3×10μl aliquots of a factor X solution (of defined protein concentration) in 3 ml of scintillation cocktail containing 100μl H_2O. All radioactivity measurements were performed in a Beckman LS1801 scintillation counter.

Phospholipids

Vesicles were either pure PC or a mixture of PSPC (30:70, w:w) and were prepared by mixing PS and PC in chloroform and drying under nitrogen. Solid n-octyl-β-D-glucopyranoside at a weight ratio of 5.6:1 was added along with a tracer quantity of [^{14}C]phosphatidylcholine (New

England Nuclear-Dupont). The mixture was dissolved in 1 ml Hepes buffer and dialyzed vs. three changes of 500 ml of the same buffer at 24°C for 72-96 hours. The vesicles were gel filtered on a 1.5 × 55 cm column of Sepharose CL-2B and eluted with Hepes buffer. The peak fractions, which were located by measuring light scattering at 280 nm, were pooled; the phospholipid concentration was determined from the ^{14}C content.

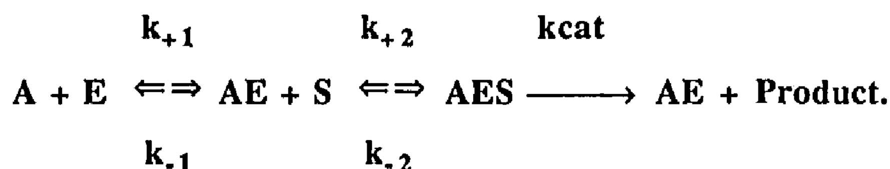
Kinetic Assays

The rate of hydrolysis of factor X by either tissue factor-factor VIIa complex or RVV-CP was measured by a radiometric assay as previously described [90]. Briefly, the radiolabeled activation peptide is extracted at appropriate intervals into 5% trichloroacetic acid. The concentration of the peptide is determined by liquid scintillation spectroscopy. Each velocity determination consisted of seven time points counted in duplicate. Reaction mixtures contained 0.025 nM tissue factor, 500 μM phospholipid (either PC or PSPC), 50 nM factor VIIa and varying amounts of [^{3}H]-factor X. Reactions were run as pairs with and without 6 μM prothrombin fragment 1. For factor X activation reactions catalyzed by Russell's viper venom similar conditions were used with 21 ng/ml RVV-CP replacing factor VIIa and Hepes/albumin buffer replacing tissue factor.

All components except factor VIIa were mixed together in Hepes/albumin buffer in the presence of 5 mM

$CaCl_2$, incubated for 30 min at 25°C and the reaction started by addition of factor VIIa. RVV-CP catalyzed reaction mixtures were assembled in the same manner at 37°C. To prevent hydrolysis of factor X by factor Xa, 2 μM dansyl-L-glutamyl-L-glycyl-L-arginine chloromethyl ketone was added. To test for the existence of an effect of prothrombin fragment 1 on the kinetics of factor X activation, the relative velocity ratios (+prothrombin fragment 1/-prothrombin fragment 1) of all reaction pairs were compared to a value of 1 (no effect) using the Wilcoxon rank sum test [91].

The hydrolysis of factor X (S) by tissue factor (A) and factor VIIa (E) is an essential activation reaction [42] (Note that the model assumes product release without requiring AE dissociation):



When enzyme is much greater than tissue factor and k_{-1}/k_{+1} (i.e., when the activator is saturated), one can model this system with an equation of the Michaelis-Menten form [92]: velocity = kcat × A_T × S/(K2+S), where K2 = $(k_{-2}+kcat)/k_{+2}$. I used 50 nM enzyme (K1 is 4.5 nM [25]) and have determined that increasing this concentration does not affect reaction velocities (reaction rates for factor X activation at 25, 50 and 100 nM factor VIIa were 0.80±0.21, 0.84±0.40 and 0.93±0.34 $nM{\cdot}min^{-1}$

respectively in the absence of prothrombin fragment 1 and 2.16±0.15, 2.66±0.29 and 2.39±0.17 $nM \cdot min^{-1}$ respectively in the presence of 6 µM prothrombin fragment 1 using 500µM PSPC). Kinetic parameters, kcat and Km, were obtained by fitting the above equation to the appropriate data using Marquardt's non-linear least squares algorithm [93]. The results are shown +/- the standard error. Note that the Km determined is only an apparent constant and depends upon which factor X concentration is used for calculation: free, phospholipid-bound, or total. Only when the appropriate concentration is used is Km equivalent to K2, a physical property of the catalytic complex.

I have ignored the effect of AES formation on the concentration of free and bound factor X. The maximum possible concentration of AES in these experiments is limited by the smaller of A or E; in this case, tissue factor at 0.025 nM. As this concentration is negligibly small with respect to any of the factor X concentrations used or calculated from binding parameters, the approximation is probably valid.

<u>Binding Studies</u>

Mixtures (0.1 ml in Hepes/albumin buffer) containing 0.025 nM tissue factor, 500 µM phospholipid, 2 µM dansyl-L-glutamyl-L-glycyl-L-arginine chloromethyl ketone, 5 mM $CaCl_2$ and various amounts of [^{3}H]-factor X added alone or with prothrombin fragment 1 (6 µM) were incubated in cellulose propionate tubes for 30 min at 25°C. Then, the

samples were centrifuged at 150,000 × g at 25°C for 20 min in a Beckman Airfuge. The phospholipid vesicles sediment accompanied by bound protein. Free factor X was assayed by removing a 25 μl aliquot from the supernatant and measuring its radioactivity. Total factor X was determined from identically prepared samples (buffer replacing phos pholipid) which were not centrifuged. Bound is the difference between total and free.

Because vesicle sedimentation is never 100% complete, some of the bound factor X remains in the free fractions. I used the ^{14}C content of all our vesicle preparations to quantify sedimentation. Total phospholipid radioactivity was determined from triplicate, uncentrifuged samples (prepared without [^{3}H]-factor X). Fractional recovery, FR, was determined from the ^{14}C content as follows:

$$FR = 1 - (^{14}C \text{ in supernatant/total } ^{14}C).$$

The following expressions were used to calculate corrected [factor $X]_{bound}$, $[X_B]$, and [factor $X]_{free}$, $[X_F]$, from the measured concentrations of bound and free factor X, $[X_{B,m}]$ and $[X_{F,m}]$. The measured total factor X, $[X_T]$, requires no correction.

$$[X_B] = [X_{B,m}]/FR$$

$$[X_F] = [X_T] - [X_B]$$

The equilibrium dissociation constant, K_d, and the maximum binding capacity, n, were determined by fitting:

$$[X_B]=n \times [\text{phospholipid}] \times [X_F]/(K_d + [X_F])$$

to the corrected data using Marquardt's non-linear least squares algorithm [93]. The results are shown +/- the standard error. As the fractional recoveries were always greater than 0.9, the effect of the data corrections was minimal.

Control experiments to validate the binding method showed that equilibrium was established within the first 10 min of the incubation period. The binding of [^{3}H]-factor X was reversible, being completely abolished by addition of large excess of unlabeled ligand. Finally, in the absence of phospholipid, less than 1% of the protein sediments.

RESULTS

Factor X binding in the presence and absence of prothrombin fragment 1

Figure 2.1 shows the binding of factor X to PSPC vesicles as a function of free factor X. Comparing samples containing equivalent total factor X concentrations, one notes that addition of prothrombin fragment 1 decreases the concentration of bound factor X at all values of total factor X measured.

K_d and n for factor X binding were 0.52 $\pm$ 0.04 μM and 8.91 $\pm$ 0.12 μmols factor X bound/mmol PSPC, respectively (without the phospholipid recovery corrections these values are 0.60 $\pm$ 0.03 μM and 8.37 $\pm$ 0.14 μmols factor X bound/mmol PSPC, respectively). The maximal binding capacity agrees quite well with reported values [64,94-96]. Reported dissociation constants range from 0.04 μM [95] -

2.5 μM [94]; our determination is in excellent agreement with the majority of these values. The addition of 6 μM prothrombin fragment 1 resulted in the following apparent parameters for factor X binding: K_d app, 1.43 ± 0.21 μM and n app, 7.32 ± 0.29 μmols factor X bound/mmol PSPC (without the phospholipid recovery corrections these values are 1.44 ± 0.06 μM and 6.75 ± 0.10 μmols factor X bound/mmol PSPC, respectively). These results are consistent with the hypothesis that prothrombin fragment 1 and factor X compete for phospholipid binding sites (i.e. a large effect on apparent binding affinity with minor effects on the maximal binding). Neither factor X nor prothrombin fragment 1 binds to pure PC vesicles.

Kinetic effects upon addition of prothrombin fragment 1

Paired reaction mixtures with and without prothrombin fragment 1 were assembled containing various total factor X concentrations, with all other constituents being constant. Figure 2.2 shows the ratio of the reaction velocities in each pair (velocity with prothrombin fragment 1 divided by the velocity without prothrombin fragment 1) plotted versus the total factor X concentration of each pair. The inset shows the effect of added prothrombin fragment 1 using a lower concentration (25μM) of PSPC.

Figure 2.1

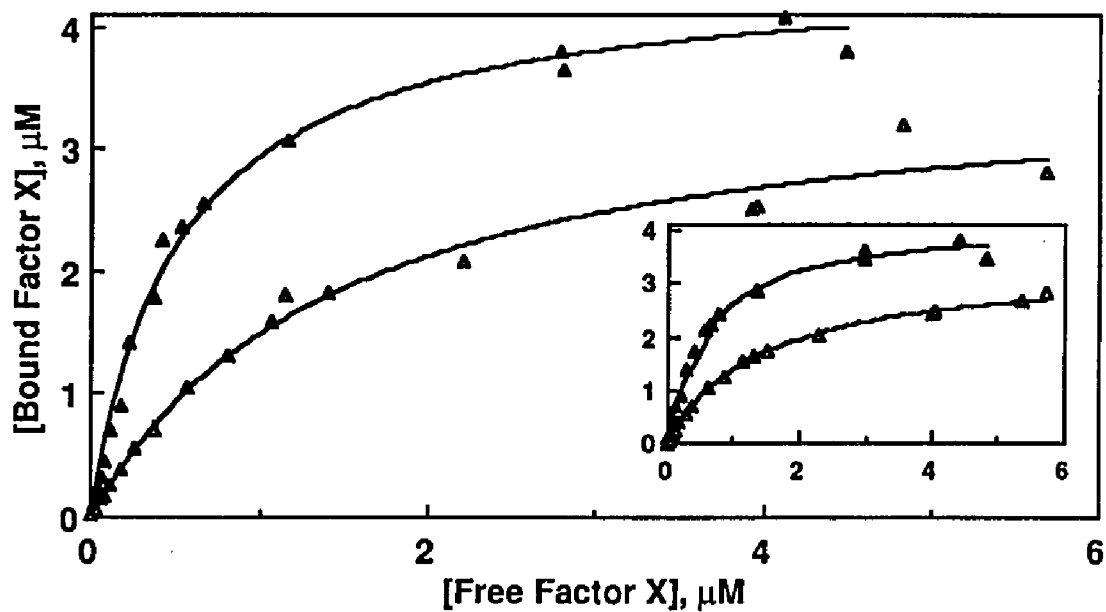


Figure 2.2

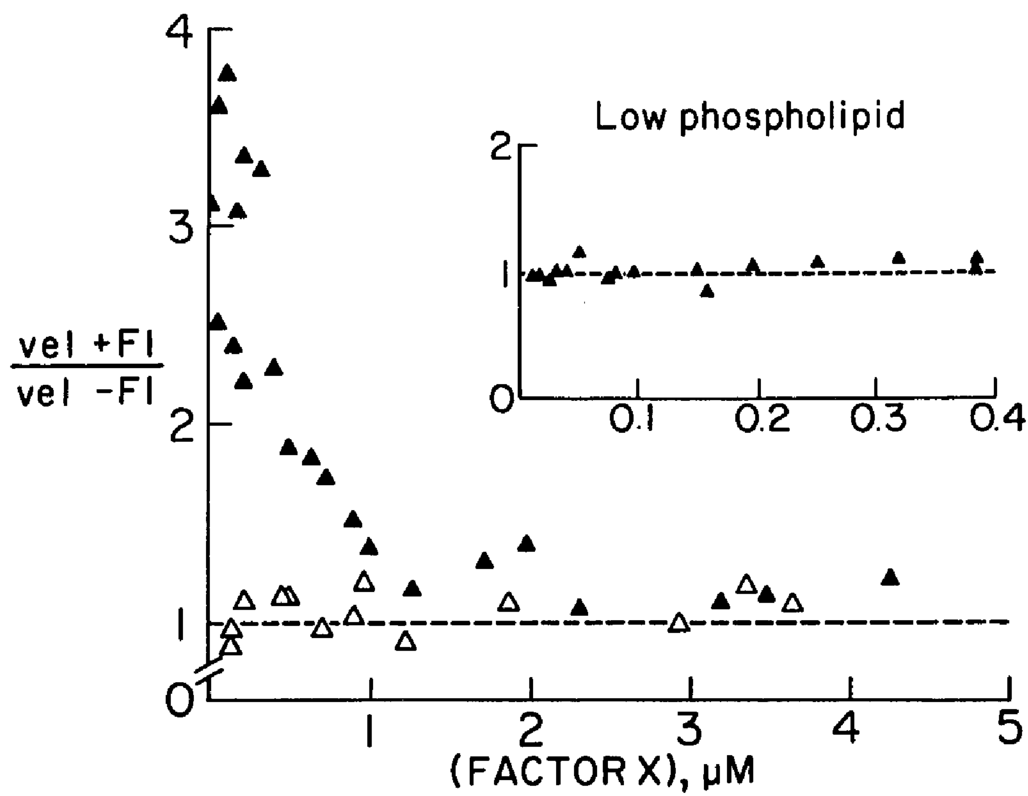


Fig. 2.1. Effect of addition of prothrombin fragment 1 on the binding of factor X to PSPC vesicles. Upper fitted curve is for data in the absence of prothrombin fragment 1. Lower fitted curve is for data in the presence of 6 μM prothrombin fragment 1. ▲, Factor X alone; △, factor X and prothrombin fragment 1. The inset shows the raw data without the phospholipid recovery corrections.

Fig. 2.2. Effect of prothrombin fragment 1 on factor X activation. Data from paired reactions are plotted as the ratio of reaction velocities (determined in the presence of 6 μM prothrombin fragment 1 divided by that in the absence of prothrombin fragment 1) versus the total factor X concentration contained in each pair. The dashed horizontal line indicates a ratio of 1 (no effect of prothrombin fragment 1). △, Tissue factor is reconstituted with PC (500μM). ▲, The PSPC (500μM) system. The inset shows the PSPC system containing 25μM phospholipid.

Using tissue factor reconstituted with PC (500μM), I found that prothrombin fragment 1 caused no significant effect on the rate of factor X activation. In 5 of 13 pairs the reaction velocity decreased with prothrombin fragment 1 (Wilcoxon rank sum T = 25, $0.1 < P < 0.2$). The kcat and Km for factor X activation in this system are shown in Table 2.1. In accord with the Wilcoxon analysis, the parameters with and without prothrombin fragment 1 are essentially the same. Note that the kinetic parameters are expressed only in terms of free factor X concentration because factor X does not bind to the neutral PC vesicles.

In contrast when tissue factor reconstituted with PSPC (500μM) was used, the addition of prothrombin fragment 1 caused a velocity increase in 22 of 22 pairs (Wilcoxon rank sum T = 0, $P < 0.0001$). At the lower concentration of PSPC (see Inset), prothrombin fragment 1 addition caused a velocity increase in 10 of 16 pairs. This difference was insignificant (Wilcoxon rank sum T = 53, $0.1 < P < 0.2$).

Table 2.1 shows the kinetic parameters, Km and kcat, for the PSPC system, calculated on the basis of either free or phospholipid-bound factor X concentrations. The parameters calculated using the free factor X concentration are essentially unaffected by prothrombin fragment 1. Note that the parameters for the 25μM PSPC system are actually calculated on the basis of total factor X concentration. However, at this low concentration of sites free factor X concentration ≅ total factor X concentration.

Table 2.1: Kinetic Parameters Calculated Using Bound and Free [Factor X].

Lipid	Frag. 1	Km_{bound} (nM)	$kcat_{bound}$ (s^{-1})	Km_{free} (nM)	$kcat_{free}$ (s^{-1})
PSPC (0.5mM)	-	625±103 [20-3100]	6.6±0.4	63±9 [2-1200]	5.8±0.3
	+	108±14 [15-2200]	6.5±0.2	41±5 [6-2100]	6.3±0.2
PC (0.5mM)	-	NA	NA	791±103 [120-3600]	6.2±0.3
	+	NA	NA	877±111 [120-3600]	6.7±0.3
PSPC (0.025mM)	-	NA	NA	39±4 [10-400]	5.6±0.1
	+	NA	NA	40±4 [10-400]	5.7±0.2

Table 2.1 - Continuation

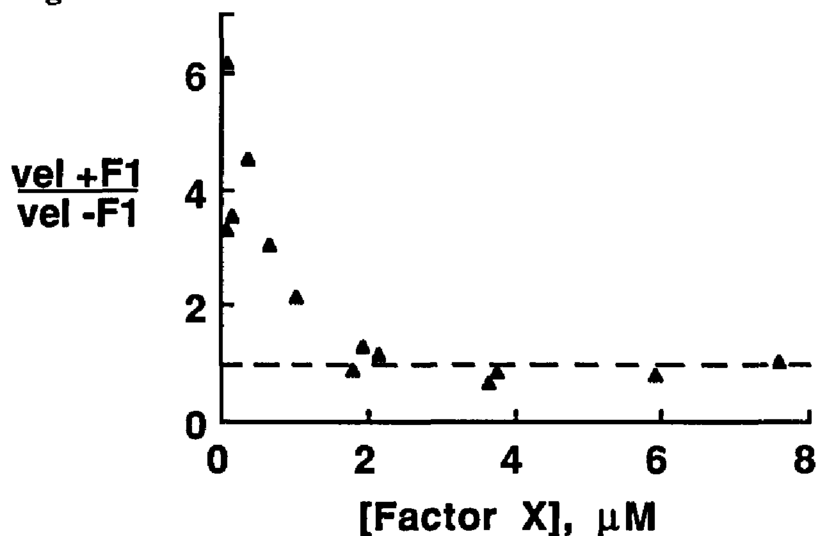
For each reaction velocity determination, bound and free factor X were calculated from:

$$[X_B] = \{(R + K_d + [X_T]) - ((R + K_d + [X_T])^2 - 4*R*[X_T])^{1/2}\}/2$$

and $[X_F] = [X_T] - [X_B]$, where R is n×[PSPC]. K_d and n are defined in the text. Frag. 1 is prothrombin fragment 1. Figures in square brackets give the range of substrate concentrations (nM) used in the determination. Because there is no binding to PC vesicles, kinetic parameters were calculated only in terms of free substrate. Similarly at 0.025mM PSPC, $[X_T] \equiv [X_F]$, therefore total factor X concentrations were assumed equivalent to free factor X in calculating kinetic parameters. NA, not applicable.

Fig. 2.3 shows the effects of prothrombin fragment 1 addition on factor X activation reactions catalyzed by fluid-phase RVV-CP in the presence of 500μM PSPC. Data from paired reactions are plotted as the ratio of reaction velocities (determined in the presence of 6μM prothrombin fragment 1 divided by that in the absence of prothrombin fragment 1) versus the total factor X concentration contained in each pair. The dashed horizontal line indicates a ratio of 1 (no effect of prothrombin fragment 1). Increased reaction velocities were obtained upon prothrombin fragment 1 addition in 9 of 13 pairs (Wilcoxon rank sum T = 16, $P < 0.05$).

Figure 2.3



Figures 2.4a-d show plots of reaction rate vs. factor X concentration for the tissue factor-factor VIIa catalyzed reactions described above. Figure 2.4a shows total [factor X] vs. reaction rate for the 500μM PSPC system. Figure 2.4b shows calculated free [factor X] vs. reaction rate for the 500μM PSPC system. Figure 2.4c shows calculated bound [factor X] vs. reaction rate for the 500μM PSPC system. Figure 2.4d shows total [factor X] vs. reaction rate for the 500μM PC system. The filled symbols are used for data in the presence of 6 μM prothrombin fragment 1. Solid curves are defined by the appropriate parameters in Table 2.1.

Figures 2.5a-d shows residual plots for the data of figures 2.4a-d obtained from the non-linear least squares curve fitting procedure. Residuals are the difference between the fitted curves in figures 2.4a-d and the actual data. The order of figures and the symbols used to represent the data are as described for figures 2.4a-d. A systematic pattern of residuals was not evident.

Figure 2.4a

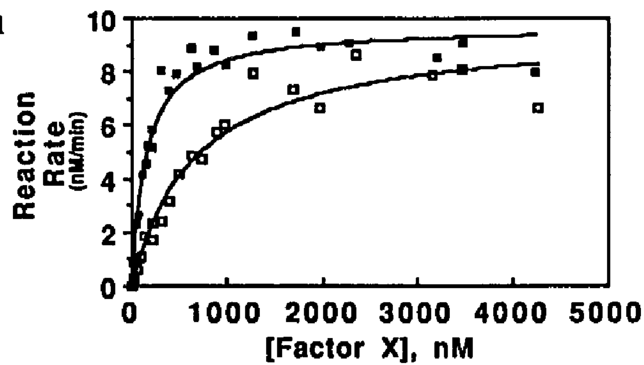


Figure 2.4b

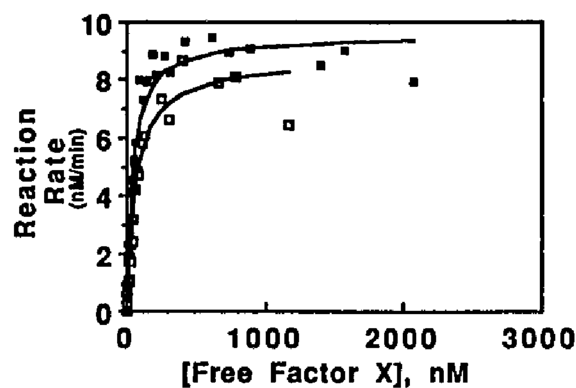


Figure 2.4c

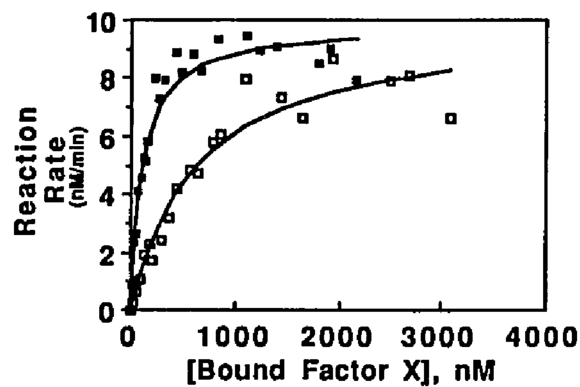


Figure 2.4d

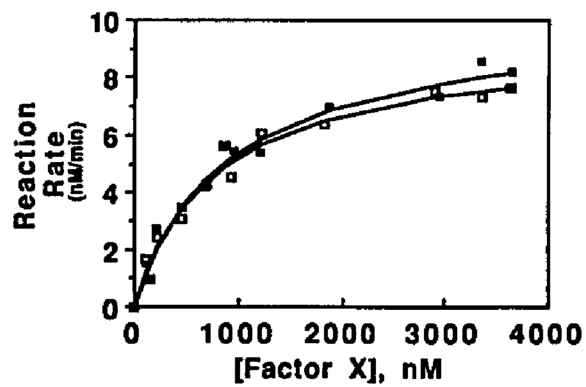


Figure 2.5a

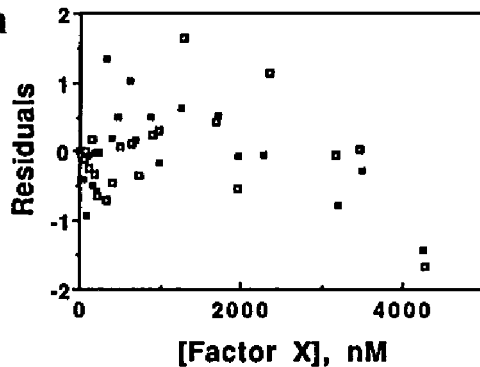


Figure 2.5b

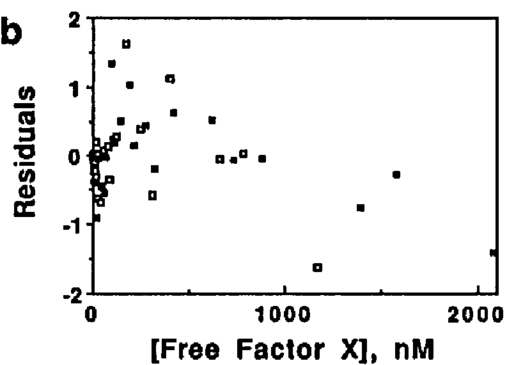


Figure 2.5c

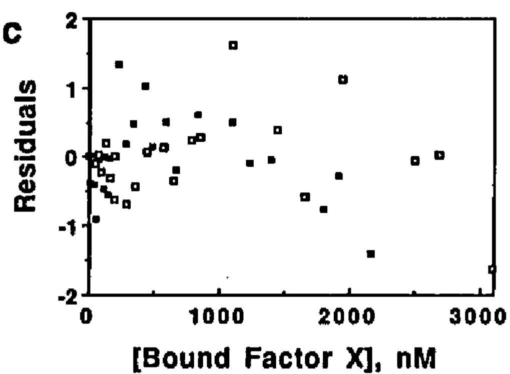
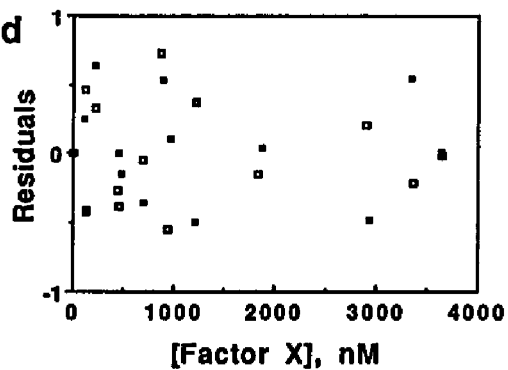


Figure 2.5d



DISCUSSION

A clear relationship exists between the concentration of factor X free in solution and reaction velocity in a system consisting of tissue factor, factor VIIa, factor X, phospholipid and calcium ions. To make this determination, I "uncoupled" the distribution of substrate (factor X) between phospholipid-bound and free pools by using prothrombin fragment 1 to perturb factor X binding to lipid without affecting the kinetic apparatus, per se. Any kinetic effects of prothrombin fragment 1 thus rest solely upon changes in phospholipid-bound and free substrate concentrations. The advantage of this approach is that phospholipid concentration and composition are kept constant, thereby avoiding potential ambiguities of phospholipid-induced changes on the kinetic parameters.

I established that prothrombin fragment 1 has no effect on the kinetic apparatus by adding prothrombin fragment 1 to a tissue factor system containing PC vesicles to which neither factor X nor prothrombin fragment 1 binds. Under these conditions, in which total substrate is equivalent to free substrate and there is no phospholipid-bound substrate to displace, prothrombin fragment 1 does not perturb the reaction velocity (Table 2.1). Therefore, I conclude that prothrombin fragment 1 has no effect on the fundamental kinetics of this reaction.

I next showed (Fig 2.1) that prothrombin fragment 1 decreases the binding of factor X to the negatively-charged

membrane surface in a tissue factor system reconstituted with PSPC. Presumably, factor X binding decreases because prothrombin fragment 1 competes with factor X for sites on the vesicle surface. Protein-protein dimerization, another possible mechanism for affecting binding, is unlikely to be significant at the protein concentrations used [97]. The phospholipid concentration, 0.5 mM, corresponds to 4.5 μM sites for factor X binding (total phospholipid $\times$ n). At this concentration of sites (nine times the K_d), most of the factor X is bound at the lower factor X concentrations. Any significant displacement of factor X from the phospholipid therefore causes a large increase in the free factor X concentration. The kinetic experiments, performed under the same conditions, are thus optimized to detect changes attributable to the free substrate pool.

From the binding data (Fig 2.1) it is clear that prothrombin fragment 1 decreased the phospholipid-bound factor X and increased the free factor X. In each kinetic pair the velocity increased with prothrombin fragment 1; indeed, at low substrate concentrations the velocity rose 3-4 fold (Fig 2.2). This observation is qualitatively consistent with free factor X controlling the rate of the reaction.

If free factor X controls the reaction velocity, one predicts that prothrombin fragment 1 addition would have no effect on reaction velocity at low concentrations of PSPC. This phenomenon was observed (see Fig 2.2 inset) and is most conveniently explained by example. Decreasing the

concentration of phospholipid will decrease the number of binding sites for factor X and hence will decrease the relative fraction of phospholipid-bound factor X. If the phospholipid concentration is decreased so that only 1% of factor X is bound, then complete displacement of the bound fraction will increase the free fraction by only 1.01%. In this latter case, one would not observe any change in reaction velocity upon addition of prothrombin fragment 1.

[Note that the same qualitative picture is observed when fluid-phase RVV-CP, rather than membrane-localized tissue factor-factor VIIa complex, is used to catalyze factor X activation. This experiment will be discussed more fully in the Introduction of Chapter 3. For now I will mention that the velocity increases observed upon prothrombin fragment 1 addition in the RVV-CP system were statistically significant in the presence of 500μl PSPC (see Fig. 2.3).]

To quantify the effects of prothrombin fragment 1 addition in terms of conventional kinetic parameters, I calculated the phospholipid-bound and free factor X concentrations for each substrate concentration (see Table 2.1) and determined the kinetic parameters using these concentrations. The Km and kcat determined using the free factor X concentration were unaffected by addition of prothrombin fragment 1. This quantitative agreement is further evidence that the free factor X concentration controls the reaction velocity and is consistent with the observation that prothrombin fragment 1 does not perturb

the "intrinsic" Km (i. e. K2). Accordingly, the decrease in the Km for the bound factor X using PSPC is actually a consequence of the fact that free factor X regulates the reaction; prothrombin fragment 1 increases the concentration of free factor X at the expense of the bound fraction. Hence, upon the addition of prothrombin fragment 1 the Km for the bound factor X will fall because of this relationship. I therefore conclude that the concentration of factor X in free solution controls the reaction rate in the tissue factor pathway.

One should note, however, that the Km derived using the concentration of free substrate in the PSPC system is still considerably lower than that obtained using tissue factor reconstituted with PC (Table 2.1). This means, in effect, that acidic phospholipids actually alter K2 and that the acceleration observed in the presence of PS is due to a change in the fundamental kinetic parameters rather than increased substrate concentration at the vesicle surface. This contrasts with the suggestion that rate accelerations are produced by localizing and concentrating substrates at the phospholipid surface [50,69,98].

The identity of the "true" substrate pool in other coagulation reactions remains controversial [68-70] partially because potential phospholipid-dependent effects on the intrinsic Km have been ignored. Pusey and Nelsestuen [68] and van Rijn et al. [70] have concluded that free substrate controls the rate of prothrombin hydrolysis in the

complete "prothrombinase complex." These conclusions were based primarily on experiments in which substrate binding was perturbed by changing phospholipid composition or concentration and then relating these changes to effects on the apparent Km. On the other hand, Nesheim et al. [69] in their computer model of prothrombin activation consider phospholipid-bound substrate to be the major contributor to hydrolysis. The assumptions of this model are that neither acidic phospholipids nor the cofactor, factor Va, induce a change in the intrinsic Km.

I have not resolved whether the intrinsic Km (K2) is determined by purely protein-protein interactions between the catalytic complex and the substrate. Nelsestuen [68] has suggested that acidic phospholipids decrease the Km in the prothrombinase system "by virtue of additive free energies of protein-protein plus protein-membrane interactions that occur at the active site..." As described by Nelsestuen, this phospholipid binding contribution is local; the reaction velocity and the Km are still determined by the concentration of free substrate. As such, our experiments cannot rule out *a priori* the possibility of coordinate binding of substrate to both the catalytic complex and lipid. However, one can estimate an upper limit for the energetic contribution from such lipid binding. First, assume that in the PC system only protein-protein interactions contribute to the binding energy (i. e. no phospholipid contribution). Then, assuming that no phospholipid groups are directly

chemically involved in the proteolytic reaction, the upper limit for the additional substrate binding energy in the PSPC system, putatively provided by interaction of factor X with phospholipid, is [99, p. 350]:

$$\Delta\Delta G_b = -RT \ln [(kcat/Km)_{PSPC}/(kcat/Km)_{PC}]$$
$$= -1.45 \text{ kcal·mole}^{-1}.$$

In contrast, the binding energy for factor X association with PSPC vesicles, calculated using the measured dissociation constant, 520 nM, is -8.54 kcal·mole^{-1}. Clearly, these binding energies are not comparable. If phospholipid interactions do contribute to the association of substrate with the catalytic complex, then either the protein-protein interaction has a tremendous negative effect on the phospholipid binding or else the local phospholipid binding has a different nature from gammacarboxyglutamic acid (Gla)-dependent binding.

It is generally accepted that Gla residues are responsible for the binding of coagulation zymogens to negatively-charged membranes [73,57]. If the Gla-dependent binding of factor X to negatively-charged membranes does not enhance the activation rate (as shown above), what purpose do the Gla residues serve? The factor X coagulant protein from Russell's Viper Venom (RVV-CP) and factor VIIa cleave the same site on factor X [38,100]. Activation of factor X by RVV-CP requires calcium ions, but occurs in the absence of phospholipid; in this fully soluble system intact factor X is activated much faster than factor

X from which the Gla-domain has been removed [101]. Thus, independent of any phospholipid binding capacity, the Gla-domain is essential for the substrate, factor X, to be activated. Further, the Gla-domain on factor VII is required for binding to tissue factor (Guha, A. and Nemerson, Y., unpublished observation). Accordingly, there is no a priori reason to invoke phospholipid-binding by substrate to explain Ca^{2+}- and Gla-mediated acceleration of blood coagulation. One speculation is that a Gla-mediated, Ca^{2+}-induced conformational change [56] may render the cleavage site on factor X more accessible to RVV-CP or factor VIIa.

The reaction of factor X with the tissue factor-factor VIIa complex is similar in many respects to the interaction of ligand with membrane-localized receptor molecules. In most membrane-associated receptor systems, the total surface area covered by receptor is a small fraction of the total membrane surface area [102]. More likely than not a diffusing ligand will first collide with a non-receptor site on the membrane. Because particles in solution move by a random walk, a ligand, having once collided with the membrane surface, has a high probability of repeat collisions [103] and more opportunities to encounter receptor. Note that the repeat collisions can occur without any binding interaction between membrane and ligand and the movement of ligand in this case is governed by fluid-phase diffusion parameters. The rate of movement of

membrane-bound ligand will be governed by surface diffusion parameters.

To use reaction velocity as an index of association rates, the tissue factor pathway must be diffusion controlled. The kcat/intrinsic Km measured in our system in the presence of negatively-charged phospholipids is approximately 1×10^8 $M^{-1}s^{-1}$, which is close to theoretical maximal values for bimolecular association rates [78, 99, p. 152]. Thus, the tissue factor pathway appears to be a good model for ligand-receptor associations. In this regard, Wiegel and DeLisi [76] have published a theoretical treatment concluding that ligand-receptor association rates are not greatly enhanced by non-specific binding of ligand to the membrane surface with subsequent surface diffusion. However, their conclusion is based on the assumption of multiple receptor sites on the membane surface - as is the case for most cellular systems. Enhancements are possible only if there are very few receptors per cell. Berg [75] also indicates that enhancements are possible under certain conditions. Our results indicate that membrane-bound factor X does not contribute to the rate of factor X activation in the tissue factor pathway. Next chapter, the ramifications of this statement regarding the fate of phospholipid-bound factor X will be explored in light of the above theories. Nonetheless, I believe the methodology in this chapter provides a valuable experimental framework

for determining the effects of ligand binding to the membrane in other ligand-receptor systems.

CHAPTER 3

INTRODUCTION

In the above work, I demonstrated that the rate of factor X activation in the tissue factor pathway is independent of the concentration of phospholipid-bound factor X. Now it seems appropriate to ask whether one would expect a dependence. As mentioned before, the kcat/intrinsic Km measured in our system in the presence of negatively-charged phospholipids is approximately 1×10^8 $M^{-1}s^{-1}$, which is close to theoretical maximal values for bimolecular association rates [78, 99, p. 152]. Thus, the activation of factor X by tissue factor-factor VIIa appears to be encounter limited. If fluid-phase factor X produces the majority of encounters with the catalytic complex, then one would expect no contribution from the bound pool. On the other hand, if the majority of enzyme-substrate collisions occur between phospholipid-bound factor X and the catalytic complex, the bound pool should dominate the reaction *unless phospholipid-bound factor X is protected* from activation.

van Dieijen et al. [97] showed that negatively-charged phospholipids inhibited the activation of factor X by the coagulant protein of Russell's viper venom (RVV-CP). In lieu of action at a distance, there are two mechanisms whereby phospholipid could inhibit the reaction: it could bind to enzyme (RVV-CP) or it could bind to substrate

(factor X). They tested whether the inhibition was caused by RVV-CP binding to phospholipid by incubating in the presence and absence of phospholipid, centrifuging both mixtures (to sediment the phospholipid), and then adding factor X to both supernatants. Factor X activating activity was the same in both supernatants. This indicates that RVV-CP is not adsorbed by the phospholipids. When RVV-CP was added before and after centrifugation in the presence of phospholipids, the rate of factor X activation was the same indicating that the free factor X concentration governed the rate of factor X activation. They suggested that RVV-CP was incapable of activating bound factor X, but did not pursue the issue further.

If phospholipid-bound factor X is less accessible to proteolytic attack by RVV-CP, then displacing bound factor X from the membrane surface using prothrombin fragment 1 should produce increased reaction rates. Figure 2.3 shows that prothrombin fragment 1 addition does increase reaction rates. Note that, because RVV-CP is fluid-phase, reaction rate enhancements through surface diffusion are not possible. The only reason why displacing phospholipid-bound factor X should increase reaction rates is if the phospholipid-bound pool is less accessible than the free pool.

Although not as extensively documented, the rate of factor X activation by Echis carinatus venom [104] and by Cerastes Cerastes venom [105] has been shown to be

decreased in the presence of negatively-charged phospholipids. Despite the appearance of these three papers, no one has published the obvious inference that phospholipid binding renders factor X generally resistant (or at least less accessible) to proteolytic activation. If the fluid-phase snake venoms were unable to approach the negatively-charged lipid surface then similar results might be expected. Possibly this alternative mechanism for the inhibition by negatively-charged lipid has prevented speculation. On the other hand there is no good reason to suppose that the snake venoms cannot approach the phospholipid surface. Charge repulsion is not likely because at the physiologic ionic strengths used in the above work, the lipid charge is largely shielded. In any event, we know that the tissue factor-VIIa complex is located at the lipid surface and therefore catalysis takes place there. Thus, if the phospholipid-bound pool has more opportunities for activation, yet still has no influence on the reaction rate, then phospholipid-binding must confer resistance to activation.

METHODS

Diffusion Models

To calculate the expected relative contributions of fluid-phase and surface diffusion pathways, I applied models originally developed to describe diffusion-limited association of ligand with membrane-localized receptor molecules. As stated above, because the pseudosecond-

order rate constant (kcat/Km) for factor X activation is approximately 1×10^8 M^{-1} s^{-1}, factor X activation in the tissue factor pathway is probably encounter limited. Both Wiegel and DeLisi [76] and Berg [75] have included surface diffusive pathways in their models of ligand-receptor association. The system described by each model is similar, consisting of large spherical membrane surface on which a circular receptor patch(es) is located. Ligand can reach the receptor patch either by collision directly from fluid-phase (specific binding) or by binding to a non-receptor portion of the membrane (non-specific binding) and sliding to the receptor. A ligand can also desorb from the membrane prior to capture by receptor. Both models include the assumptions of steady-state and diffusion-limited association to the receptor; i. e. the receptor is perfectly absorbing. In both models, the relative contributions of fluid-phase and surface diffusion to the rate of ligand-receptor association depend on the fluid-phase diffusion coefficient, the surface diffusion coefficient, the radius of the receptor-containing vesicle, the radius of the receptor, the radius of ligand binding sites on the membrane surface and the macroscopic dissociation constant for binding to the membrane surface. The models are complementary in that Berg's model describes a single receptor per vesicle (albeit of variable size), but allows variation of the on- and off-rates for non-specific binding. The Wiegel/DeLisi model considers the effects of N uniformly distributed, circular

receptor patches on the membrane surface, but assumes that non-specific binding occurs rapidly with respect to other processes.

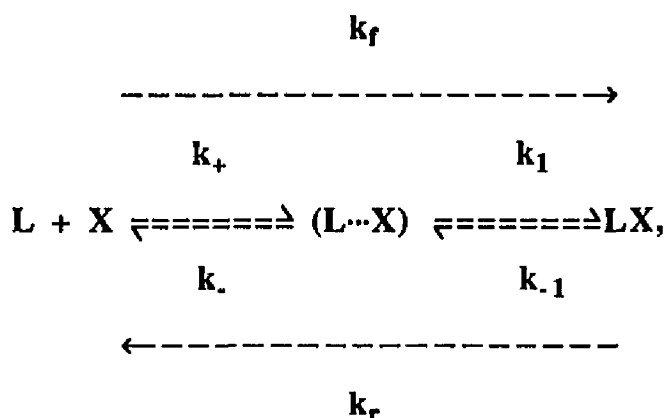
The actual equations will be discussed below; the Basic source code implementations of the models are in the Appendices. Most of the results are plots of Ψ_s (the fractional contribution of surface diffusion pathways to the total ligand capture rate by the receptor) as a function of various diffusion or geometric parameters. Because the Basic language cannot distinguish some of the parameter names as used below in the text, I have added the name of the corresponding Basic variable in curly brackets wherever necessary.

The following expression for Ψ_s {PSISURFACE} is derived from the Wiegel and DeLisi solution for total ligand current divided by ligand current in absence of surface diffusion:

$$\Psi_s = 1 - \frac{1 + (N \cdot S/\pi \cdot R)[1+(\pi \cdot R^2/N \cdot S^2)(Ds \cdot K^*/Df)]}{[1+(\pi \cdot R^2/N \cdot S^2)(Ds \cdot K^*/Df)][1+N \cdot S/\pi \cdot R]},$$

where N is the number of free receptors of radius, S, distributed uniformly on the surface of a cell (vesicle) of radius, R. Ds is the surface (or lateral) diffusion coefficient and Df is the fluid-phase diffusion coefficient. K^* {K.star} is the nonspecific affinity of the ligand for the phospholipid surface after the diffusive contribution is removed, i. e. it

is the affinity after ligand has moved within a ligand's radius, D, of the membrane surface. Thus, K^* is not equivalent to the measured affinity constant, K' {K.prime}, for binding to the phospholipid surface; Wiegel and DeLisi provide the following relationship (without derivation): $K' = (4\pi D^3/3) \times K^*$. However, a similar relationship was previously derived by DeLisi and Wiegel [106] in the following fashion. To determine the diffusive contribution, the formation of a lipid-factor X (LX) complex was represented as a two-stage event; diffusion to an encounter complex (L···X) followed by unimolecular reaction,



where k_+ and k_- are the forward and reverse diffusive rate constants and k_1 and k_{-1} are the reaction rate constants. By inspection the following relationship holds:

$$K' = k_f/k_r = (k_+/k_-) \times (k_1/k_{-1})$$
$$= K_D \times K^*.$$

The forward diffusive rate constant, k_+, equals $4\pi RDf \times [D/(D+\pi R)]$ and the reverse diffusive rate constant, k_-, equals $3\pi RDf/[D^2(D+\pi R)]$. In this case the geometrical factor, K_D, comes to $4D^3/3$, rather than $4\pi D^3/3$ as used by

Wiegel and DeLisi. I have been unable to determine the source of the latter figure and only note that both expressions are on the order of the volume occupied by factor X in the encounter complex. In any event simulations based on either diffusive factor (K_D) produce similar results (compare Figs. 3.3 a & b). [N. B.: K' is determined on a per site, rather than on a per vesicle basis.]

The solution to Berg's model is somewhat more complicated:

$x_0 = \cos(\theta_0)$

$Dr = kT/(8\pi\cdot\eta\cdot R^3)$, k is Boltzman's constant, η is viscosity of the solution, T is temperature

$K' = 1/K_d$

$K = K'\cdot(4R^2/D^2)$

$k_A = K\cdot k_D$

$k_{SR} = k_A\cdot(4\pi R\cdot Ds)/(4\pi R\cdot Ds - k_A)$

$\lambda = k_{SR}/K$

$\kappa = k_{SR}/(4\pi\cdot R^2)$

$\alpha = Ds\cdot\kappa/(Df\cdot\lambda\cdot R) = Ds\cdot K/(4\pi\cdot R^3\cdot Df)$

$\beta = Df/(\kappa\cdot R)$

$\gamma = Dr\cdot R^2/Df$

$1/C_n \cong \beta + 2/[1+(2n+1)\cdot(1+\gamma)^{1/2}]$, this approximation holds to within 7% for all n and γ (Berg, O., personal communication)

$$\text{SUMNUM} = \sum[1+\alpha n(n+1)/C_n]^{-1}P_n(x_0)\cdot[P_{n-1}(x_0)-P_{n+1}(x_0)]$$

$$\text{SUMDEN} = \sum[1+\alpha n(n+1)/C_n]^{-1}\cdot(2n+1)^{-1}[P_{n-1}(x_0)-P_{n+1}(x_0)]^2$$

$$\Psi_S = [1+(1-x_0+\text{SUMNUM})/(1+x_0-(1-x_0)^{-1}\cdot\text{SUMDEN})]^{-1}$$

where $P_{n-1}(x_0)$, $P_n(x_0)$ and $P_{n+1}(x_0)$ are Legendre polynomials [{PLGNDR(n-1)}, {PLGNDR(n)} and {PLGNDR(n+1)}] solved by the recurrence formula [107]: $(n+1)P_{n+1}(x) = (2n+1)\cdot x\cdot P_n(x)-nP_{n-1}(x)$. The parameters required (additional to those required for the Wiegel/DeLisi model) are: Dr is the rotational diffusion coefficient of the vesicle; k_D {Kd.rate} is the macroscopic dissociation rate constant of ligand from phospholipid, including probability of reassociation (discussed further below); K is the affinity constant for nonspecific binding on a per vesicle basis (= K' $\times$ nonspecific sites per vesicle); k_A {Ka.rate.ves} is the macroscopic association rate constant of ligand to the vesicle; $4\pi R\cdot Df$ {Dif.lim.ves} is the diffusion limited association rate of ligand to the vesicle; k_{SR} {k.surf.reactivity} is the surface reactivity per vesicle (takes partial diffusion control into account); λ {LAMBDA} is the local dissociation rate constant; κ {KAPPA} is the local reactivity per unit area for nonspecific association to the lipid surface; α {ALPHA}, β {BETA} and γ {GAMMA} are combination parameters which describe respectively the influence from

surface sliding, the departure from diffusion control for the nonspecific binding due to a reaction step, and the influence of rotational motion of the target vesicle; finally, θ_0 is half the angle subtended by the receptor patch.

RESULTS AND DISCUSSION

Estimates of Parameters

Before starting detailed discussion of the results, I will consider what we know, or can infer, about the values of the parameters governing association in this system.

Diffusion: The fluid-phase diffusion coefficient, Df, for prothrombin [108] is $4.8 \times 10^{-7} cm^2 s^{-1}$. Because factor X is slightly smaller than prothrombin, I have assumed a Df of $5 \times 10^{-7} cm^2 s^{-1}$ for factor X diffusion. I have no information about the magnitude of the surface diffusion coefficient, Ds, for coagulation factors adsorbed on a negatively-charged membrane. Ds is a relative translational diffusion constant; it is the sum of the surface diffusion coefficients of bound factor X and of the tissue factor-factor VIIa complex. Various diffusion coefficients between $5 \times 10^{-9} cm^2 s^{-1}$ and $10^{-12} cm^2 s^{-1}$ have been determined for surface diffusion of integral membrane proteins (labelled with fluorescent-tagged antibody) in a variety of different membrane systems [109]. Generally, artificially reconstituted membrane systems have produced the largest rates of surface diffusion. Thus, in this system one expects a Ds on the order of $10^{-10} cm^2 s^{-1}$ or greater. The effects of various Ds values were simulated.

Non-specific binding: I measured the K_d for factor X binding to be 520nM (see Fig. 2.1). Wei et al [110] used dilution-jump experiments in a stopped-flow light scattering apparatus to determine that prothrombin dissociates from PSPC vesicles at a rate of 3 s^{-1}. In the absence of any other information about the rate of dissociation of factor X from PSPC vesicles, I have used this value for the macroscopic off-rate, k_D. Given this dissociation rate constant and the measured equilibrium binding constant (520 nM), the association rate constant to the vesicle is 1.725×10^{-11} cm^3 s^{-1}. This value is 36% of the theoretical maximum predicted from $4\pi RDf$. Thus, k_D cannot be much larger than 3 s^{-1}.

Geometric factors: The entire tissue factor-factor VIIa complex has a molecular weight of ~96,000 (43,000 for tissue factor [23] and 53,000 from factor VIIa [21,35]). Using hydrodynamic data [59], I estimate that 6-7 $\times$ 10^{-7} cm is probably an upper limit for receptor radius, S, (probably only a portion is really involved in recognition and I have generally used the range 1-10 nm for calculations). I measured a maximal binding capacity of 8.91 μmols factor X/mmol total phospholipid. Thus, there are 56 phospholipid molecules per factor X binding site (assuming 50% of the phospholipids are on the exterior leaflet). Given 0.7 nm^2 per phospholipid molecule [88], I calculate $3.92 \times 10^{-13} cm^2$ per site. Assuming a circular site, the factor X binding site radius, D, would be $3.5 \times 10^{-7} cm$.

Although I did not determine the size of the vesicles used for the studies in Chapter 2, Bach et al. [25] measured identically prepared vesicles as having a mean radius, R, of 75 nm. They measured the size by gel exclusion chromatography on a Sephadex G-1000 column calibrated by a series of monodisperse latex beads. A vesicle with a 75 nm radius contains approximately 200,000 phospholipid molecules (calculated from 2× total surface area and 0.7 nm^2 per phospholipid molecule [88]). The molar ratio of tissue factor/phospholipid used in these studies was approximately 1/100,000. Thus, two tissue factor molecules are expected to be incorporated/vesicle. Because, however, tissue factor molecules are randomly oriented in the vesicle [25], on average only one faces the outside solution. Thus, I assume that our system consists primarily of vesicles containing one "effective" tissue factor molecule/vesicle. Results from both models will be described first for the case of one receptor/vesicle. Then, the Wiegel/DeLisi model will be used to generalize to multiple receptors/vesicle.

Convergence: The Wiegel/DeLisi model possesses a simple analytical solution; Berg's model requires an iterative solution. For the latter, one must first determine how many iterative steps are needed to converge to a solution. Preliminary runs showed that the summation term in the numerator, SUMNUM, would be "rate limiting" in terms of convergence; the denominator summation term,

SUMDEN, was well behaved and converges rapidly. Figure 3.1 shows the effects of iteration on the calculated value of Ψ_s as a function of surface diffusion coefficient, Ds, given a receptor radius of 1 nm, k_D of 3 s^{-1}, and K_d of 520 nM. The discrepancy between the value obtained after 100 iterations *vs.* that obtained after 1500 iterations becomes larger as Ds decreases. Thus, more steps are required for convergence as Ds becomes lower. In Figure 3.1a, a vesicle radius of 75 nm was assumed; Figure 3.1b shows the same simulation using a vesicle radius of 35 nm. Comparison of these two figures shows that the discrepancy between the value obtained after 100 iterations *vs.* that obtained after 1500 iterations was larger at 75 nm radius, suggesting that the number of steps to convergence increases with increasing vesicle radius. Figure 3.2 shows the effect of receptor radius on convergence of the numerator summation term, SUMNUM, as a function of the number of iteration steps. This figure shows that convergence is inversely related to receptor radius. This simulation was made using the same conditions as Figure 3.1 with Ds = $10^{-14} cm^2 s^{-1}$ and R = 75 nm, which were the worst cases in Figure 3.1 with respect to convergence. 750 iteration steps seem sufficient to achieve convergence under these conditions. Therefore, this figure, together with the previous figure, indicates that 750 iteration steps are

ample to obtain SUMNUM (and thus Ψ_s) within an error of 1% for the following limits of parameters:

$Ds \geq 10^{-14} cm^2 s^{-1}$

$Df = 5 \times 10^{-7} cm^2 s^{-1}$

Receptor radius, $S \geq 1$ nm

Vesicle radius, $R \leq 75$ nm

Radius of nonspecific site, $D = 3.5 \times 10^{-7} cm^2$

$k_D = 3\ s^{-1}$

$K_d = 520$ nM.

Unless otherwise mentioned R = 75 nm and at least 750 steps of iteration were used for simulations (in Figures 3.4 and 3.5 where k_D or K_d varied, certain points required as many as 3000 iterations before adequate convergence was achieved - monitored by visual inspection of the SUMNUM vs. iterations plot as in Fig. 3.2).

Although in principle increasing the number of iteration steps should improve the accuracy of the calculation, in practice one must also cope with accumulating round-off errors as the number of iterations increases. I have tested for round-off error by doubling the number of iterations and measuring the relative change of the variable of interest at each doubling. As the relative change continued to decrease with each doubling (at least, beyond the numbers of iteration steps used in these simulations), round-off error is probably not significant in these calculations and convergence is confirmed.

Ψ_s as a Function of Ds

Variation with receptor radius: Figure 3.3a shows the variation of Ψ_s with receptor radius as a function of surface diffusion coeficient obtained from the Wiegel/DeLisi model (using $K_D = 4\pi D^3/3$). Figure 3.3b shows the same model with $K_D = 4D^3/3$. Figure 3.3c shows the equivalent simulation obtained from the Berg model. All three simulations show a similar pattern of variation. The contribution of surface diffusion increases with increasing surface diffusion coefficient and with decreasing receptor radius.

Figures 3.3a-c were calculated using the closest parameter estimates available for our tissue factor system. The main point of these figures is that surface diffusion dominates the rate of ligand capture for all values of receptor radius as long as Ds $\geq 10^{-12} cm^2 s^{-1}$. As there is no published information about the value of Ds in this system, I cannot unambiguously conclude whether surface diffusion provides the majority of collisions with the catalytic complex. However, measured lateral diffusion coefficients have generally exceeded this value by more than 2 orders of magnitude (at least in other artificial membrane systems). Thus, surface diffusion should contribute the majority of encounters and bound factor X concentration should control the reaction velocity in the tissue factor pathway. As this contradicts the experimental results, I infer that surface-derived encounters with the catalytic complex are "silent". The phospholipid-bound factor X is

protected from proteolytic cleavage by tissue factor-factor VIIa complex.

Variation with k_D: Figure 3.4 shows the variation of Ψ_s vs. Ds at various dissociation rate constants using a receptor radius of 4 nm. Note that even at the highest possible dissociation rate (obtained by assuming that the association rate is 100% diffusion limited), Ψ_s exceeds 0.5 for surface diffusion coefficients greater than $10^{-11} cm^2 s^{-1}$.

As the K_d was fixed at 520 nM, the association and dissociation rate constants varied in parallel. Increasing both the dissociation and association rate constants causes the curves to shift downward. In other words the influence of surface diffusion decreases as the dissociation rate increases even though the association rate is also increasing. This occurs because the membrane-bound molecule is more likely to desorb before laterally diffusing a significant distance. In a situation where the dissociation rate increases without a corresponding increase in the association rate (i. e. an increased K_d), the contribution from surface diffusion would be decreased further.

Variation with K_d: Figure 3.5 shows Ψ_s vs. Ds as a function of receptor radius. With a receptor radius of 10 nm (which almost certainly exceeds the true radius) surface diffusion provides the majority of encounters as long as Ds exceeds $10^{-10} cm^2 s^{-1}$.

In contrast to Figure 3.3c the K_d used in this simulation is the highest value published: 2.5 μM [93]. Also, the

dissociation rate was maximized by setting the association rate at the diffusion limit. In other words, conditions were chosen to minimize the contribution from surface diffusion. Even under these conditions (which are far less conducive to a surface diffusion contribution than those in our actual experimental system), surface diffusing species would provide the major contribution to encounter frequency for experimentally reasonable values of the surface diffusion coefficient.

Ψ_S as a Function of Free Receptor Number

Figure 3.6 shows the effect of free receptor number on Ψ_S as a function of surface diffusion coefficient using the Wiegel/DeLisi model. The receptor radius, S, was 4 nm. The K_d was 520 nM. Regardless of the magnitude of the surface diffusion coefficient, the contribution of surface diffusion rapidly decreases with increasing numbers of receptors.

The physical basis of diffusive search implies geometrical constraints imposed by localization of receptor at the membrane surface. With few receptors the capture rate increases linearly with increasing receptor number. At high numbers of receptors the capture rate asymptotically approaches $4\pi RDf \times$ [free ligand], the capture rate for a totally absorptive cell of radius, R. This hyperbolic dependence was first explained by H. C. Berg and E. M. Purcell [103] who described a system of uniformly distributed receptors on a non-ligand-adsorbing cell

surface. Because of the Brownian nature of diffusive search, a ligand, having once struck a non-receptor portion of the membrane surface, has a high probability of repeat encounters. Therefore, an isolated receptor has an "effective capture radius" greater than its physical radius because it can capture "near misses" on subsequent excursions. However, as the number of receptors increases and the spacing between receptors decreases, the receptors begin to interfere with one another by depressing the concentration of ligand in each other's vicinity. Once the "effective capture radii" of the receptors begin to overlap, increasing the number of receptors does not increase the capture rate very much. H. C. Berg and E. M. Purcell showed that this occurs when only a very small fraction of the cell surface is occupied by receptor. The capture rate is half maximal when the fraction of the cell surface covered by receptor reaches $\pi S/4R$, where S is the receptor radius and R is the radius of the cell. Given the large size differential between the typical cell and its typical receptor, half-maximal capture rate will be achieved at the expense of less than 1% of the cell surface.

Adsorption of ligand to the phospholipid surface with subsequent lateral diffusion can increase the "effective capture radius" of the individual receptors over that obtained in the absence binding. In effect, binding increases the average surface area explored by ligand and thus receptor captures more ligands which have traveled

farther from their initial touch-down point. However, surface diffusive pathways can increase the capture rate only if there are very few, widely-spaced receptors. If the receptors are spaced such that the increase in "effective capture radii" produces overlap, then surface diffusion cannot produce a significant increase in capture rate. By analogy with the Michaelis-Menten equation, surface diffusive pathways to receptor can decrease the number of receptors required to achieve half-maximal capture rate, but the maximal capture rate is unchanged.

The hyperbolic dependence of capture rate on receptor number has relevence in assessing the potential of various tissue factor-containing cells to initiate blood coagulation. Rodgers et al. [111] showed that cultured, intact non-vascular cells have more procoagulant activity than vascular cells. The vascular cells did not express tissue factor. In a later paper Rodgers et al. [112] showed a hyperbolic relationship between the tissue factor activity expressed by various non-vascular cell types and the number of receptors for factor VII. As the number of factor VII molecules bound per cell increased two orders of magnitude (from 2400 to 880,000), the tissue factor activity increased only five-fold. To explain this dependence they speculated that there may be non-functional receptors for factor VII or there may be other cellular factors that modulate tissue factor activity. I submit that their results instead reflect the biophysics of

receptor-mediated association and that the receptors measured are normal, functional tissue factor. As monoclonal antibodies to human tissue factor become available and sensitive assays for tissue factor antigen are developed, one should remember that the relationship between tissue factor level and relative coagulant potency of cells may be linear only at very low levels of tissue factor/cell.

In summary, there is mounting evidence for receptor-mediated initiation of coagulation by tissue factor in complex with factor VII(a). The role of negatively-charged phospholipids in the tissue factor pathway is two-fold. On the one hand, negatively-charged lipids have a direct effect on the tissue factor-factor VIIa complex, lowering the intrinsic Km for activation of fluid-phase factor X. This accelerates the activation of factor X. On the other hand, the binding of factor X to the phospholipid surface protects the Arg-Ile bond from cleavage, thus inhibiting the reaction. Whether negatively-charged lipids produce a net velocity increase or decrease over neutral lipids depends on fraction of factor X bound to the lipid surface.

By using a ligand, prothrombin fragment 1, to compete with factor X for phospholipid binding sites, I was able to determine that fluid-phase substrate controls the rate of reaction. Because the protocol required no assumptions about the effect of a negatively-charged phospholipid

environment on the intrinsic Km, I could determine that the intrinsic Km was enhanced. This approach may be of use in other receptor-mediated systems where surface diffusion pathways potentially contribute to ligand-receptor (or substrate-enzyme) association.

Negatively-charged phospholipids inhibit the activation of factor X by RVV-CP [95]. Phospholipid produces similar inhibition of factor X activation by Echis carinatus venom [104] and by Cerastes Cerastes venom [105]. Addition of prothrombin fragment 1 accelerated the rate of factor X activation in a system containing RVV-CP and negatively-charged phospholipids. This latter finding is consistent with van Diejien's hypothesis that RVV-CP cannot activate phospholipid-bound factor X. Simulations based on two different models for receptor-mediated association indicate that the majority of enzyme-substrate encounters involve phospholipid-bound factor X (as long as the lateral surface diffusion coefficient exceeds $10^{-12} cm^2 s^{-1}$, which is likely). Because phospholipid-bound factor X probably has more encounters with enzyme, yet does not contribute to the rate of activation, I assert that phospholipid-bound factor X is protected from activation in our system (and, by extension, the tissue factor pathway in general). Alternatively, if one believes that fluid-phase and phospholipid-bound factor X are equally available for proteolysis, then one must conclude that the above theoretical treatments of ligand-receptor association are

invalid. Definitive resolution of this conundrum, alas, remains for the future; requiring an independent, direct means for measuring the rate of substrate-enzyme encounters.

Finally, the experimental hyperbolic relationship between tissue factor activity and factor VII binding in cells seemingly agrees with the theoretical dependence of ligand capture rate on the receptor number. Thus, tissues with widely differing levels of tissue factor expression may have similar physiological procoagulant potential.

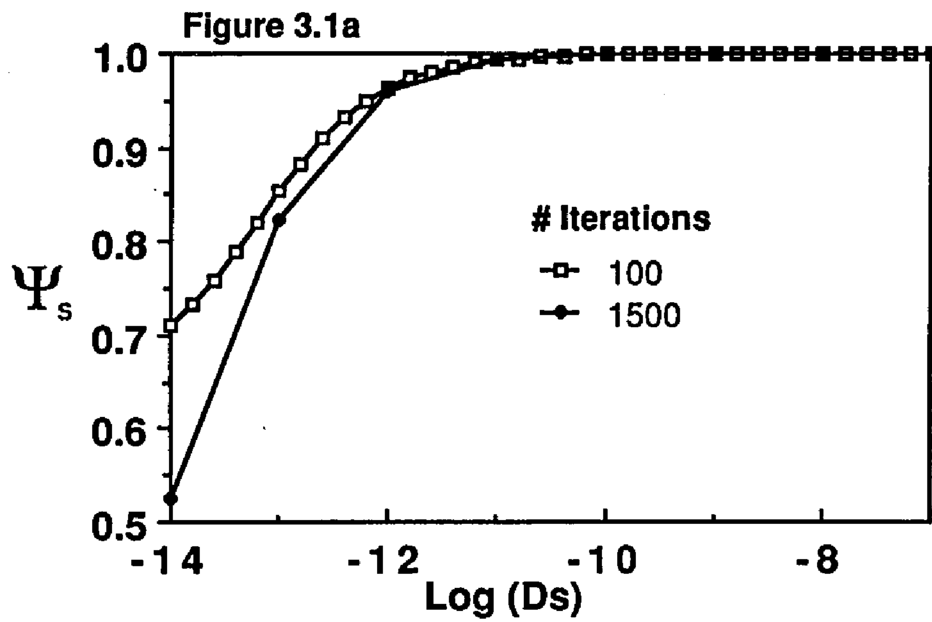


Figure 3.1a
1.0
0.9
0.8
0.7
0.6
0.5
Ψ_s
Iterations
100
1500
-14
-12
-10
-8
Log (Ds)

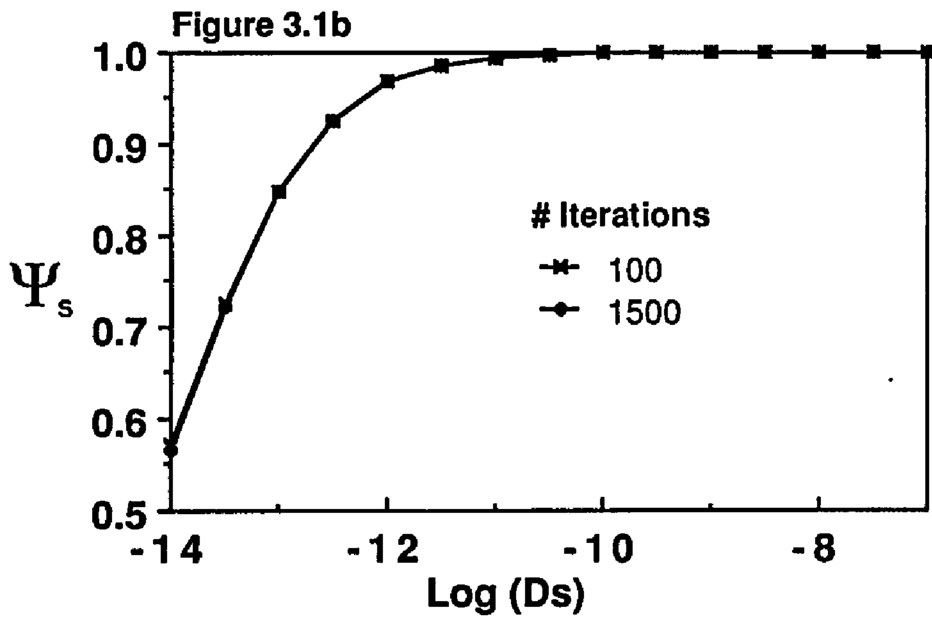
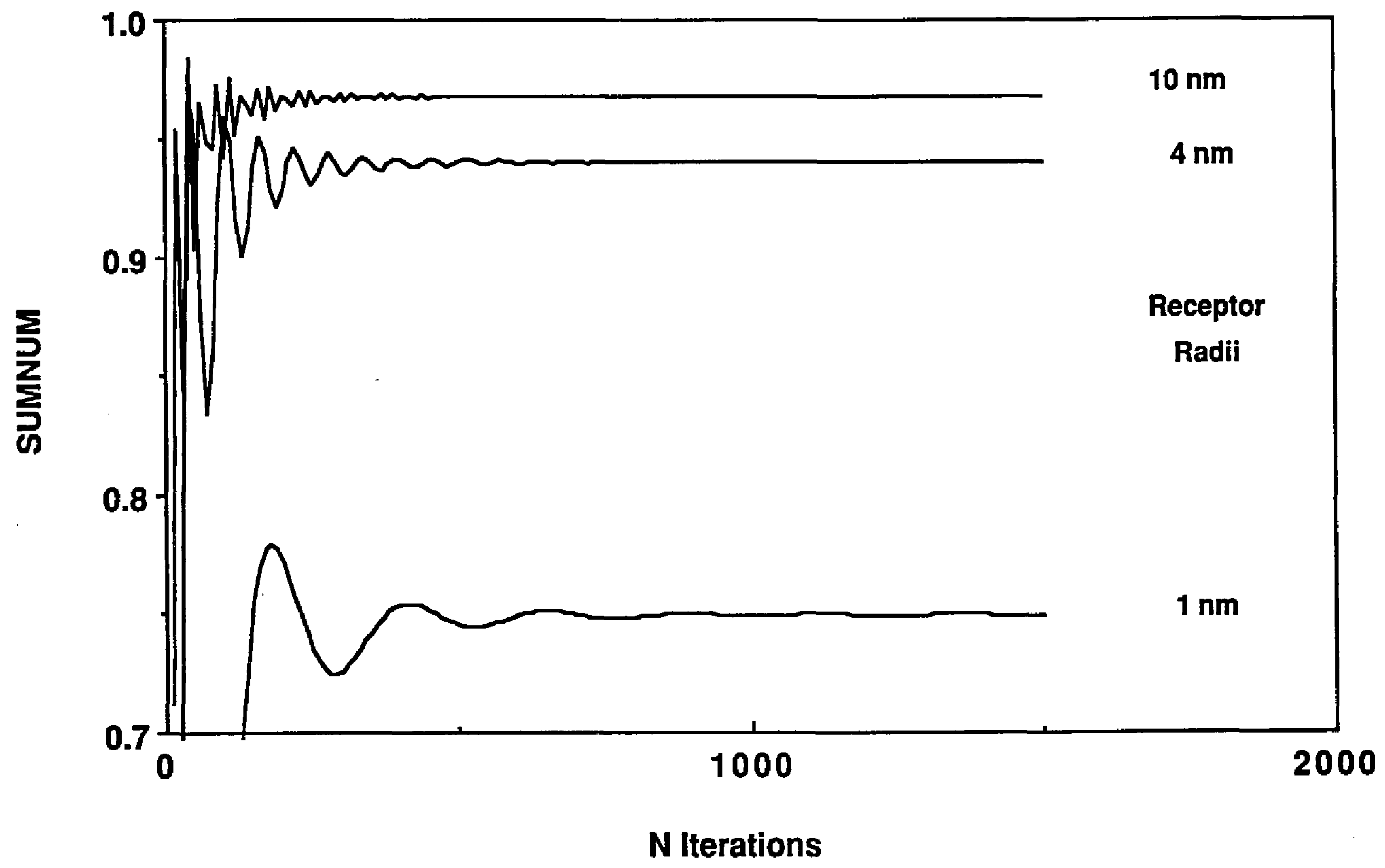


Figure 3.1b
1.0
0.9
0.8
0.7
0.6
0.5
Ψ_s
Iterations
100
1500
-14
-12
-10
-8
Log (Ds)

Figure 3.2



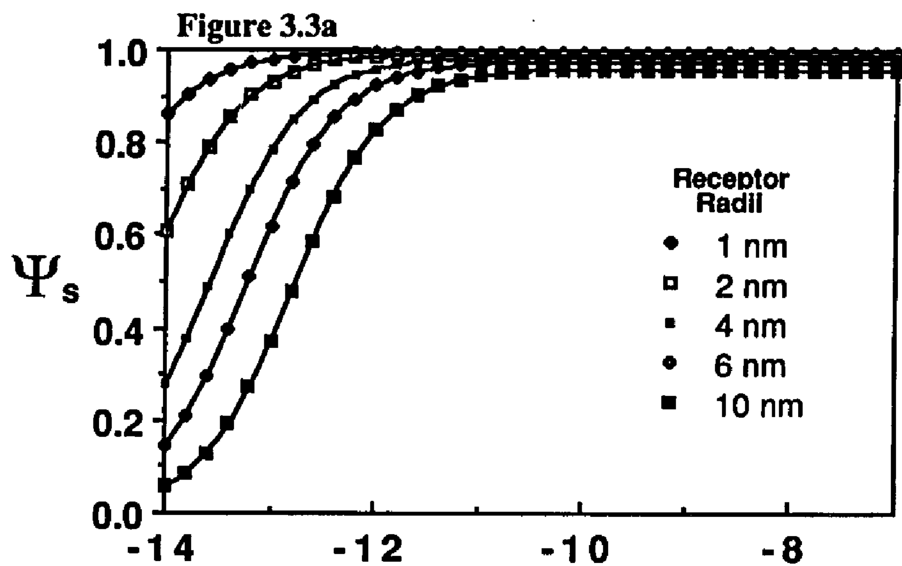


Figure 3.3a
1.0
0.8
0.6
0.4
0.2
0.0
-14
-12
-10
-8
Ψ_s
Receptor Radii
1 nm
2 nm
4 nm
6 nm
10 nm

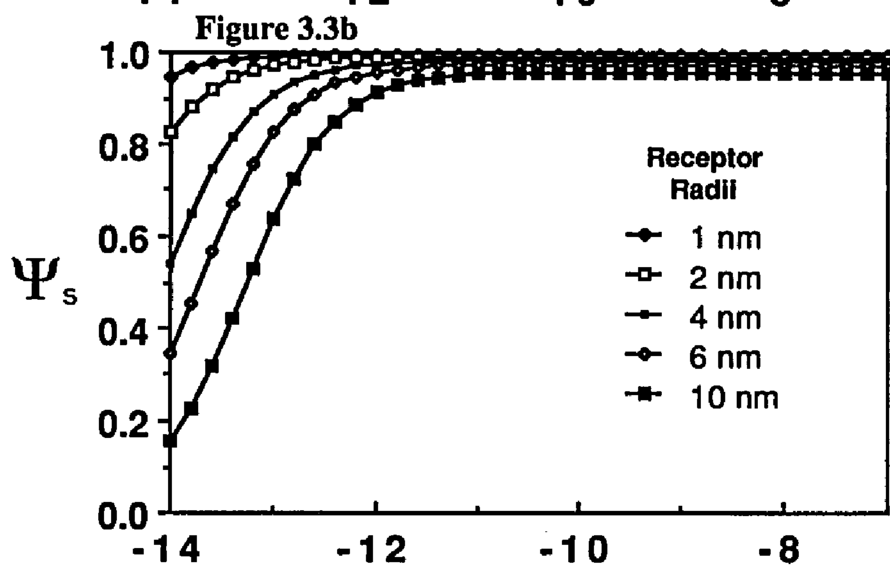


Figure 3.3b
1.0
0.8
0.6
0.4
0.2
0.0
-14
-12
-10
-8
Ψ_s
Receptor Radii
1 nm
2 nm
4 nm
6 nm
10 nm

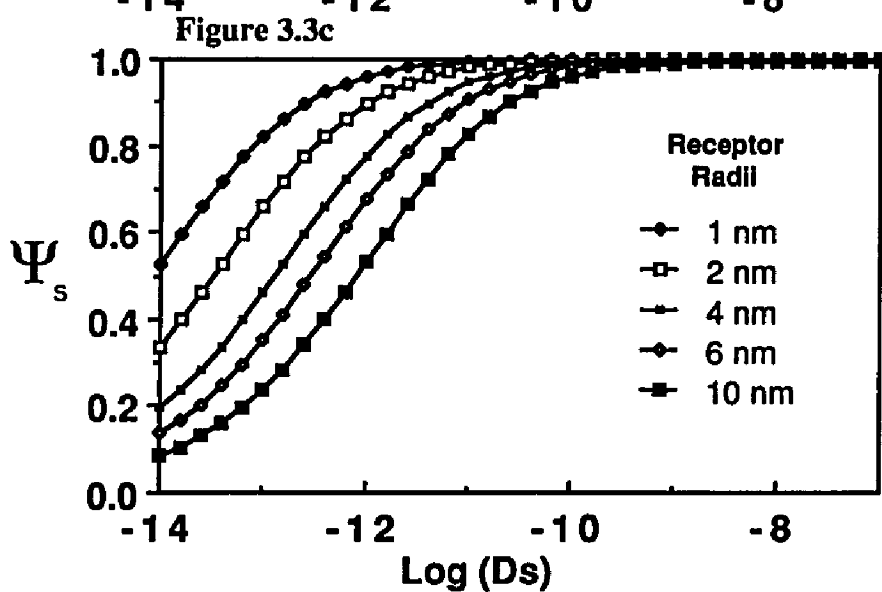


Figure 3.3c
1.0
0.8
0.6
0.4
0.2
0.0
-14
-12
-10
-8
Log (Ds)
Ψ_s
Receptor Radii
1 nm
2 nm
4 nm
6 nm
10 nm

Figure 3.4

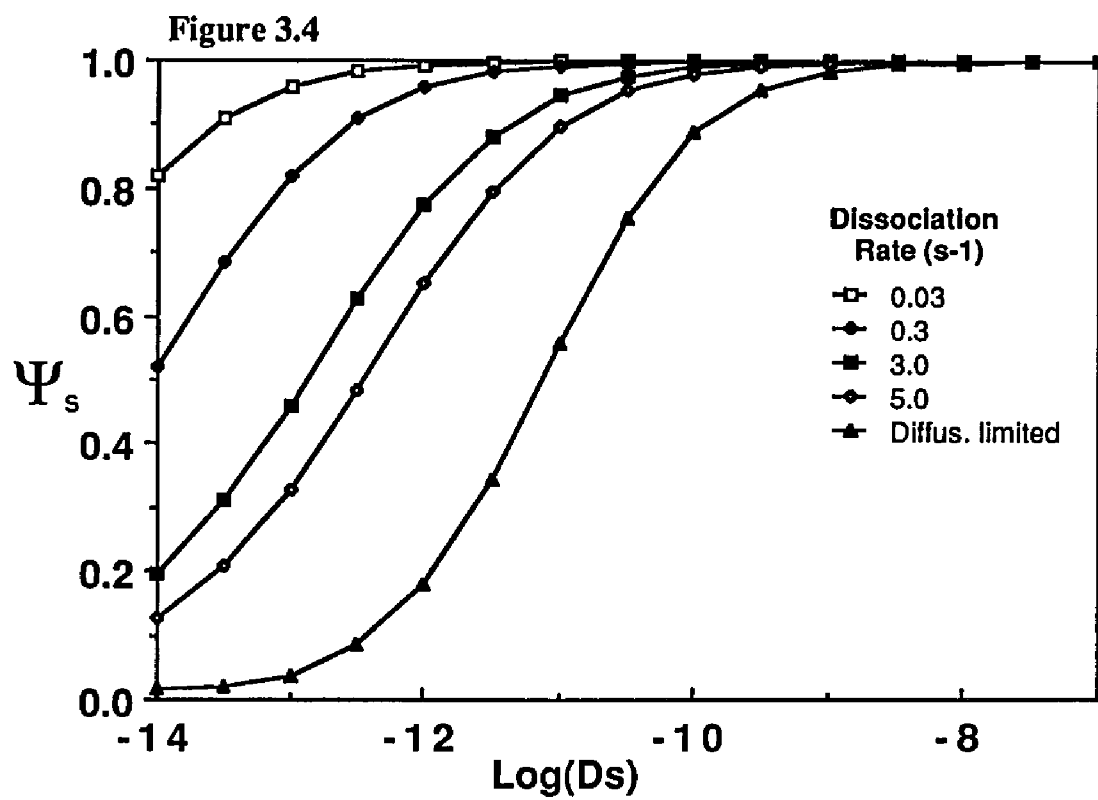


Figure 3.5

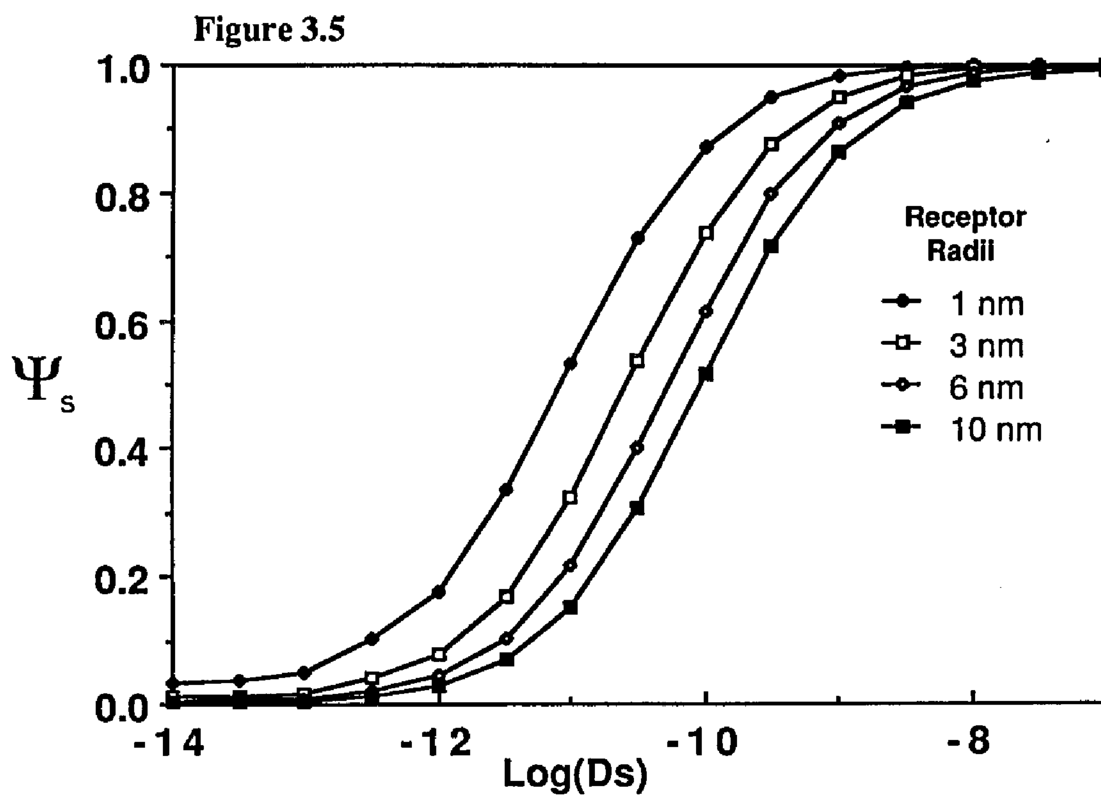
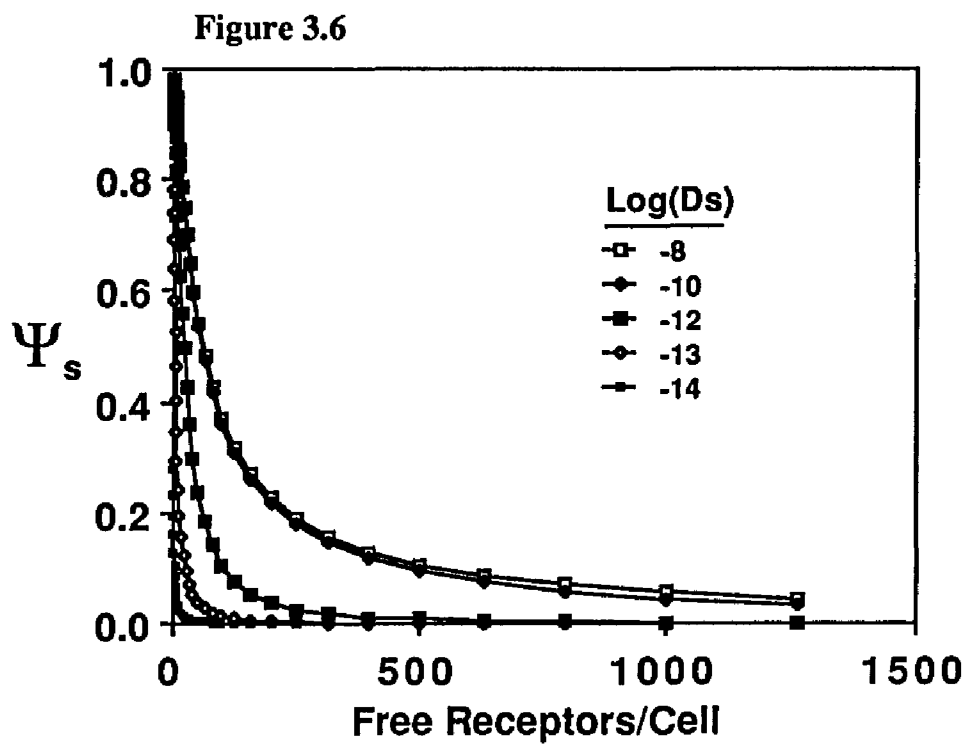


Figure 3.6



Appendix A - Basic Code for Wiegel/DeLisi Model Implementation

```
REM---Simulation of Ligand-Receptor Association
REM---Based on multiple-receptor model of F. W. Wiegel and C.
DeLisi (1982), Am. J. Physiol. 243:R475
REM---Version 1.0 for Microsoft Basic 2.1 on the Macintosh
DEFDBL a-z
pi = 3.1415927#

    REM---Routine to open datafile "log(Ds) w/N=1/R=75/S=6
        Wiegel Model1.0" on Disk "THESIS DATA"
    REM---Outputs Ascii data string in form readable by Statworks
OPEN "THESIS DATA:log(Ds) w/N=1/R=75/S=6 Wiegel Model1.0" FOR
OUTPUT AS #1
   PRINT #1,"*";CHR$(13);
    PRINT#1, "log(Ds)";CHR$(9);"Current.ratio";CHR$(9);
                "Psisurface";CHR$(13);

FOR N5%=-140 TO -70 STEP 2 :REM  Initialize loop, 10x larger than
                necessary so that I can use integer arithmetic
  N4=N5%/10
  N = 1  : REM   number of free receptors/cell
  R = .0000075  :REM   radius of vesicle (cm)
  S = .0000006 :REM   radius of receptor site (cm)
  D = 3.5E-07    :REM   radius of nonspecific ligand
                   binding site on vesicle (cm)
  Df = .0000005  :REM  fluid-phase diffusion coefficient (cm2 s-1)
  Ds = 10^N4 :REM  lateral surface diffusion coefficient (cm2 s-1)
  Kd.eq.molar = 5.2E-07  :REM  measured non-specific
                   equilibrium dissociation constant (per site
                       basis)  (M)
     K.prime = (1/Kd.eq.molar)  :REM non-specific affinity constant
                   (reciprocal of dissociation constant)   (M-1)
     K.prime.cm3.units = K.prime/(6.02E+20)  :REM divided by
                   Avogadro's number*liters per  cm3  (cm3)
     K.star = K.prime.cm3.units/(4*pi*D*D*D/3)  :REM non-specific
                   affinity with diffusive contribution removed

    Current.ratio = (1+(pi*R*R/(N*S*S))*(Ds*K.star/Df))*
        (1+N*S/(pi*R))                    :REM numerator only
     Current.ratio=Current.ratio/(1+(N*S/(pi*R))*(1+(pi*R*R/(N*S*S))*
```

```
        (Ds*K.star/Df)))   :REM  Total current divided by current
                               in absence of non-specific pathways
    Psisurface = 1 - 1/Current.ratio  :REM  Fraction of total
                           encounters due to surface diffusion pathways

PRINT #1,CSNG(N4);CHR$(9);CSNG(Current.ratio);CHR$(9);
     CSNG(Psisurface);CHR$(13);
NEXT N5%
   CLOSE #1
END
```

Appendix B - Basic Code for Berg Model Implementation

```
REM---Simulation of Ligand-Receptor Association
REM---Based on single-receptor model of O. Berg (1985), Biophys.
     J. 47:1
REM---Version 3.0 for Microsoft Basic 2.1 on the Macintosh
REM---Version used w/Kd.rate=.03,.3,3 s-1/6-14-86
DEFDBL a-z
       N% = 750  :REM   one less than the # of LGNDR Poly's to
              compute; therefore max # of iterations of partial
              summations
       DIM PLGNDR(N%+1)
    REM---Routine to open datafile "log(Ds)/Kd.rate=3s-
                 1/S=4nm/Berg3.0"  on Disk "THESIS DATA III"
    REM---Outputs Ascii data string in form readable by
                 Statworks
OPEN"THESIS DATA III:log(Ds)/Kd.rate=3s-1/S=4nm/Berg3.0" FOR
OUTPUT AS #1
    PRINT #1,"*";CHR$(13);
    PRINT #1,
            "SUMNUM";CHR$(9);"SUMDEN";CHR$(9);"SUM_ka"
                 ;CHR$(9);"Psisurface";CHR$(9);
                 "Eff.Ass.Ratio"; CHR$(9);"Log(Ds)";CHR$(13);

FOR N5%=-140 TO -70 STEP 2  :REM   Initialize loop, 10× larger than
                      necessary so that I can use integer arithmetic
   N4=N5%/10
        R = .0000075  :REM    radius of vesicle (cm)
        S = .0000004 :REM    radius of receptor site (cm)
        D = 3.5E-07    :REM    radius of nonspecific ligand binding
                               site on vesicle (cm)
        Df = .0000005  :REM   fluid-phase diffusion coefficient
                                      (cm2 s-1)
        Ds = 10^N4 :REM  lateral surface diffusion coefficient
                                      (cm2 s-1)
        Dr = 4E-14/(8*3.1415927#*.01*R*R*R)       :REM rotational
                      diffusion coefficient   [4e-14 is energy of
                      thermal fluctuation (ergs) and 0.01 is
                      viscosity of water at room temperature
                      (poise)]  (rad2 s-1)
```

```
        Kd.rate = 3  :REM   macroscopic dissociation rate constant of
                          ligand from nonspecific site;includes
                          probability of reassociation   (s-1)
        Kd.eq.molar = 5.2E-07   :REM   measured nonspecific
                          equilibrium dissociation constant (per site
                          basis)   (M)
      K.prime = (1/Kd.eq.molar)   :REM non-specific affinity constant
                          (reciprocal of dissociation constant)     (M-1)
        K.prime.cm3.units = K.prime/(6.02E+20)  :REM divided by
                          Avogadro's number*liters per cm3   (cm3)
      K = K.prime.cm3.units*(4*R*R/(D*D))   :REM nonspecific affinity
                          constant on a per vesicle basis; 4R2/D2 are
                          the non-specific sites/vesicle    (cm3)
      Ka.rate.ves = K*Kd.rate   :REM   macroscopic association rate
                          constant of ligand to the vesicle    (cm3 s-1)
          Dif.lim.ves = 4*3.1415927#*R*Df   :REM   diffusion limited
                          association rate to the vesicle    (cm3 s-1)
          k.surf.reactivity  =  Ka.rate.ves*(Dif.lim.ves)/
                (Dif.lim.ves-Ka.rate.ves)  :REM   surface reactivity
                              per vesicle (takes partial diffusion
                              control into account) (cm3 s-1)
          KAPPA  =  k.surf.reactivity/(4*3.1415927#*R*R)  :REM
                              local association rate per unit area
                                                (cm  s-1)
      ALPHA = Ds*K/(Df*4*3.1415927#*R*R*R)
     BETA =  Df/(KAPPA*R)
     GAMMA =  Dr*R*R/Df
   DEF FNC(N1) = 1/(BETA + 2/(1 + (2*N1+1)*SQR(1 +
      GAMMA)))  :REM   inverse of eq. A8b
   DEF FNCOEF(N2) = 1/(1 + ALPHA*N2*(N2+1)/FNC(N2))
                 :REM    1st term in both summations in eq. A14
   X0 = (SQR((R+S)*(R-S)))/R  :REM    cosine of THETAo; angle which
                 receptor site takes up -- used in LEGENDRE
                 evaluation

CALL LEGENDRE(N%,X0,PLGNDR())  'CALCULATES ALL VALUES OF
LEGENDRE POLYNOMIALS NEEDED

REM        Computes partial summations in the numerator and
denominator
   SUMNUM=0: SUMDEN=0 : SUMKA=0  :REM INITIALIZE
                                              SCRATCH VARIABLES
    FOR J% = 1 TO N%
```

```
        SUMNUM=FNCOEF(J%)*(PLGNDR(J%)*(PLGNDR(J%-1)-
              PLGNDR(J%+1)))+SUMNUM  :REM   numerator summation
                                            term in eq. A1
              SUMDEN=FNCOEF(J%)*(PLGNDR(J%-1)-PLGNDR(J%+1))*
                   (PLGNDR(J%-1)-PLGNDR(J%+1))/(2*J%+1) + SUMDEN
                                            :REM denominator summa-
                                            tion term in eq. A14
      NEXT J%
              PSISURFACE = 1/(1 + (1 - X0 + SUMNUM)/(1 + X0 -
                   SUMDEN/(1-X0)))    :REM   Fraction of total flux
                                       contributed by surface diffusion
       FOR L% = 1 TO N%
              SUMKA = ((PLGNDR(L%-1)-PLGNDR(L%+1))/
                   (FNC(L%) + ALPHA*L%*(L%+1)))*(PSISURFACE*(1-
                   X0)*PLGNDR(L%)+(1-PSISURFACE)*(PLGNDR(L%-1)-
                   PLGNDR(L%+1))/(2*L%+1))+SUMKA :REM  summa-
                                            tion term in eq. A15
       NEXT L%
              EFFEC.ASSOC.RATIO= 1/(1 + BETA + SUMKA/((1-X0)*
                   (1-X0)))  :REM  ka/4πDR

PRINT#1,CSNG(SUMNUM);CHR$(9);CSNG(SUMDEN);
CHR$(9);CSNG(SUMKA);CHR$(9);CSNG(PSISURFACE);CHR$(9);CSNG(
EFFEC.ASSOC.RATIO);CHR$(9);CSNG(N4);CHR$(13);
       NEXT N5%
        CLOSE  #1
END

SUB LEGENDRE(M%,X,P(1)) STATIC
   P(0)=1: P(1)=X
         FOR I% = 2 TO M%+1
        P(I%)= ((2*I%-1)*X*P(I%-1)-(I%-1)*P(I%-2))/I%
   NEXT I%
END SUB
```

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