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ISOLATION AND PHYSICO-CHEMICAL CHARACTERIZATION OF ACTIVATED BOVINE FACTOR X

by

© ROBERT D. RADCLIFFE

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "ISOLATION AND PHYSICO-CHEMICAL CHARACTERIZATION OF ACTIVATED BOVINE FACTOR X" submitted by ROBERT D. RADCLIFFE in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

The partially purified zymogen form of bovine factor X was converted to enzymatically active forms by incubation either with Russell's viper venom, with a trypsin derivative, with the assembled components of the "extrinsic" and the "intrinsic" clotting pathways, or in 25% w/v sodium citrate. Each of the five activated factor X products was purified by DEAE-cellulose chromatography. A low-molecular weight contaminant was removed by dialysis and gel filtration. A single major band was obtained for each preparation in disc gel electrophoresis.

No significant differences in the physico-chemical properties of the five species were observed. In each case, amino acid analysis revealed a high content of glutamic and aspartic acids. Amino terminal alanyl and isoleucyl residues were found for each species. Approximately 1.6 moles of arginine per mole of protein was released by carboxypeptidase B from the two species so treated. For each of four species examined, a single peak was obtained in sedimentation velocity centrifugation and the slope of $S_{20,w}$ against protein concentration was positive with an $S^{\circ}_{20,w}$ of 3.90 ± 0.03 S. With a $\bar{v}$ of 0.715 calculated from the amino acid content and a $D^{\circ}_{20,w}$ of 6.75×10^{-7} cm^2 sec^{-1}, a molecular weight of 50,000 was calculated from the Svedberg equation. High and low speed sedimentation equilibrium centrifugation molecular weight estimates of 45,000 - 50,000 were considered only approximate due to failure to obtain linear ℓn y against r^2 plots. Gel filtration on Sephadex G-200 indicated molecular weights of approximately 48,000 and the elution profiles were characteristic of associating-dissociating systems.

Analysis of the venom-activated species for hexoses, amino sugars and neuraminic acids were negative. SDS-acrylamide gel electrophoresis of the five species after reduction with mercaptoethanol revealed two peptide bands (estimated molecular weights of 31,000 and 19,000) for each. The larger component incorporated ^{32}P after inhibition of the enzymatic activity with (^{32}P) DFP.

To more fully evaluate the extent of physical similarity of the five species of activated factor X, three techniques were used. When the five species were mixed together in various combinations and then subjected to disc gel electrophoresis, a single major band was always obtained. Secondly, when such mixtures of the five species were reduced and subjected to SDS-acrylamide gel electrophoresis, only two major bands could be discerned. Thirdly, peptide maps of tryptic digests of the five species revealed only minor and probably insignificant differences.

It was concluded that the activated factor X products obtained with these five systems are most likely identical molecular species. Presumably therefore, zymogen factor X contains one or more particularly susceptible peptide bonds which are cleaved similarly by the five activating systems.

Experiments designed to elucidate the mechanism of "citrate activation" of factor X revealed that activation requires the presence of contaminant enzymes, one of which was identified as thrombin. Another is probably factor VII.

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ABBREVIATIONS

A	absorbance
BSA	bovine serum albumin
CPA	carboxypeptidase A
CPB	carboxypeptidase B
$D_{20,w}$	diffusion coefficient corrected to water at 20° C
$D^{\circ}_{20,w}$	intrinsic diffusion coefficient at 20° C
DEAE	diethylaminoethyl
DFP	diisopropylphosphorofluoridate
$E^{1\%}_{1\ cm}$	absorbance of a 1% w/v solution in a 1 cm light path
EDTA	ethylenediaminetetraacetate
factor X_a^{Cit}	factor X activated in 25% w/v sodium citrate
factor X_a^{IMET}	factor X activated with IMET
factor X_a^{Int}	factor X activated by the "intrinsic" pathway
factor X_a^{RVV}	factor X activated with RVV
factor X_a^{TF}	factor X activated with "tissue factor"
IMET	water-insoluble copoly-(maleic acid-ethylene)-trypsin
K_m	Michaelis constant
k_o	k_{cat}, k_3 in the equation $V_{max} = k_3$ (Enzyme)

(Continued)

ABBREVIATIONS (Continued)

ℓn y	natural logarithm of protein concentration in fringe displacement units
nm	nanometer
RVV	Russell's viper venom
S	Svedberg unit
$S_{20,w}$	sedimentation coefficient corrected to water at 20° C
$S^{\circ}_{20,w}$	intrinsic sedimentation coefficient at 20° C
SDS	sodium dodecyl sulfate
SNP	standard normal human plasma
STI	soybean trypsin inhibitor
TAME	tosyl-arginine methyl ester
TCA	trichloroacetic acid
TLCK	tosyl-lysine chloromethyl ketone (1-chloro-L-3-tosylamido-7-amino-2-heptanone)
TPCK	tosyl-phenylalanine chloromethyl ketone (1-chloro-L-3-tosylamido-4-phenyl-2-butanone)
tris	tris-(hydroxymethyl) aminomethane
$\bar{v}$	partial specific volume

CHAPTER I

INTRODUCTION

A. Biological Characterization of Factor X

Factor X* is the zymogen form of a plasma coagulant protein. Its enzymatically active form was postulated by Morawitz in 1905 (2) but bleeding disorders due to factor X deficiency were not reported until 1956 (1,3-5). These patients suffered severe external bleeding after injury and spontaneous internal bleeding into joints.

Zymogen factor X may be activated by two distinct clotting pathways and by numerous enzymes not normally present in blood. During normal clotting only a small proportion of the factor X available is activated, due to a limiting level of factor VIII (6). Activated factor X converts prothrombin to thrombin in a reaction greatly accelerated by the presence of calcium ions, phospholipids and a protein cofactor, factor V (7). It hydrolyses TAMe (5), and activates chymotrypsinogen slowly (9) but possesses little or no caseinolytic activity (10). Activated factor X has been reported to initiate platelet aggregation *in vitro* (11).

The synthesis of factor X in the liver is vitamin K dependent. This is also true for factors II, VII and IX (12).

---

* The International Committee on the Nomenclature of Blood Clotting Factors approved the name factor X in 1959 (1). For synonyms see Table I.

Activated factor X (but not zymogen factor X) is cleared from the blood both by a circulating inhibitor, variously called antithrombin III, heparin co-factor, or anti-X_a (13) and by the liver (14). It has been suggested that inhibition of activated factor X by anti-X_a constitutes a primary mechanism for prevention of disseminated intravascular coagulation following injury (13).

B. The Blood Clotting Process

Lorand (15) and Bettelheim and Bailey (16) established that thrombin cleaves the peptide chains of fibrinogen. Subsequently it has become accepted that blood clot formation involves a sequence of proteolytic hydrolyses (for a review see Esnouf and Macfarlane (17)). Table I lists the factors known to participate in clotting and shows some of their common synonyms. The "cascade-waterfall" hypothesis (Fig. 1) proposed by Macfarlane (18) and Davie and Ratnoff (19) in 1964 incorporates a sequence of zymogen to enzyme conversions, some steps involving calcium ions and phospholipids. More recent investigations have confirmed the basic "cascade-waterfall" pattern, except that in the present model factors V (7) and VIII (20,21) are regarded as co-factors, not enzymes (Fig. 2). The clotting process may be initiated either by contact of factor XII with an appropriate ionic surface (the "intrinsic" pathway) or by the release of damaged tissue components into the blood (the "extrinsic" pathway). Unfortunately, the mechanisms of these initiating steps are not well understood, nor has their relative importance *in vivo* been fully evaluated.

TABLE I

BLOOD CLOTTING FACTOR NOMENCLATURE

Roman Numeral	Preferred Name	Synonyms
I	Fibrinogen	
II	Prothrombin	
IV	Calcium ions	
V	Proaccelerin	Accelerator globulin (AcG), Labile factor
VII	Proconvertin	SPCA, Stable factor, Autoprothrombin I
VIII	Antihaemophilic factor (AHF)	Antihaemophilic globulin (AHG), Platelet cofactor I
IX	Plasma thrombo-plastin component (PTC)	Christmas factor, Autoprothrombin II
X	Stuart factor	Prower factor, Autoprothrombin III, Prothrombokinase
XI	Plasma thrombo-plastin antecedent (PTA)	Platelet factor II
XII	Hageman factor	Glass factor
XIII	Fibrin stabilizing factor (FSF)	

(From Gaston (22), Kline (23) and Milstone (24)).

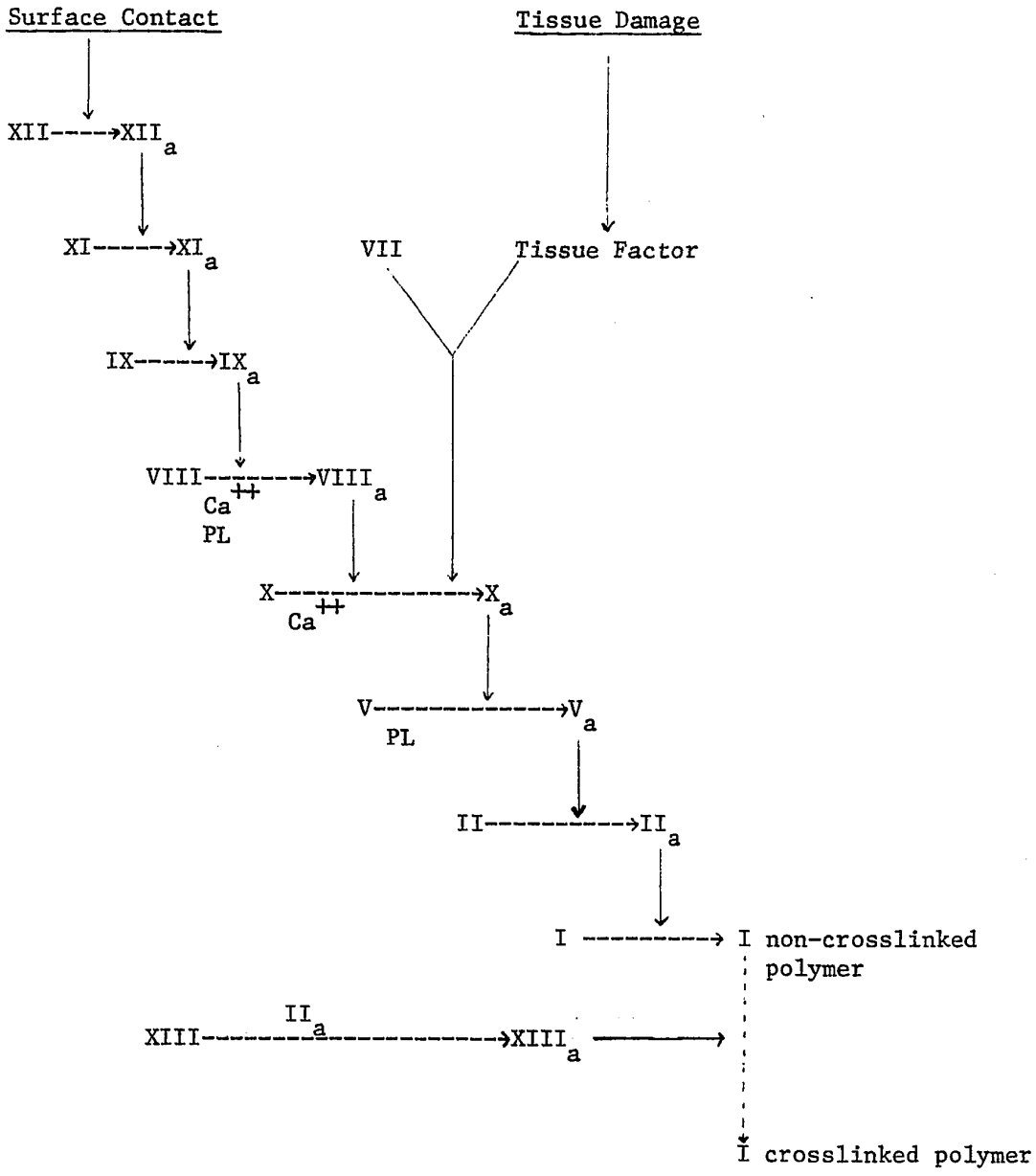


Figure 1. Cascade-Waterfall proposal 1964 modified from Macfarlane (18), and Davie and Ratnoff (19).

a - Subscript "a" indicates enzymatically active forms.

-----→ Transformation

——→ Action

PL - Phospholipids

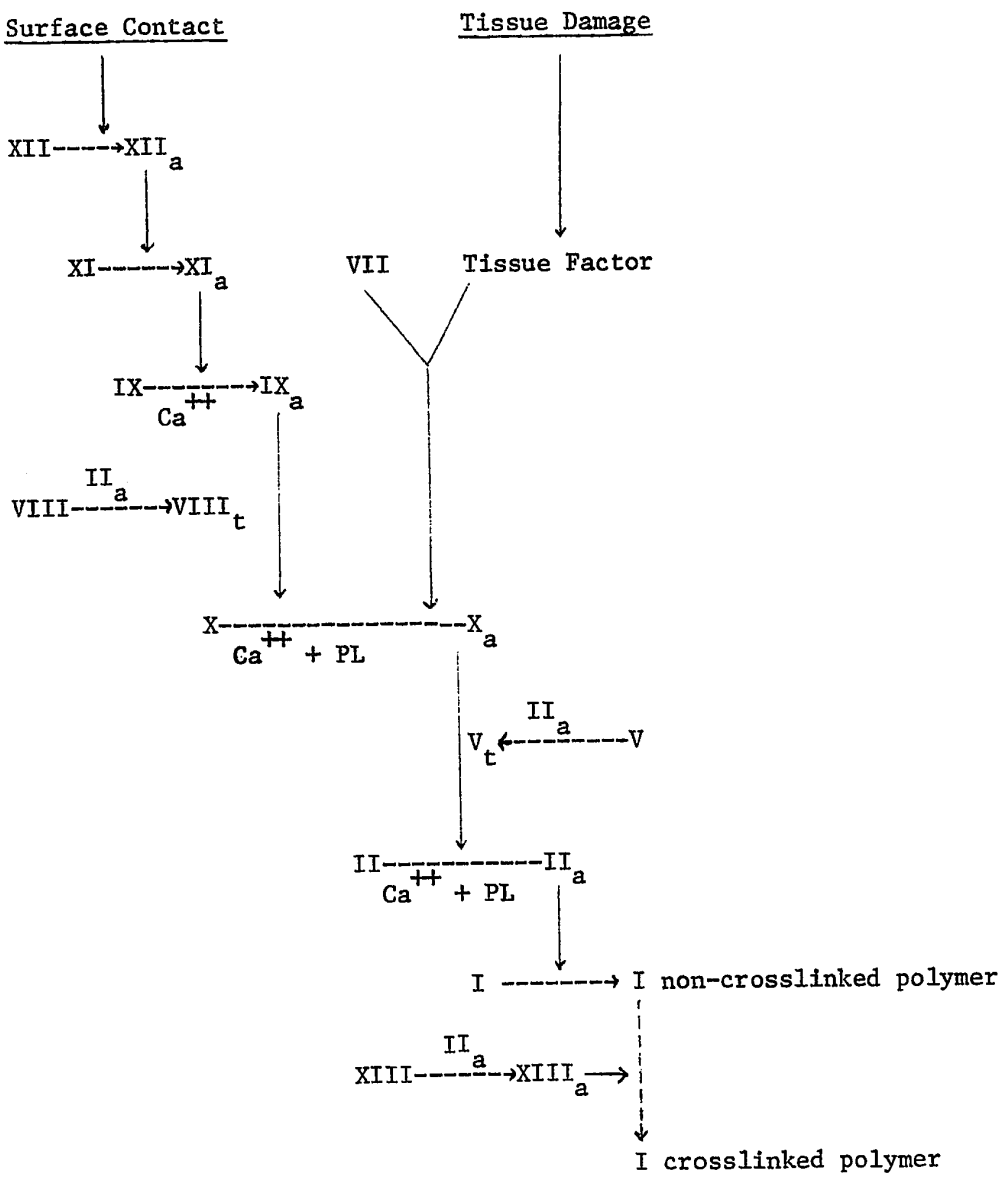


Figure 2. Revised blood clotting sequence, 1973

a - Subscript "a" indicates enzymatically active forms

----- Transformation

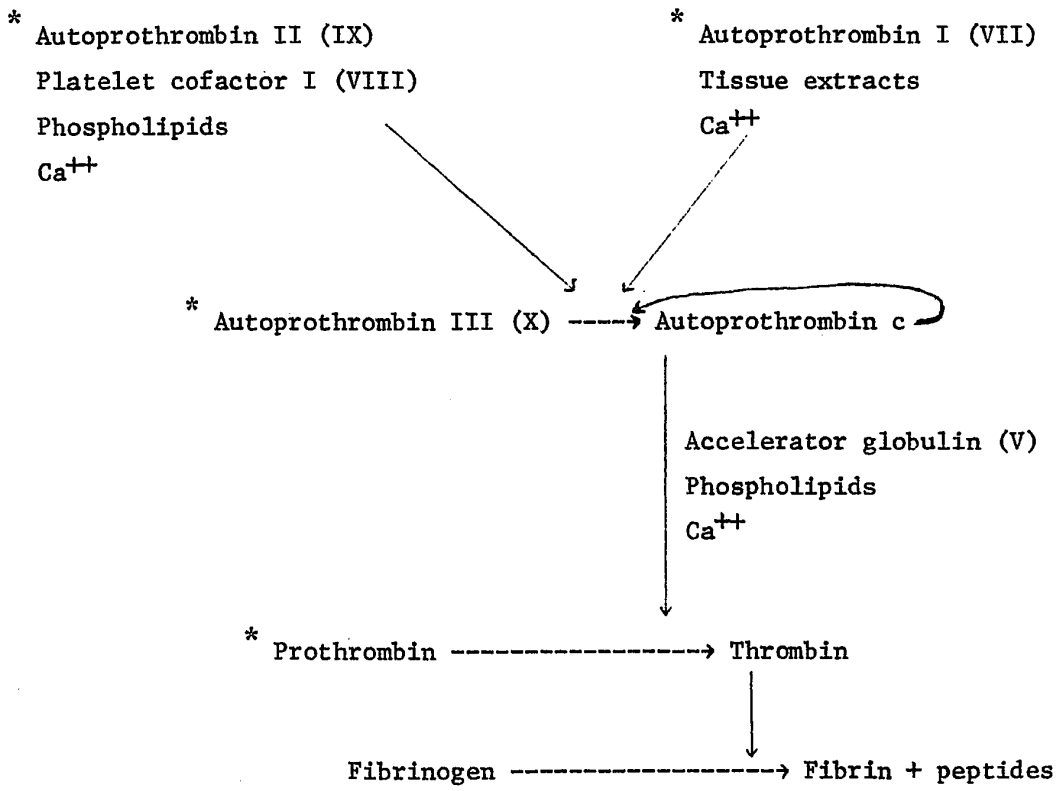
——— Action

PL - Phospholipids

The intrinsic and extrinsic pathways converge at the point of conversion of factor X to activated factor X. Referring to Fig. 1, it should be noted that factor VIII is now considered by many as a cofactor rather than as the zymogen form of an enzyme (20,21). In the more recent view (Fig. 2) activated factor IX cleaves factor X to form activated factor X with factor VIII as cofactor. Calcium ions and phospholipids are required for a rapid reaction (20).

A striking analogy can be seen between the above reaction and the subsequent cleavage of prothrombin to thrombin by activated factor X in that the latter reaction is also greatly accelerated by the presence of calcium ions, phospholipids and a protein cofactor, factor V (Fig. 2). It has been postulated that the phospholipids provide a surface for the assembly of the clotting factors (25). Thrombin, once formed, functions most importantly to cleave fibrinogen to fibrin monomers. Thrombin also acts on factors V (26) and VIII (27), transiently increasing their cofactor activity; and on factor XIII, converting it from a zymogen to an active enzyme (28). Final clot formation involves polymerization of fibrin monomers and formation of ε-lysyl-γ-glutamyl cross-links by activated factor XIII, stabilizing the clot (28).

In opposition to the "cascade-waterfall" scheme, Seegers has proposed the "prothrombin derivatives" theory (29), shown in Fig. 3. His group observed that a "prothrombin" preparation, prepared without the use of chromatography or electrophoresis (30), generated several clotting activities besides thrombin. These



* From Prothrombin Complex

Figure 3. Prothrombin derivatives theory of blood coagulation, Adapted from Seegers et al. (31).

-----> Transformation

——> Action

activities were said to arise from the prothrombin molecule by "autocatalysis" under appropriate conditions. The "cascade-waterfall" school would say that these activities arose not from the prothrombin molecule but from contamination of the prothrombin by factors VII, IX, X and perhaps other factors. In more recent publications from Seegers' laboratory the term "prothrombin complex" has been substituted for "prothrombin" (31). This terminology change may reflect recognition that the material earlier called prothrombin by this group was in fact not pure. The presence of factors VII, IX and X in Seegers' "prothrombin" has been well documented (32-36).

C. Physico-Chemical Characterization of Factor X

Attempts to isolate factor X (8,31,33,34) have generally involved adsorption of oxalated plasma with $BaSO_4$, followed by washing of the $BaSO_4$ with dilute saline and subsequent elution of the factor X with sodium citrate solution at a minimum concentration of 0.06 M (37). $BaSO_4$ is reported to adsorb factors II, VII, IX and X (38). Elution was followed by chromatography on DEAE-cellulose or DEAE-sephadex or both. Careful gradient elution led to a nearly pure product. Jackson et al. (37) and Seegers et al. (31) included a final Sephadex G-100 chromatography. The former authors found that DFP must be added at each step to prevent degradation of factor X, presumably by thrombin, while the latter group employed STI similarly.

Jackson and Hanahan (39) reported that zymogen factor X, judged pure by disc electrophoresis, eluted from DEAE-Sephadex A-50 in two peaks that showed identical amino acid compositions but different

carbohydrate contents (personal communication from C.M. Jackson). These were called variants X_1 and X_2. One mole of factor X_1 contains 8 moles of hexose, 8 moles of sialic acid and 6 to 7 moles of hexosamine as compared with 10 moles of hexose, 9 to 10 moles of sialic acid and 7 to 9 moles of hexosamine per mole of factor X_2. In all other chemical respects examined X_1 and X_2 appeared the same.

Jackson and Hanahan (39) calculated a molecular weight of 54,000 from an intrinsic sedimentation coefficient, $S^{\circ}_{20,w}$, of 3.56 S and a diffusion coefficient, $D^{\circ}_{20,w}$, of 5.6×10^{-7} cm^2 sec^{-1}. This molecular weight was in agreement with their value from sedimentation equilibrium centrifugation. Seegers' group found $S^{\circ}_{20,w}$ values of 3.4 and 3.58 S in two studies (31,40). However, Esnouf and Williams (8) reported a molecular weight of 87,000 calculated from an S_{20} value of 4.23 S and a D_{20} value of 4.57×10^{-7} cm^2 sec^{-1}. They obtained a value of 85,000 from sedimentation equilibrium centrifugation. Plots of $S_{20,w}$ against zymogen protein concentration showed a normal negative slope (39), in contrast with the active enzyme (see Chapter V).

Sephadex gel filtration molecular weight estimates have been reported in the range of 82,000 to 90,000 (31,39,41). Jackson and Hanahan (39) argued that this value is estimated too high due to an axial ratio of about 10 which they found for factor X.

Both variants of factor X were found to contain two polypeptide chains in SDS-acrylamide gel electrophoresis (personal communication from C.M. Jackson). After isolation of the reduced, carboxymethylated chains on Sephadex G-25, the larger chain of molecular weight 38,000 was found to contain all the carbohydrate. The

smaller chain of molecular weight 15,000 is highly acidic, suggesting a possible role in the calcium-dependent binding of activated factor X to phospholipid (7). The small chain showed an N-terminal alanyl residue while the N-terminal amino acid of the large chain may be glutamic acid. Seegers et al. (31) found N-terminal glycyl and seryl residues for autoprothrombin III. Tishkoff et al. (34) reported a molecular weight of 38,000 for what they called "monomer" X, a species observed in 6 M guanidine-HCl, 0.5% mercaptoethanol. Presumably this corresponds to the large chain observed by Jackson and Hanahan (39).

The sedimentation coefficient of factor X shows a decrease with increasing ionic strength of the supporting buffer (39). It may be of particular relevance to the project reported in this thesis that citrate solutions produced a greater decrease in sedimentation coefficient than did saline solutions of the same ionic strength (39).

The highest specific activity reported for bovine factor X is about 140 units per A_{280} unit (39)*.

D. Methods of Activating Factor X

1. Activation by Russell's Viper Venom

Russell's viper venom (RVV) was shown to be a powerful coagulant by Macfarlane (43) in 1934. Further work in his laboratory established a requirement for calcium ions and phospholipids in this reaction (44). Hougie (45) found that RVV was unable to correct the clotting defect of his factor X deficient patient, R. Stuart, in contrast with the ability of RVV to correct factor VII deficiency.

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*A unit of factor X is defined as the amount present in 1 ml of normal human plasma using the standard assay (42).

Peden and Peacock (46) suggested that RVV functions as an enzyme acting on a serum substrate. Calcium ions, phospholipids, factor V and factor X were essential for activation of prothrombin with RVV. After reaction of RVV with serum, the venom could be neutralized with a specific antibody and this technique was used by Macfarlane (47) to demonstrate the enzymatic action of RVV and to show that the substrate probably was factor X. Finally, Macfarlane (48) established the RVV substrate to be factor X by showing that factors VII and IX deficiencies but not factor X deficiency were corrected by RVV.

Williams and Esnouf (49) isolated from RVV a protein which activated purified factor X ($S_{20,w}$ = 4.23 S at approximately 5 mg protein per ml) in the presence of calcium ions, yielding two products with smaller sedimentation coefficients ($S_{20,w}$ = 3.72 and 1.90 S at approximately 5 mg protein per ml). At the same time there appeared a new N-terminal residue, leucine or isoleucine, not present on the zymogen, which had been reported to have N-terminal alanyl and glycyl residues (8). In contrast with the zymogen, the activated factor X (called factor X_a^{RVV} hereafter) possessed TAMe esterase activity.

Williams and Esnouf (49) reported that the coagulant fraction they had isolated from RVV also possessed TAMe esterase activity. They suggested that the coagulant and esterase activities reside in a single protein species. Later workers (50,51) have separated the factor X activating protein of RVV from the TAMe esterase activity. The factor X activating species is a glycoprotein dimer of molecular weight 104,000 with no observed esterase or glycosidase activity (personal communication from C.M. Jackson).

It did not degrade α-casein or gelatin and was not inhibited by DFP. No enzymatic activity was found other than its activity toward factor X.

Factor X_a^{RVV} is inhibited by STI (52,53) and by DFP at concentrations of 10^{-2} M or greater (54). Leveson and Esnouf (54) reacted (^{32}P) DFP with factor X_a^{RVV} and found ^{32}P bound covalently to one serine residue located in the classic Gly-Asp-Ser-Gly sequence.

It has been shown by several groups (7,41) that factor X_a^{RVV} binds reversibly to phospholipids in the presence of calcium ions, and more weakly in the presence of other divalent cations. Kinetic data yielding a Michaelis-Menton constant of 25 mM for TAMe hydrolysis suggest that factor X_a^{RVV} is a relatively poor enzyme for catalysis of ester hydrolysis (37,55).

2. Activation by Trypsin

(a) Native trypsin

The ability of trypsin to enhance clotting was reported by Douglas and Colebrook (56) in 1916. Calcium ions (57) and "cephalin" (58) were found to potentiate the effect. Stormorken (59), in 1956, found that factor V was needed for rapid thrombin generation after treatment of plasma with trypsin. Factor VII was apparently not essential. The latter conclusion was substantiated by Alexander (60) using factor VII-rich and factor VII-poor plasma, but this conclusion was contested by others (61,62). Pechet and Alexander (63) found that trypsin activated a purified factor X preparation.

Yin (64) reported that factor V-deficient plasma and factors VII- and X-deficient (Seitz-filtered) plasma clotted slowly in the presence of trypsin and calcium ions, but factor VII-deficient plasma clotted rapidly. Factor X activated by trypsin was found to require calcium ions, phospholipids and factor V for rapid prothrombin conversion. Factor VII was destroyed, not activated, by trypsin (64).

Papahadjopoulos et al. (41), using purified factor X, found that trypsin activation yields activity quantitatively equal to that generated from the same factor X preparation by RVV. Also, factor X fully activated with RVV could not be further activated with trypsin and vice-versa. Despite these similarities, one major difference between RVV- and trypsin-activation of factor X was observed. While the trypsin activation mixtures rose to a maximum of factor X_a activity and then began to lose activity, RVV apparently did not degrade factor X_a^{RVV} significantly once formed. This can be rationalized on the grounds of a broader specificity of trypsin than of RVV. On gel filtration factor X_a^{RVV} appeared to elute slightly before factor X_a activated by trypsin, suggesting non-identical molecular species.

(b) Water-insoluble trypsin derivatives

Binding of trypsin to water-insoluble matrices permits removal of the enzyme from a reaction mixture by centrifugation or filtration as opposed to more complex techniques involving the selective inhibitors benzamidine-HCl or soybean trypsin inhibitor (64). Water-insoluble copoly-(maleic acid-ethylene)-trypsin (IMET) activates factor X but not factors II or VII (65). Water-insoluble polytyrosyl-trypsin (IPTT) activates factor X and factors II and VII (65).

Both matrix-bound forms of trypsin consume prothrombin. None of these activities requires calcium ions.

3. Activation by Tissue Extracts (The Extrinsic Pathway)

Howell (66), in 1912, reported the presence in aqueous extracts of tissues of a clot-accelerating factor that contained protein and lipid. Chargaff et al. (67) found the accelerating factor to be insoluble in aqueous solutions. Flynn and Coon (68) reacted brain extracts with serum and obtained a sedimentable product capable of accelerating prothrombin conversion in the presence of factor V. Hecht et al. (69) used centrifugation at 100,000 x g for 1 hour to separate a saline extract of brain (which they called "brain extract thromboplastin") into two components. The two appeared as separate layers on the bottom of the centrifuge tube. The lower grey layer possessed little pro-coagulant activity. The upper yellow layer, which they termed "brain thromboplastin", possessed "thromboplastic" activity and upon intravascular injection into mice resulted in death. From results of a qualitative biuret assay, they concluded that the "brain thromboplastin" contained no protein. They attributed the "thromboplastic" activity to a unique lipid structure. Refluxing with ethanol for 1-1/2 hours destroyed this activity while refluxing with ether for 1-1/2 hours or boiling in water for 1-1/2 hours did not.

Hougie (70) showed that factor X-deficient and factor VII-deficient sera when incubated with brain extracts, factor V and calcium ions failed to develop prothrombin-activating activity, in contrast with normal sera. Deficiency in factor X led to low yields of prothrombin-converting activity and deficiency in factor VII led to a

slow rate of formation of this activity. Straub and Duckert (71) also found calcium ions, factor VII and factor X to be essential for activity development with tissue extracts. Nemerson and Spaet (72) confirmed the observation that factor VII is essential to the activation of factor X by tissue extract and calcium ions using factor VII- and X-deficient sera. From kinetic studies they concluded that tissue factor acts as an enzyme on substrate factor X with factor VII acting as "accelerator". However the factor X-activating activity was not eliminated by reaction of the tissue factor with DFP. Williams (73), after extensive centrifugal purification of the microsomal component of bovine lungs, found it to contain 48% protein, 38% phospholipid and 10% cholesterol (w/w). After interaction with serum, additional protein was localized in the particles. Later Williams (74,75), separating factor VII from factor X on DEAE-cellulose, found that factor X could be activated by tissue factor and calcium ions only when contaminated with factor VII. By incubating lung microsomes with purified factor VII, he was able to show that factor X-converting activity was generated in less than ten seconds. Moreover, both yield of activity and rate of generation were dependent on the concentration of both reactants. Microsomes, after exposure to factor VII and calcium ions, showed an increased density in the centrifuge. Neither EDTA, citrate nor oxalate (chelating agents) released the activity into the supernatant. This data did not appear to support either a mechanism involving activation of a zymogen factor VII by the tissue factor or activation of a tissue component zymogen by factor VII. It was, however, noticed that one preparation of factor VII,

after long storage at -20°C, could activate factor X without addition of tissue factor, and this suggested that factor VII was the immediate source of the enzyme. Reaction of 1 mM DFP with factor VII, before addition of tissue extract, prevented activity generation but no effect was noted when tissue factor was pre-treated with DFP. DFP eliminated the activity of the factor VII-tissue factor complex (76).

The necessity for phospholipids in the clot-promoting activity of tissue factor is shown by the fact that removal of phospholipids by butanol (77) or phospholipase C treatment (78) of tissue factor destroyed activity. Adding back phospholipids restored the activity. Østerud et al. (79) reported an "activated factor VII" which passed through a Millipore filter claimed to retain tissue factor particles. This "activated factor VII" was formed by interaction of factor VII with tissue factor. The factor X-converting activity of the "activated factor VII" was destroyed by phospholipase C, but then was only restored by tissue factor and not by phospholipids alone. An "activated factor VII"-phospholipid complex was concluded to be the active principle.

The mechanism by which tissue factor and factor VII activate factor X is not well understood. No physico-chemical data have been previously reported on the resultant factor X_a^{TF} species.

4. Activation by Surface Contact (The Intrinsic Pathway)

The observation (80) that the clotting of factor VII-deficient plasma is normal suggested that blood may clot by a route distinct from the "extrinsic" pathway. Biggs et al. (81) found that when platelets, $CaCl_2$, serum and $Al(OH)_3$-adsorbed plasma were incubated for 5 minutes in a siliconized vessel, an activity developed which clotted recalcified plasma in 8 to 10 seconds, but did not clot fibrinogen. The activity did not develop if factor VIII-deficient plasma was used. A preparation of factor VIII corrected this deficiency. The same group (82) later observed the clotting of normal blood in siliconized tubes when various additions were made. When platelets plus normal sera were added to 10 volumes of blood, maximum thrombin levels were attained in 4 minutes. When sera from a patient with Christmas disease (factor IX-deficient) was substituted, thrombin generation required 12 minutes, the same as for the saline control. For comparison, addition of "brain extract thromboplastin" led to a maximum thrombin level in 1 minute. Observing that maximal prothrombin-converting activity evolved from blood after 3 minutes in glass, but only after 12 minutes in siliconized tubes, they concluded that glass contact activated factor IX, which in conjunction with factor VIII evolved a "thromboplastic" activity or the ability to activate prothrombin in the presence of factors X, V, $CaCl_2$ and phospholipids.

Bergsagel (83) found the active component of platelets to be removed by ether, suggesting it was lipid. Factor VIII was observed to disappear during generation of "thromboplastic" activity. The concentration of factor VIII affected primarily the rate of reaction,

rather than the yield (84). Bergsagel and Hougie (85) used deficient sera to study the development of "thromboplastic" activity when $CaCl_2$, factor VIII and normal sera were incubated together. The resulting activity was called "Intermediate Product I". Substitution of factor VII-deficient sera did not alter these results. $Al(OH)_3$-adsorbed sera or partially purified factor IX, free of factor VII, were found to substitute completely for the normal sera. The resultant activity was found to be sedimentable. It was destroyed by sodium citrate and Amberlite. "Intermediate Product I" did not activate prothrombin in factor V-deficient plasma. This suggested that factor IX, platelets, factor VIII and $CaCl_2$ produce a product which activates prothrombin in a later step requiring factor V.

Factor XII (Hageman factor), discovered in 1955 (86), was soon shown to be essential for the clot-promoting effect of glass contact (87). Biggs et al. (88), from studies of factor XII- and factor XI-deficiencies, proposed that glass contact "activates" factor XII followed by activation of factor XI by "activated" factor XII. Calcium ions are not essential to this process (89). Nossel (90) found that activated factor XI is produced after contact activation of factor XII in proportion to the disappearance of plasma factor XI. This is in keeping with the notion that zymogen factor XI is converted enzymatically to activated factor XI.

Macfarlane (47,91) showed that if a mixture of factors XI and XII were "contact activated" and then reacted with a mixture of factors VIII, IX and X in the presence of calcium ions, an activity was generated which was similar to that of factor X activated by RVV.

However, it was not immediately clear which of these "intrinsic" pathway factors was the enzyme directly responsible for the activation of factor X. Two schools of though developed, as shown in Fig. 1 and Fig. 2. In one view, this enzyme was thought to be activated factor VIII, itself formed under the influence of activated factor IX (53). In a second model, activated factor IX was considered to act directly on factor X with factor VIII serving as a cofactor (20,21). The activity of factor VIII is greatly stimulated by thrombin, but even after this treatment it is still considered as a cofactor in this model (27). Østerud and Rapaport (92) showed that the "intrinsic" factor X-activating species could be neutralized by antibodies either to factor IX or factor VIII. This elminated the possibility that a hypothetical factor $VIII_a$ species alone might convert factor X to activated factor X (53,93). When purified factor IX_a, factor VIII treated with thrombin (factor $VIII_t$), phospholipids and calcium ions were combined, an activity able to activate factor X developed instantly (92). Proof that these various factors formed an active complex came from the work of Hougie (94) and Chuang et al. (95), who demonstrated that the "intrinsic" factor X-activating species was eluted in the void volume of a Sephadex G-200 column after factors IX_a, $VIII_t$, phospholipids and calcium ions were incubated together and subjected to chromatography in the presence of calcium ions. However, if calcium ions were omitted during chromatography the factor IX_a and factor $VIII_t$ activities eluted separately and neither alone could activate factor X in the presence of calcium ions and phospholipids.

Their recombination regenerated the factor X-converting activity. These data are consistent with a model for the "intrinsic" factor X activator as a calcium ion-dependent, reversible complex of factors IX_a and $VIII_t$ with phospholipids.

The purification and characterization of factor X activated by the "intrinsic" pathway (X_a^{Int}) have not been reported hitherto.

5. Activation by Concentrated Sodium Citrate and Ammonium Sulfate Solutions

In 1962, Marciniak and Seegers (96) reported that incubation of non-chromatographed prothrombin with 25% w/w sodium citrate solution led to the generation of thrombin and an additional activity similar to that of activated factor X. This activity they called autoprothrombin c. Purification and characterization of autoprothrombin c were reported the following year (97). Purity was claimed on the basis of a single peak observed during sedimentation in the ultracentrifuge. A molecular weight of 21,500 was calculated from an $S_{20,w}$ of 2.27 S, $D_{20,w} = 8.4 \times 10^{-7}$ cm^2 sec^{-1}, and $\bar{v} = 0.695$. Plots of sedimentation coefficient against protein concentration showed a negative slope. Analyses for carbohydrate revealed 7% hexose and 3.8% hexosamine. Specific activities ranged from 2140 to 4300 units per mg tyrosine and in exceptional cases as high as 17,000 units per mg tyrosine (97).

Unfortunately, later work indicated the material thus characterized was not pure (98-100). It had been prepared by chromatography on DEAE-cellulose. Contaminant protein had been eluted with 0.1 M sodium phosphate buffer, pH 7.4. Autoprothrombin c had been eluted by an abrupt increase to 0.4 M phosphate buffer.

Later Seegers et al. (100) altered the chromatography to include an intermediate ionic strength (0.175 M sodium phosphate) washing before elution of autoprothrombin c with 0.4 M buffer. The material applied in this case had been activated with concentrated $(NH_4)_2SO_4$. This material normally showed a higher specific activity (4,000-8,000 units per mg tyrosine) but was polydisperse in the ultracentrifuge. The authors claimed that DEAE-cellulose degrades autoprothrombin c yielding multiple peaks but this seems unlikely in view of the impressive stability claimed for the preparation in earlier chromatographic experiments. Landaburu and Albado (98) isolated three main protein peaks from autoprothrombin c by chromatography on IRC-50 XE 97. Only one peak showed autoprothrombin c activity. Thus, the physicochemical data cited above (97) likely do not relate to the active enzyme present. A very recent report from Seegers' laboratory (31) claimed the molecular weight of autoprothrombin c (the authors use the same term although in this case activation was with RVV) to be 53,500 and 58,000 by two gel filtration procedures.

The mechanism of action of concentrated sodium citrate or ammonium sulphate is not well understood. However, it has been established that prothrombin, chromatographed to purity, is activated in concentrated salt solutions only after addition of autoprothrombin c (factor X_a) (34,35,98,100,101). Secondly, if the chromatographed precursor of autoprothrombin c, called autoprothrombin III, is incubated in strong salt solution, autoprothrombin c is not generated (41,102). One or more contaminant enzymes must be present at least in trace amounts.

The nature of such postulated enzymes is yet uncertain. Kipfer and Seegers (103) reported that generation of autoprothrombin c in 25% sodium citrate solutions of chromatographed autoprothrombin III could be "primed" by initially including a trace of autoprothrombin c. However, as discussed above, the autoprothrombin c preparation used by them was impure, so that no firm conclusion can be drawn. Marciniak and Seegers (96) followed the generation of thrombin from non-chromatographed prothrombin in 25% sodium citrate "primed" with additions of autoprothrombin c or thrombin or both. Priming with autoprothrombin c alone or with thrombin alone greatly accelerated thrombin formation and these effects were additive. Again, interpretation of this data is uncertain since the effects of the added thrombin and impure autoprothrombin c could be on either autoprothrombin III or prothrombin. In the preferred terminology, the possible "citrate activators" of factor X include factor X_a itself, factors VII and IX as possible contaminants of the autoprothrombin c preparation and finally, thrombin.

There is available only indirect evidence on the mechanism by which concentrated salts can affect the conformation of a substrate such as factor X so as to allow its more rapid proteolysis by enzymes (104).

6. Activation by Snake Venoms other than RVV

Nahas et al. (105) tested eight venoms for coagulant activities. In five cases it was concluded that the venoms activated factor X directly. These were V. Russellii, B. Atrox, B. Jararaca, E. colorata and A. Rhodostoma. Interestingly, none of the venoms tested was found to activate any factor occurring before factor X in the postulated sequence of interactions.

7. Activation by Papain and Ficin

Pechet and Alexander (106,107) observed that papain and ficin activated factors X and VII but not prothrombin. Numerous other proteases tested by them had no activating effects.

8. Activation by Factor X_a

Papahadjopoulos et al. (41) observed that preparations of factor X, purified by chromatography, developed low levels of factor X_a activity "spontaneously" after three months at -20°C in siliconized tubes. Moreover, when this "spontaneously formed" factor X_a, factor X_a^{RVV} or trypsin-activated factor X was incubated with zymogen factor X, factor X_a activity evolved. However, it can be questioned whether the trypsin or RVV, initially used to prepare $X_a^{Trypsin}$ and X_a^{RVV}, had been entirely removed by Sephadex G-200 chromatography and benzamidine-HCl respectively. Also, trace contaminants in the factor X preparation are difficult to rule out.

9. Activation by Cathepsin C

Purcell and Barnhart (108) found that if non-chromatographed prothrombin was treated with a preparation of cathepsin C, thrombin activity evolved. If "cephalin" was added with cathepsin C, autoprothrombin c activity also evolved. However, it is not certain that the autoprothrombin c activity arose due to the direct action of cathepsin C, since the rate of generation of autoprothrombin c was approximately the same whether cathepsin C plus "cephalin", or only "cephalin" alone, was added to the prothrombin preparation.

E. Purpose of this Study

The various mechanisms of activating factor X appear to yield qualitatively similar activities, which in the presence of phospholipids, calcium ions and factor V activate prothrombin rapidly. On the grounds of functional similarities alone, numerous authors (17,101,109,110) have suggested that the factor X_a products are identical. However, it is conceivable that different proteins exhibiting very similar biological activities may have distinct physical and chemical structures. A thorough examination of the identity or non-identity of the activated species of factor X must therefore include physico-chemical characterization. Such limited physico-chemical data as are available with respect to autoprothrombin c (97), X_a^{RVV} (8) and $X_a^{Trypsin}$ (41) are at variance. The work described in this thesis was undertaken with the aim of establishing the identity or non-identity of factors X_a^{RVV}, X_a^{Cit}, X_a^{IMET}, X_a^{TF} and X_a^{Int} by investigation of the physical and chemical properties of the purified proteins.

CHAPTER II

MATERIALS AND GENERAL METHODS

A. Materials

Inorganic chemicals, organic solvents, sucrose, sodium dodecyl sulfate (SDS), trichloroacetic acid (TCA), celite, 1,6 hexanediamine and sodium diethyl-barbiturate were ACS certified reagent grade obtained from Fisher Scientific Co. or from Baker Chemical Co. SDS was recrystallized from ethanol before use. $BaSO_4$ was a U.S.P. product of Merck and Co. obtained from Taylor Chemical Co., St. Louis, Mo. Diisopropyl phosphorofluoridate (DFP) was from Aldrich Chemical Co. and (^{32}P) DFP from Amersham-Searle Corp., Don Mills, Ontario. N-ethyl morpholine and 2-mercaptoethanol were from Eastman Organic Chemicals. N-ethyl morpholine was distilled before use. N-α-p-Tosyl-L-lysine chloromethyl ketone HCl (TLCK), Trizma base (reagent grade), ethylene diamine tetraacetic acid (EDTA), hemoglobin, myoglobin, carboxypeptidases A and B (DFP inhibited), ovalbumin, and factor VII- and X-deficient plasma were purchased from Sigma Chemical Co. Seitz-filtered plasma (factors VII- and X-deficient) was also prepared according to reference (42). Trypsin (TPCK-inhibited) was a Worthington product obtained from Winley-Morris Co. Ltd., Montreal, P.Q.. Urea (ultra-pure) was from Schwarz-Mann, Orangeburg, N.J., dansyl chloride from Pierce Chemical Co., Rockford, Ill., ethylene-maleic acid (EMA) from Monsanto Chemical Co., and formic acid (98-100%) from BDH Ltd., Poole, England. Canal Industrial Corporation supplied Coomassie Brilliant Blue protein stain, acrylamide, N,N-bis methylene acrylamide, N,N,N',N'-

tetramethyl-ethylene-diamine, riboflavin, and ammonium persulfate. Dithiothreitol and bovine albumin, crystalline A grade, were obtained from Calbiochem., Los Angeles, Calif. Standard Normal Plasma (SNP) was from DADE Division, American Hospital Supply, Miami, Fla. A bovine factor VIII preparation was purchased from Maws Pharmacy Supplies Ltd., Barnet, Herts, England. Thrombin, Topical was from Parke-Davis, Detroit, Mich., U.S.A. Factor VII-deficient human plasma was a gift from E.T. Yin. Russell's viper venom (Stypven) was a Wellcome reagent obtained from Warner-Chilcott, Scarborough, Ontario. Chymotrypsinogen A and Sephadex gels were obtained from Pharmacia (Canada), Montreal, P.Q. DEAE-cellulose (Whatman microgranular, DE -32 exchange capacity 1.0 m-equiv/g) was obtained from Reeve-Angel, Clifton, N.J. ε-Polycaprolactam layer sheets (Cheng Chin Trading Co.) were obtained from Gallard-Schlesinger Chemical Corp., Carle Place, N.Y. and a collodion bag concentrator, #100, from Schleicher and Schuell, Inc., Keene, N.H.

Brain lipid extract ("cephalin") was prepared according to Bell and Alton (111) as a stock solution containing 120 μg phosphorus per ml and diluted with Michaelis buffer, pH 7.35, to a concentration of 2 μg of phosphorus per ml for use in coagulation assays. Tissue extract was prepared by suspending 30 g of rabbit brain acetone powder (Pel-Freez Biologicals Inc., Rogers, Ark.) in 100 ml of 0.14 M NaCl and allowing large particles to settle for five minutes.

B. Preparation of Bovine Plasma

In routine preparations, 15 gallons of bovine blood was collected into 1.5 gallons of 1.7% sodium oxalate at the slaughter house and transported immediately to the laboratory. The blood was centrifuged in 1 liter polypropylene bottles at 4500 r.p.m. for 10 minutes in the HG-4L rotor of a Sorvall RC-3 centrifuge at 15°C. The plasma was removed from the bottles by gentle aspiration.

C. Regeneration of DEAE-cellulose

For chromatography of factor X, the DEAE-cellulose was washed with 0.5 N HCl, then with water until the washings were neutral, then with 0.5 N NaOH and again with water until neutral. The cellulose was then equilibrated with 0.04 M phosphate buffer, pH 7.45, and stored with 0.03% toluene as preservative. For chromatography of activated factor X species, DEAE-cellulose was washed according to Yin and Wessler (112). It was found that for chromatography of activated factor X, which utilizes citrate buffers, the washing procedure involving concentrated citrate and citric acid solutions could not be replaced with conventional HCl and NaOH washings.

D. Concentration of Protein Solutions

Aqueous protein solutions were concentrated at 4° C by use of a collodion bag apparatus from Schleicher and Schuell.

E. Coagulation Assays

Dilutions of samples to be assayed were made in 0° C veronal-acetate buffer, pH 7.35, (Michaelis buffer) containing 19.28 g sodium acetate trihydrate, 29.528 g sodium diethyl-barbiturate and 34 g sodium chloride in a final volume of four liters. The pH was adjusted to 7.35 with 1 N HCl (87 ml).

Factor X was assayed by the method of Bachmann et al. (42). The following reagents were incubated together for 20 seconds at 37° C in a glass tube (8 mm x 75 mm) previously cleaned in sodium dichromate solution: (1) 0.1 ml of the sample diluted in Michaelis buffer, (2) 0.1 ml of factor X-deficient bovine plasma, and (3) 0.1 ml of "Bell and Alton cephalin" suspension (2 µg phosphorus per ml) containing a 1/40,000 dilution of RVV (w/v). After incubation, 0.1 ml of $CaCl_2$ solution (0.025 M) was blown in and at the same time a stopwatch was started. The tube was rocked in a 37° C water bath until clot formation impeded flowing of the solution. Clotting times were converted to units per ml of factor X by reading from a log-log plot of the clotting times of various dilutions of standard normal human plasma (SNP) on the ordinate against dilution on the abscissa. One unit of factor X was taken as the amount present in 1 ml of SNP.

Activated factor X was assayed identically except that the RVV was omitted from the "cephalin" suspension. A unit of factor X_a was arbitrarily taken as one hundred times that amount which gave a clot in seventeen seconds. Dilutions were made with a Hamilton micro-liter syringe into Michaelis buffer at 0° C to obtain clotting times as near to 20 seconds as was practical.

Factor VII was assayed by incubating together 0.1 ml of factor VII-deficient human plasma (a gift from E.T. Yin), 0.1 ml of "tissue extract" and 0.1 ml of appropriately diluted test solution for 20 seconds at 37° C, then blowing in 0.1 ml $CaCl_2$ solution (0.025 M) and timing clot formation. Calibration was performed as above using SNP as standard. Thrombin was assayed by recording the clotting time of a mixture of 0.2 ml of fibrinogen solution (2 mg per ml), 0.1 ml of appropriately diluted test solution and 0.1 ml $CaCl_2$ solution (0.025 M) at 37° C. NIH Standard Thrombin Lot. No. B-3 was used as a standard. Prothrombin was assayed by the one stage RVV method of Hjört *et al.* (113).

F. Disc Gel Electrophoresis

Disc gel electrophoresis was performed according to Ornstein (114) using a Tris-glycine buffer (0.025 M Tris, 0.192 M glycine, pH 8.3) (115). Gels were stained with 1% buffalo black in 7-1/2% acetic acid and destained electrophoretically.

G. SDS-Acrylamide Gel Electrophoresis

Earlier runs were by the method of Maizel (116), using 5% or 10% acrylamide gels in 12 cm x 5 mm tubes. Later runs were by the method of Laemmli (117), using a stacking gel and 10% separating gels. Staining was with Coomassie Brilliant Blue, and destaining by passive diffusion in 7-1/2% acetic acid. Gel scanning was performed using a Gilford Model 240 spectrophotometer fitted with scanning accessory.

H. Amino Acid Analysis

Amino acid analyses were performed by the technique of Spackman, et al. (118) on a Beckman 120 C amino acid analyzer. Protein solutions were dialyzed against water to remove halide ions and hydrolyzed in evacuated tubes with 6 N HCl for 24, 48, and 72 hours at 105° C. Extrapolations were made to correct for losses of serine and threonine and for incomplete hydrolysis of leucine, isoleucine and valine. Cysteine and methionine were measured after performic acid oxidation (119). Peak areas were determined manually or with electronic integration.

I. Amino and Carboxyl Terminal Amino Acid Determinations

For N-terminal amino acid determinations, 1 to 2 mg of protein in 1 ml 0.1 M sodium citrate, pH 7.0, was treated for 12 hours at 20° C with 1/100 volume of 2 M DFP in 2-propanol, then dialyzed against several changes of 0.5 M sodium bicarbonate, pH 8.5, at 4° C over 24 hours. The dialyzed sample was made to 8 M with urea and an equal volume of dansyl chloride in acetone (20 mg per ml) was added and allowed to react several hours at 37° C (120). This was dialyzed for 48 hours against 3 changes of 2 liters of water, and blown dry in a hydrolysis tube with N_2. After hydrolysis with 6 N HCl for 18 hours at 105° C, the dansyl residues were dissolved in 50% pyridine and determined by chromatography on ε-polycaprolactam layers (5 cm x 5 cm) (121,122).

C-terminal amino acid residues were also determined on DFP-inhibited samples. About 5 mg of inhibited protein was digested with CPA or CPB in 6 M urea followed by amino acid analysis of the material soluble in 7-1/2% TCA (123,124).

J. Carbohydrate Determinations

Neutral sugars were estimated by the orcinol-sulfuric acid procedure as described by Neuberger and Marshall (125) using mannose, galactose and ovalbumin as standard reference materials. 2-amino sugars were estimated using Ehrlich's reagent after hydrolysis in 3.8 N HCl at 100° C for 4 hours according to the same reference (125). Galactosamine, glucosamine and ovalbumin were used as reference materials. N-acetyl-neuraminic acid and N-glycolylneuraminic acid were estimated by the resorcinol procedure of Svennerholm (126) as described in reference (125). N-acetyl-neuraminic acid and thyroglobulin were used as standard reference materials.

K. Peptide Mapping of Tryptic Digests of Activated Factor X

Samples of activated factor X (0.02 to 0.05 μmoles) were inhibited with DFP as described for N-terminal residue determinations and then dialyzed for 48 hours at 4° C against three changes of 0.05 M N-ethyl morpholine-acetic acid buffer, pH 8.0. Then, 1/100 mole of trypsin (TPCK-inhibited) per mole of activated factor X was added and digestion allowed to proceed for 6 hours at 20° C. The mixture was frozen, lyophilized, re-dissolved in water and again lyophilized to more completely remove N-ethyl morpholine.

Digests were applied as 1 cm bands on Whatman No. 1 paper (45 cm x 55 cm) and electrophoresed in glass tanks using toluene as coolant (127) using the pH 6.5 buffer system of Ambler (128) containing pyridine:acetic acid:water (100:3:900 v/v/v). Runs were performed at 3000 volts for 45 minutes. The resulting strips were cut from the paper

sheet, sewn to a second sheet of Whatman No. 1 and run in the second direction in a Gilson Electrophoretor Model D (Gilson Medical Electronics, Middletown, Wisconsin, U.S.A.) (128A) at pH 1.8 using formic acid:acetic acid:water (1:4:45 v/v/v). Peptides were located with the cadmium-ninhydrin reagent of Heilmann et al. (129).

L. Ultracentrifugal Analysis

Sedimentation-velocity measurements were routinely carried out using a Beckman-Spinco Model E Analytical Ultracentrifuge (An-D rotor) operating at a speed of 59,780 r.p.m. with 2°, 12 mm Kel F cells fitted with sapphire windows in conjunction with a Schlieren optical system. The analyses were performed at 20° C over a concentration range of 0.8 to 8 mg per ml on samples dialyzed at 4° C for 24 or 48 hours against 0.1 M NaCl, 0.01 M sodium acetate buffer, pH 7.0. Where specified, $CaCl_2$ was introduced by dialysis. Photographs were taken at 8 or 16 min intervals. The photographic images were measured with a comparator (Gaetner Scientific Corp., Chicago, Ill.). Values of $S_{20,w}$ were calculated as described by Schachman (130).

Diffusion coefficients were measured using a double sector, charcoal-filled Epon cell with sapphire windows in an An-H rotor. The centrifuge was operated at 5,200 r.p.m. and photographs taken at 8 minute intervals using Rayleigh optics. Protein samples in the range of 1 to 5 mg per ml were dialyzed for two days at 4° C versus 0.1 M NaCl, 0.01 M acetate buffer, pH 7.0, before centrifugation. Values of D^{obs} were calculated from the rate of fringe spreading as measured at the 1/4 and 3/4 positions (131), and were converted to $D_{20,w}$ values (130).

Sedimentation equilibrium measurements on dialyzed protein solutions were carried out at 20° C in an An-H titanium rotor with a 2-1/2° charcoal filled Epon 12 mm double sector cell using Rayleigh optics. Low speed runs (132) were at 12,000 r.p.m. for 30 hours. High speed runs (133) were performed at 28,000 to 34,000 r.p.m. for 24 hours. Attainment of equilibrium was indicated by constancy of fringe patterns observed in photographs taken after 20 hours of centrifugation. Calculations of $\ell n\ y$ versus r^2 were done by computer using a program kindly supplied by Mr. William Wolodko.

M. Partial Specific Volume Calculation

Since several preparations of factor X_a, each requiring ten days labor for isolation, would have been required for a pycnometric determination of $\bar{v}$, partial specific volumes were calculated from the amino acid compositions using the partial specific volumes of constituent amino acids according to Cohn and Edsall (134).

N. Extinction Coefficient

Extinction coefficients, $E^{1\%}_{1\ cm}$, were measured by drying down solutions of extensively dialyzed samples of precisely known absorbance ($A_{280\ nm}$) at 110° C *in vacuo* until a constant weight was obtained.

O. Gel Filtration

Sephadex gels were prepared by boiling for several hours in water followed by equilibration in 0.1 M NaCl, 0.01 M acetate buffer, pH 7.0, for several days. Pharmacia K 9/60 columns were packed and operated at a 10-15 cm hydrostatic pressure head to give a flow rate of 4-8 ml per hour. Applied samples contained approximately 1 mg protein in

exactly 1 ml of buffer. Fraction size was estimated by weighing the tubes. Myoglobin, ovalbumin and bovine serum albumin were used for molecular weight calibration. Factor X_a molecular weights were estimated by interpolation of elution volumes at peak concentration (135).

CHAPTER III

PREPARATION AND PURIFICATION OF ACTIVATED FACTOR X SPECIES

A. Activation with Russell's Viper Venom

1. Partial Purification of Zymogen Factor X

Approximately 40 preparations of bovine plasma (routinely 30-40 liters) were required for isolation of the factor X used during studies of factor X_a^{RVV}. Freshly prepared plasma was stirred with $BaSO_4$ (10 mg per ml) at 20° C for 30 minutes and the $BaSO_4$ with adsorbed protein allowed to settle overnight at 4° C. The supernatant, including the precipitated fibrinogen, was poured off and the $BaSO_4$ transferred to a Waring blendor. The $BaSO_4$ was homogenized for 5 minutes with 2 liters of 0.45% NaCl, 0.001 M sodium citrate buffer, pH 6.5, and the homogenate transferred to 2 x 1 liter polypropylene bottles. These were centrifuged at 4500 r.p.m. in a Sorvall RC-3 centrifuge for 5 minutes. The supernatant washings were discarded. Homogenization and centrifugation were repeated six more times, discarding the washings.

Factor X and contaminant protein were eluted from the $BaSO_4$ by homogenizing twice with 1.5 liters of 0.08 M citrate buffer, pH 6.5. After centrifugation as above the eluates were pooled, diluted with 1 volume of water and 10 ml of packed wet DEAE-cellulose was added. This was stirred for 20 minutes at 20° C. The DEAE-cellulose was collected by centrifugation as above and applied to the top of a glass chromatography column (1.7 cm diameter) previously packed to a height of 30 cm with DEAE-cellulose in 0.04 M sodium phosphate buffer, pH 7.45.

The column was washed with 400 ml of 0.175 M sodium phosphate buffer, pH 7.45 at 20° C, and the factor X activity eluted with a linear gradient from 0.175 M sodium phosphate to 0.175 M sodium phosphate containing 0.2 M NaCl (300 ml per chamber). An elution profile is shown in Fig. 4. Fractions showing specific activities greater than 50 units per A_{280} unit were pooled and frozen for later use. Approximately 11,000 units of factor X was obtained from 35 liters of plasma. To check that factor X activity was stable to freezing and thawing, one aliquot was frozen and thawed 10 times. No activity loss was observed.

2. Activation with Russell's Viper Venom

Prior to activation with RVV, the above factor X solution was dialyzed against 4 x 4 liters of 0.14 M NaCl, 0.005 M barbital-acetate buffer, pH 6.2, for 48 hours at 4° C. Less extensive dialysis led to precipitation of calcium phosphate in the following activation step. A pH of 6.2 was chosen to minimize possible degradation of factor X by thrombin or other enzymes present (39). The dialyzed factor X solution was adjusted to pH 7.5 with 1.0 N HCl, 1/5 volume of 0.025 M $CaCl_2$ and 0.1 mg of RVV powder added, and the mixture stirred at 20° C for three hours, by which time generation of factor X_a^{RVV} activity had reached a plateau. When activation was complete, two volumes of water and 3 ml of packed wet DEAE-cellulose were added and the suspension stirred for 20 minutes. The DEAE-cellulose with adsorbed protein was collected on a Buchner funnel and transferred to the top of a glass column (0.9 cm diameter) previously packed to a height of 20 cm

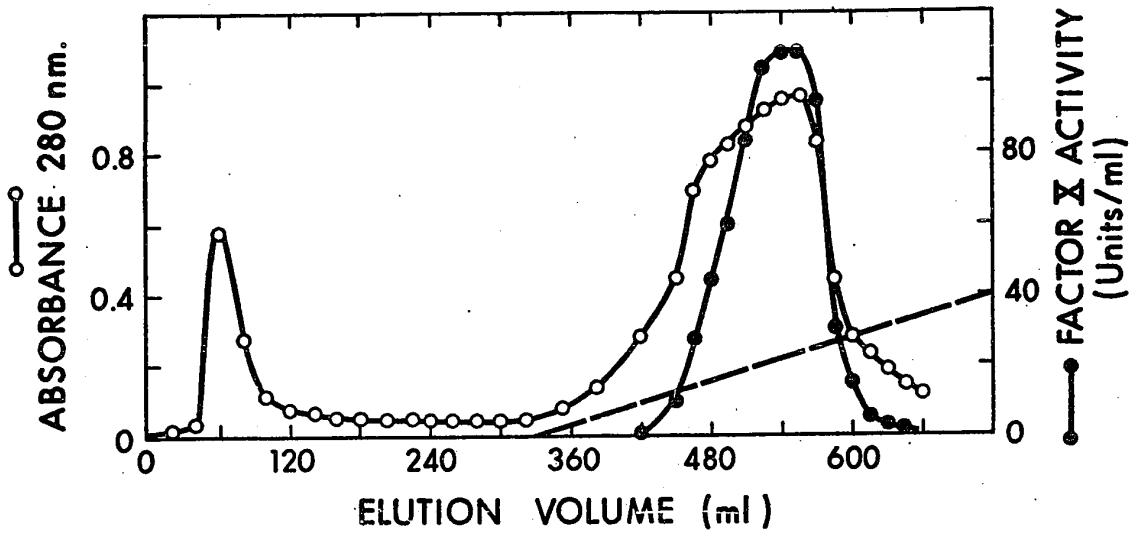

Figure 4. Chromatography of $BaSO_4$ eluate on DEAE-cellulose. A glass column (1.5 cm diameter x 50 cm height) was used at 20° C with a hydrostatic pressure of 130 cm. Fractions of 10 ml were collected at a flow rate of about 1 ml per minute. Elution was with 0.175 M sodium phosphate, pH 7.45 plus a linear gradient from 0.0 to 0.25 M NaCl, indicated by the dashed line.

with DEAE-cellulose in 0.04 M sodium citrate, pH 7.0. Chromatography was begun with 0.09 M sodium citrate, pH 7.0, using a hydrostatic pressure head of 1 meter at 4° C. Elution was continued with this buffer until factor X_a^{RVV} activity began to elute, after approximately 120 ml. When the factor X_a^{RVV} activity level in the elute increased to approximately 50 units per ml, a linear gradient was begun from 0.09 M sodium citrate to 0.15 M sodium citrate, pH 7.0 (60 ml per chamber). The upper panel of Fig. 5 shows the elution profile. Fractions showing minimal specific activity of 1000 units per A_{280} unit were pooled and rechromatographed exactly as before. The elution profile from the rechromatography is shown in the lower panel of Fig. 5. Rechromatography removed trace contaminants detected as weak bands by disc gel electrophoresis. Fractions of highest specific activity (1200 to 1600 units per A_{280} unit) were used for further studies.

Referring to the elution profiles, it must be mentioned that much of the material absorbing at 280 nm is not protein. Even peak factor X_a^{RVV} fractions contained up to 30% of this unidentified non-protein material. The absorbing species readily passes through collodion bags and dialysis tubing, does not stain with protein stains (Buffalo Black or Coomassie Brilliant Blue) and shows an absorbance profile that is not typical of proteins containing a significant content of tryptophan and tyrosine. Fig. 6A shows the absorbance profile of material which passed through a collodion concentrator bag during concentration of a factor X_a^{RVV} sample which showed one major band in disc gel electrophoresis (Fig. 7A). During this concentration, no factor X_a^{RVV} activity appeared to pass out of the concentrator bag though 27% of the absorbing material did. Fig. 6A also shows the absorbance profile of a

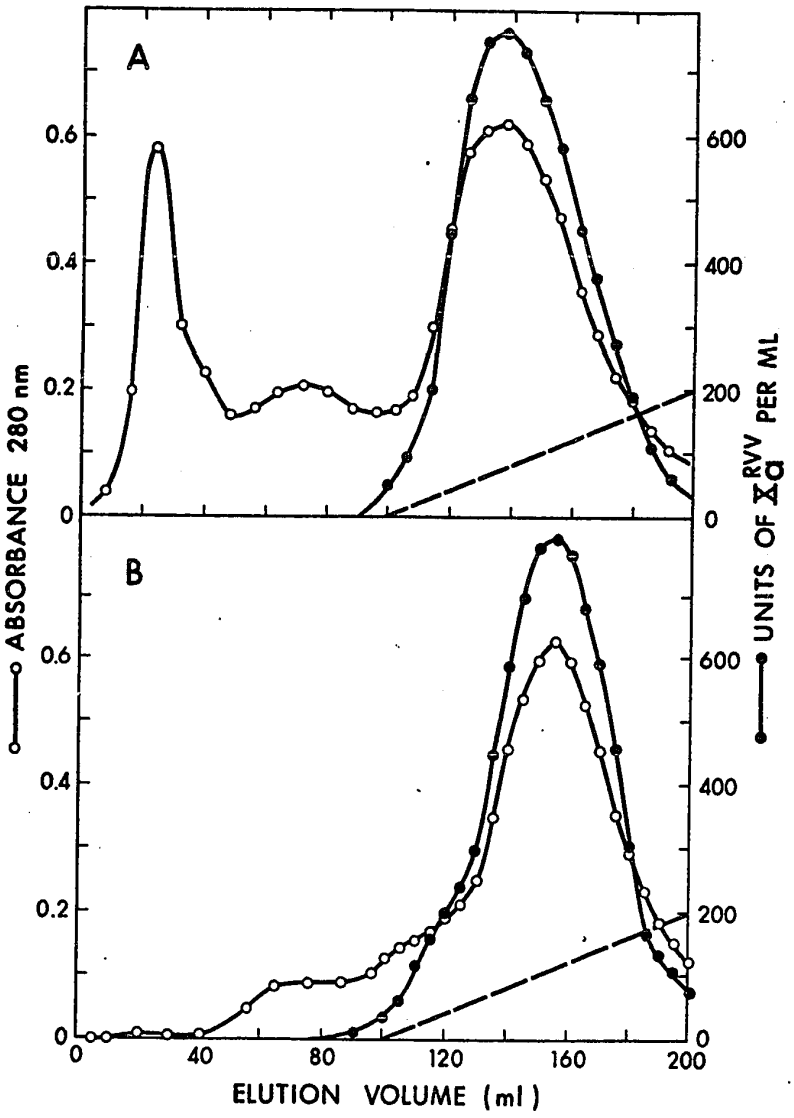

Figure 5. Chromatography of factor X_a^{RVV} activation mixture (A) and rechromatography (B) of selected fractions of (A) on DEAE-cellulose. A glass burette (0.9 cm diameter x 45 cm height) was used at 4° C with a hydrostatic pressure of 130 cm. Fractions of 2 ml were collected at a flow rate of 0.25 ml per min. The dashed line indicates a linear gradient from 0.09 to 0.15 M sodium citrate, pH 7.0.

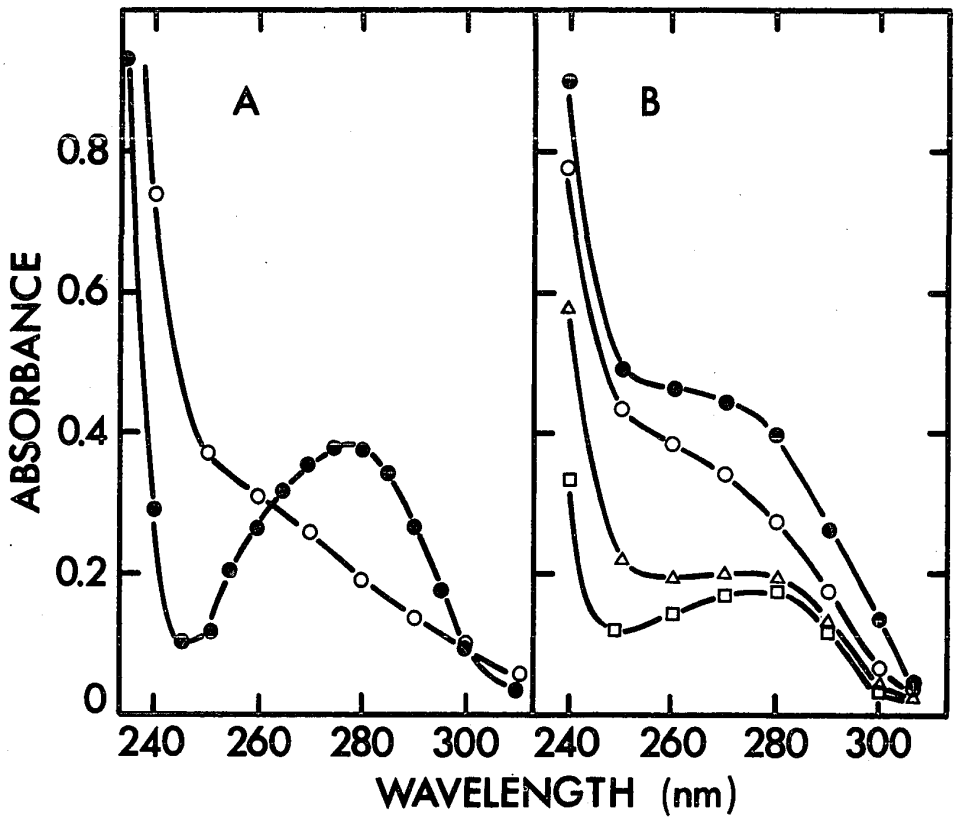

Figure 6. A, absorbance profile of factor X_a^{RVV} freed of contaminant by chromatography on Sephadex G-200 and extensive dialysis (●——●); and the absorbance profile of the contaminant which passed through a collodion bag concentrator (o——o). B, absorbance profiles of fractions from Fig. 5A which eluted at 50 ml (□——□), 100 ml (Δ——Δ), 130 ml (●——●) and 180 ml (o——o).

factor X_a^{RVV} sample after removal of contaminant by Sephadex G-200 chromatography and dialysis against 0.14 M NaCl, 0.01 M sodium acetate, pH 7.0, for 72 hours at 4° C. It displays a profile similar to that expected for a protein. Fig. 6B shows the absorbance profiles of fractions selected from the DEAE-cellulose chromatography shown in Fig. 5A. The early fractions display a protein-like profile, while later fractions can be seen to contain a progressively larger contribution from contaminant. This suggests that the impurity elutes continuously from the column, but more strongly as the salt concentration increases. This contaminant presented no great problems for further studies, since it is removed by dialysis. However, the contribution of this dialyzable material to the total absorbance makes interpretation of the DEAE-cellulose profiles uncertain. Thus the factor X_a^{RVV}, as shown later, is far more free of protein contamination than the elution profile would suggest. This contaminant was encountered during purification of all five species of activated factor X. Disc gel electrophoresis of this factor X_a^{RVV} preparation shows one major band (Fig. 7A). Concentrated aliquots of the preparation ($\sim$1000 units per ml) failed to clot equal volumes of fibrinogen (2 mg per ml in Michaelis buffer) after 3 hours at 37° C.

B. Activation in 25% Sodium Citrate Solution

Activation was initiated by combining equal volumes of factor X solution and 50% w/v sodium citrate, pH 7.5. Fig. 8 shows the course of factor X_a generation in 25% citrate from factor X chromatographed as described for preparation of the venom-activated species.

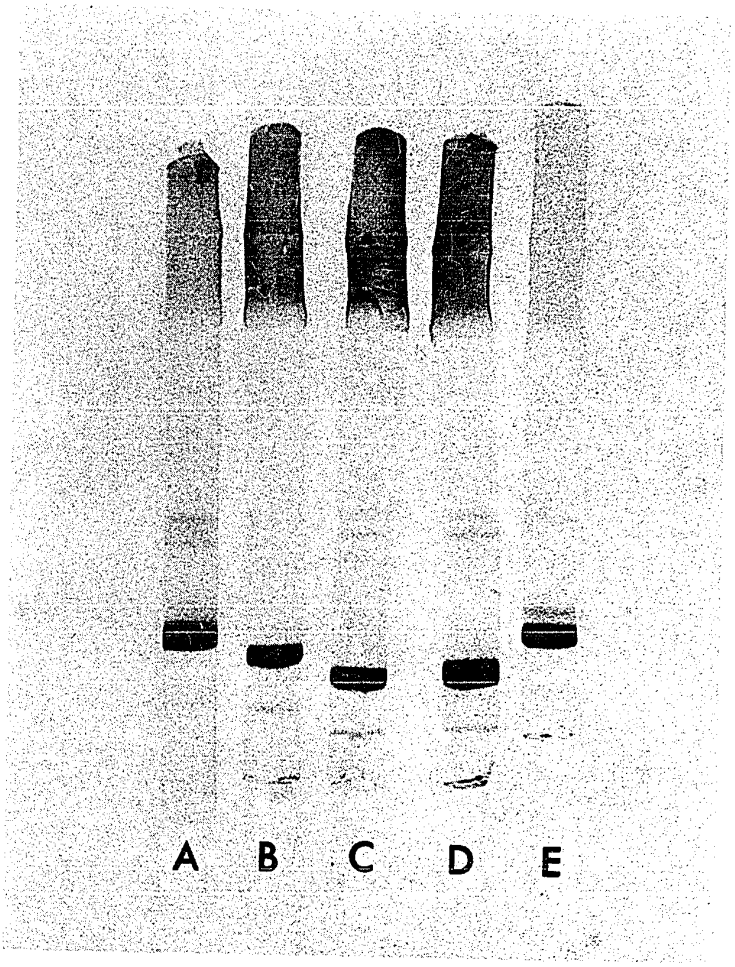

Figure 7. Disc gel electrophoresis of activated factor X preparations at pH 9.5. A, factor X_a^{RVV}; B, factor X_a^{Cit}; C, factor X_a^{IMET}; D, factor X_a^{TF}; E, factor X_a^{Int}. Protein loadings were in the range of 50 - 100 µg. Gels were stained with Buffalo Black.

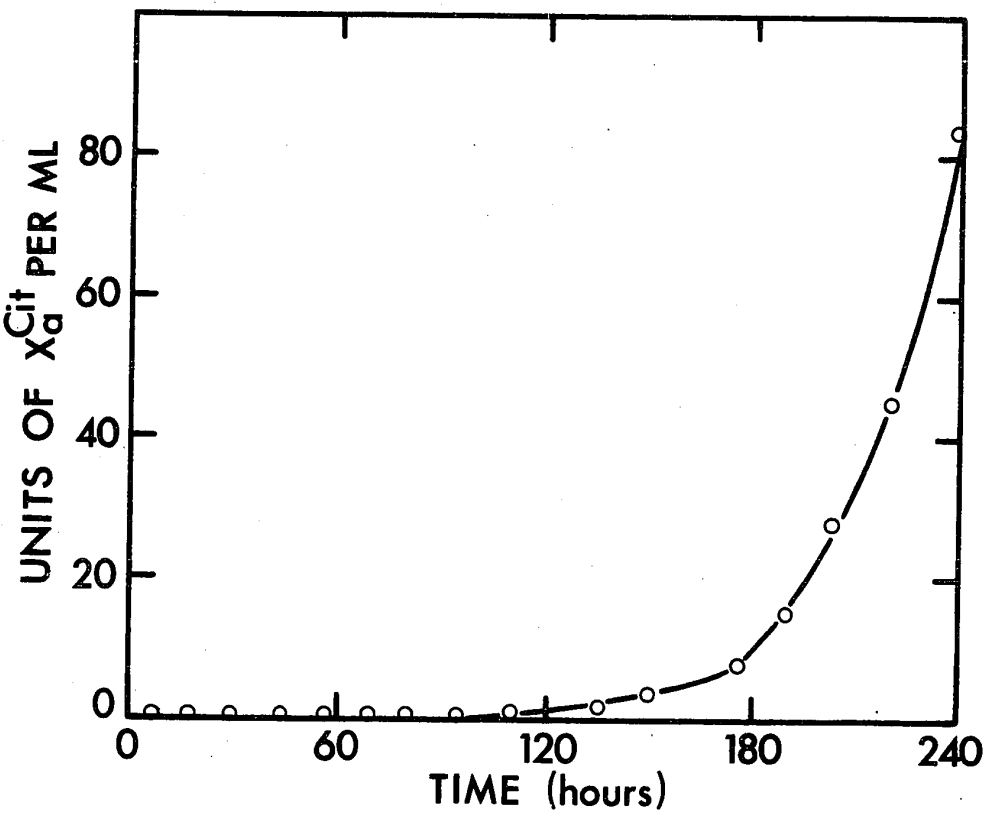

Figure 8. Time dependence of activation of factor X (Preparation I) in 25% w/v sodium citrate, pH 7.5 at 20° C.

A control aliquot of factor X in Michaelis buffer generated no activity. Activation was not complete after 10 days, while Marciniak and Seegers (96) reported that their prothrombin preparation gave maximum levels of autoprothrombin C (factor X_a) after 16 hours. Since our preparation of zymogen factor X in 25% citrate activated too slowly for routine use in generation of factor X_a^{Cit}, several preparations were made using altered procedures, the aim being to establish the minimum impurity level required for rapid activation in 25% citrate.

1. Preparation I

The routine preparation of zymogen as used for venom activation will be called preparation I. It was prepared, as described earlier, by DEAE-cellulose chromatography wherein 400 ml of washing buffer (0.175 M sodium phosphate, pH 7.45) was run through the column before eluting the factor X with a linear gradient from 0.175 M sodium phosphate to 0.175 M sodium phosphate containing 0.25 M NaCl, pH 7.45.

2. Preparation II

In a second chromatographic procedure the normal washing and eluting buffers were replaced by a longer gradient designed to separate the "impurity" proteins which eluted together in the washing buffer in preparation I. A linear gradient from 0.0175 M sodium phosphate, pH 7.45, to 0.175 M phosphate containing 0.25 M NaCl, pH 7.45, (450 ml per chamber) was begun at fraction 1. The resulting elution profile is shown in Fig. 9. Assays indicated that peak II-A contained prothrombin, peak II-B contained factor VII and peak II-C contained

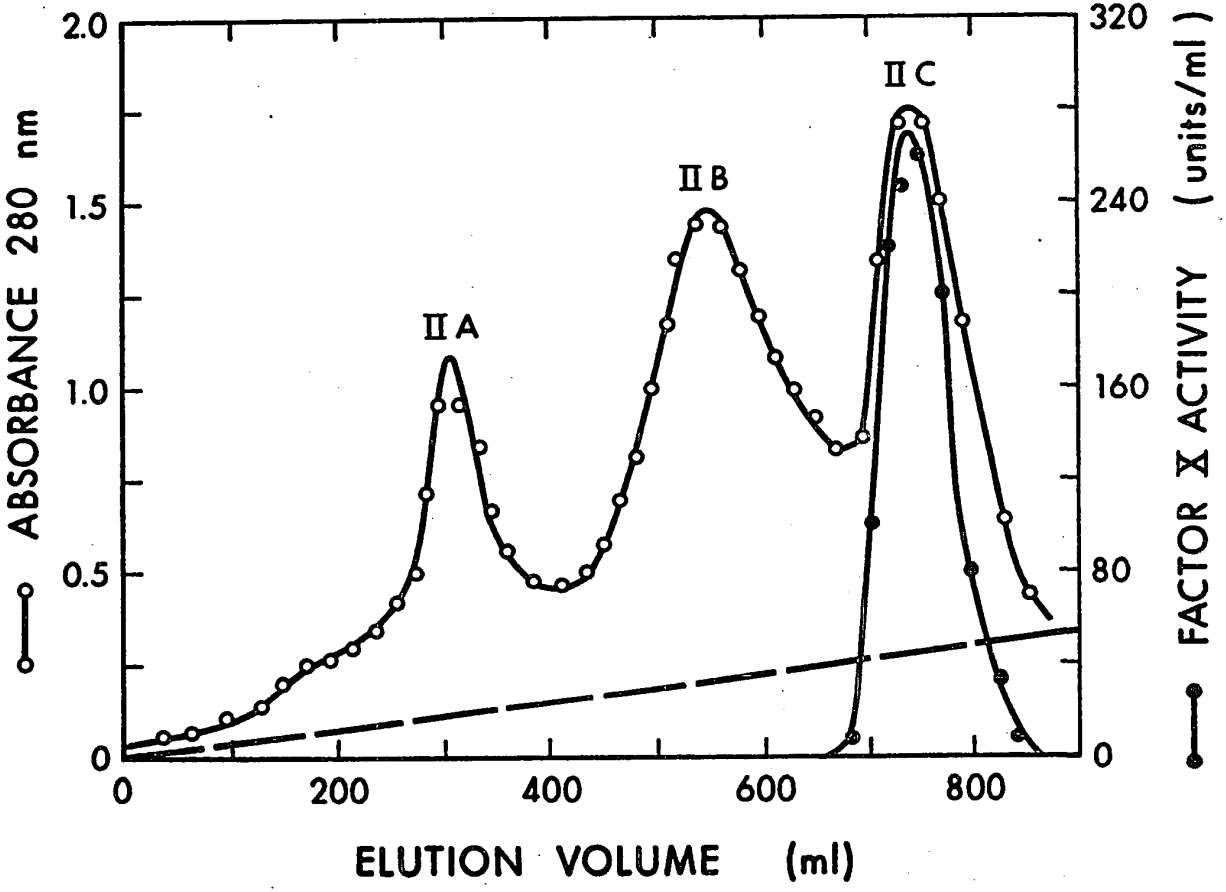

Figure 9. Chromatography of $BaSO_4$ eluate on DEAE-cellulose (Factor X, Preparation II). A glass column (1.5 cm diameter x 50 cm height) was operated with a hydrostatic pressure of 130 cm at 20°C. Fractions of 10 ml were collected at a flow rate of about 1 ml per minute. A linear gradient fom 0.0175 M sodium phosphate to 0.175 M sodium phosphate containing 0.25 M NaCl, pH 7.45 (450 ml/chamber) is indicated by the dashed line.

factor X. Assay for factor IX was not performed, but this factor was expected to elute after factor X (41,136).

3. Preparation III

The aim of this procedure was to obtain a factor X preparation containing large amounts of prothrombin and other adsorbable factors. Therefore the amount of $BaSO_4$ used to adsorb the plasma was increased to 100 mg per ml.

Bovine plasma (20 liters) was adsorbed with $BaSO_4$ (100 mg per ml), the $BaSO_4$ washed seven times with 0.45% w/v NaCl, 0.001 M sodium citrate, pH 7.0, and eluted twice with two portions (3 liters each) of 0.08 M sodium citrate, pH 7.0. The protein was precipitated from 65% saturated ammonium sulfate solution and the precipitate dissolved in 0.175 M sodium phosphate, pH 7.45.

4. Preparation IV

Preparation IV was the most extensively chromatographed. The procedure was identical to that used for preparation I except that 800 ml of washing buffer was run before beginning the gradient, rather than the routine 400 ml.

5. Preparation V

Preparation V was prepared identically to preparation I except that no washing buffer was run before beginning the gradient. The resultant factor X preparation is the least pure of those subjected to DEAE-cellulose chromatography.

When the above preparations were combined with an equal volume of 50% sodium citrate and evolution of factor X_a activity studied, it was found that the rate of activation was proportional to the level of contamination of the factor X. The most highly purified preparation (preparation IV) showed no activation in 25% citrate after 1 week at 20° C. As discussed above, the second most purified factor X preparation (preparation I) was not completely activated after 10 days. The least carefully chromatographed preparation (preparation V) activated completely after 36 hours.

6. Preparation VI

Since even the most poorly chromatographed factor X preparation V) required an inconveniently long time for activation, in the procedure finally adopted for preparation of factor X_a^{Cit}, non-chromatographed factor X was used. This was prepared by batchwise adsorption of the normal $BaSO_4$ eluate (10 mg per ml) onto 10 ml packed wet DEAE-cellulose followed by batchwise elution with 200 ml of 0.175 M sodium phosphate, pH 7.45 containing 0.25 M NaCl. When combined with an equal volume of 50% sodium citrate, pH 7.5, this material activated completely overnight at 20° C. A large amount of precipitate formed during incubation. After activation the solution was diluted 20-fold with water at which point the precipitate redissolved, and 5 ml packed DEAE-cellulose slurry was stirred in. Chromatography and rechromatography were performed as for factor X_a^{RVV}. Elution profiles are shown in Fig. 10. This material showed only very weak impurity bands in disc gel electrophoresis at heavy loadings (Fig. 7B).

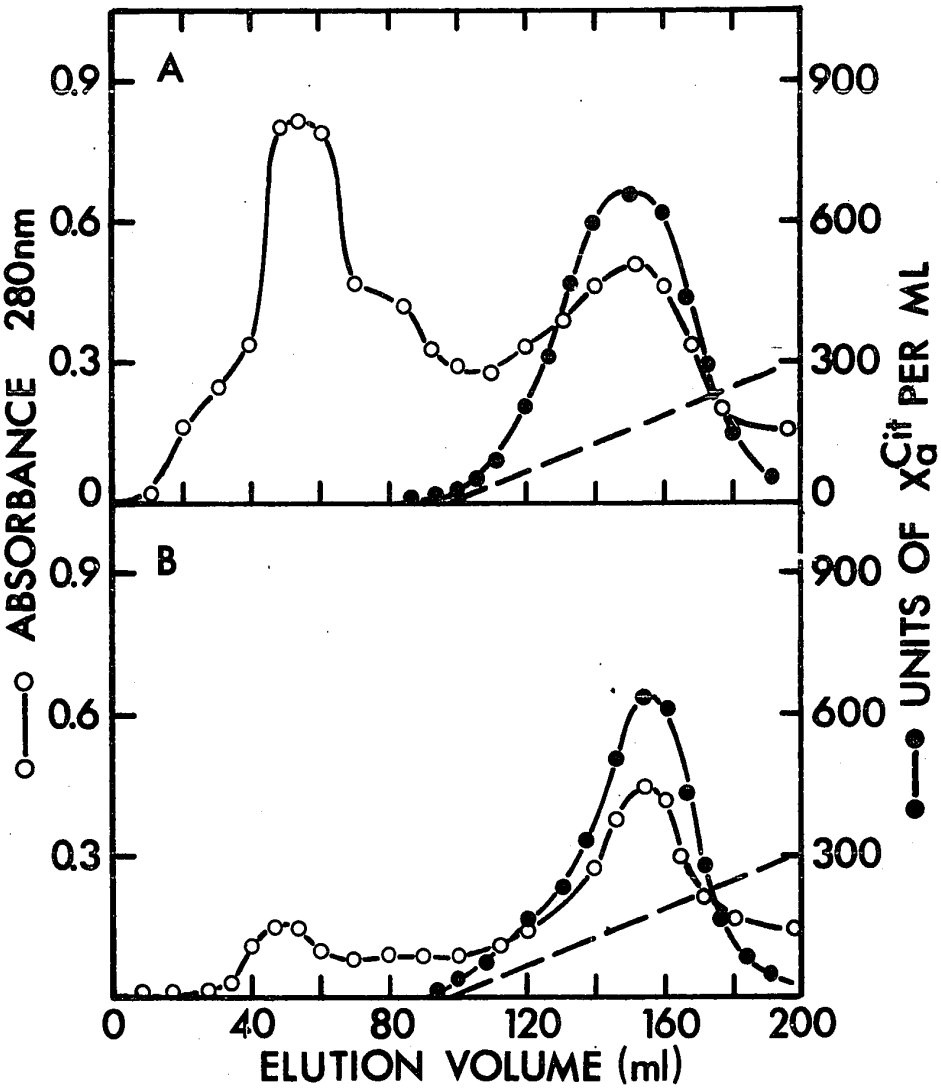

Figure 10. Chromatography of factor X_a^{Cit} activation mixture (A) and rechromatography (B) of selected fractions of (A) on DEAE-cellulose. A glass burette (0.9 cm diameter x 45 cm height) was used at 4° C with a hydrostatic pressure of 130 cm. Fractions of 2 ml were collected at a flow rate of 0.25 ml per min. The dashed line represents a linear gradient from 0.09 to 0.15 M sodium citrate, pH 7.0.

Several experiments were then performed with the aim of identifying the enzyme(s) responsible for activation of factor X in 25% citrate solution.

(i) Purified factor X_a^{Cit} (final concentration 20 units per ml) was added to an aliquot of factor X, preparation I (final concentration 100 units per ml) and the mixture made to 25% citrate, pH 7.5. Generation of additional factor X_a activity proceeded at the same very slow rate over 90 hours as the control which lacked added factor X_a^{Cit}. This lack of effect of the added factor X_a is in contrast to the findings of Kipfer and Seegers (103), who found a marked increase in the rate of activation of autoprothrombin III (factor X) when autoprothrombin c (factor X_a) was added to the incubation mixture. As discussed in Chapter I, their preparations were most likely impure.

(ii) In a second experiment aliquots of the contaminant peaks in Fig. 9 were recombined with aliquots of the factor X peak with the aim of identifying which component was responsible for factor X activation in 25% citrate solution. Aliquots (1 ml) of various fractions were added to aliquots (1 ml) of the factor X peak (peak II-C), each mixture made to 25% citrate concentration and generation of factor X_a activity in plastic tubes at 20° C measured at intervals over a 48 hour period. The results are shown in Fig. 11. Inclusion of the peak II-A material caused a 15-fold rate increase in evolution of factor X_a. Inclusion of peak II-B material caused a 5-fold rate increase. After 96 hours, each incubation mixture contained about 130 units per ml of factor X_a. The peak II-A mixture also contained 20 units per ml of thrombin. The peak II-B mixture and the control mixture displayed traces

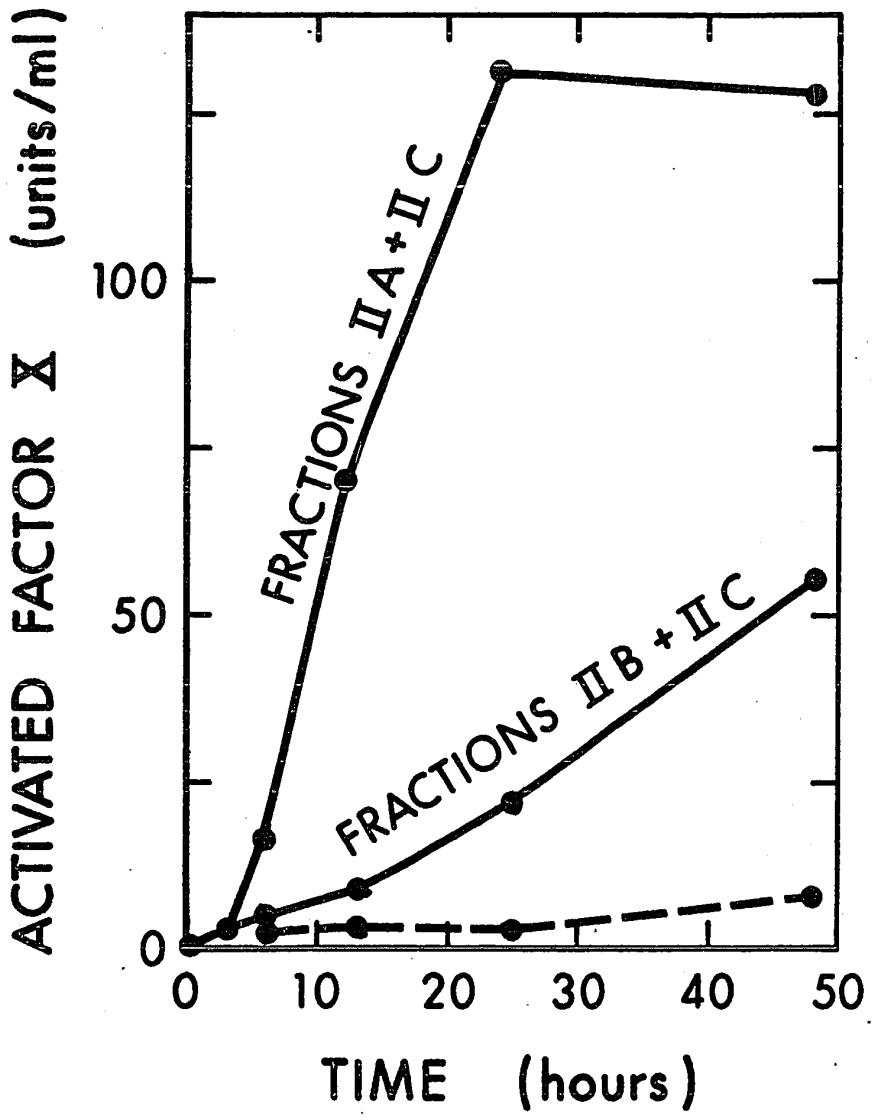

Figure 11. Time dependence of activation of factor X (Preparation II) in 25% w/v sodium citrate, pH 7.5, at 20° C. Aliquots (0.5 ml) of fraction II-C from Fig. 9 were mixed with aliquots (0.5 ml) of fraction II-A or II-B (solid lines) or 0.5 ml of 0.175 M sodium phosphate, pH 7.45 (dashed line), and each mixture was made to 2.0 ml with 50% w/v sodium citrate, pH 7.5. Activation was in plastic tubes.

of thrombin. These data suggest that thrombin, derived from the added prothrombin of peak II-A, as well as factor VII from peak II-B, can activate factor X in 25% citrate.

(iii) An attempt was made to confirm the above suggestion that thrombin activates factor X in 25% sodium citrate solution. Thrombin, isolated from Parke-Davis Topical Thrombin as described by Yin and Wessler (112), was added to chromatographed factor X (Preparation I) in 25% sodium citrate and generation of factor X_a activity was followed at 20° C. Fig. 12 indicates that the rate of generation of factor X_a was greatly increased by the presence of thrombin and that the amount of the increase was proportional to the amount of thrombin added.

From this limited data, it seems likely that thrombin can cause activation of factor X in 25% sodium citrate solution. It is also probable that factor VII, but not factor X_a itself, can bring about generation of factor X_a^{Cit} from zymogen factor X.

C. Activation with a Water-Insoluble Trypsin Derivative (IMET)

Water-insoluble copoly-(maleic acid-ethylene)-trypsin (IMET) was prepared with a 1:1 ratio of trypsin (TPCK-treated) to polymer as follows (137):

(i) 0.1 ml of 1% hexanediamine in 0.2 M sodium phosphate, pH 7.5, was stirred with 1 ml of 1% ethylene maleic anhydride in the same buffer at 0° C for 2-1/2 minutes.

(ii) After 2-1/2 minutes 10 mg of trypsin in 0.5 ml of the same buffer was added and the mixture stirred for 20 hours at 4° C.

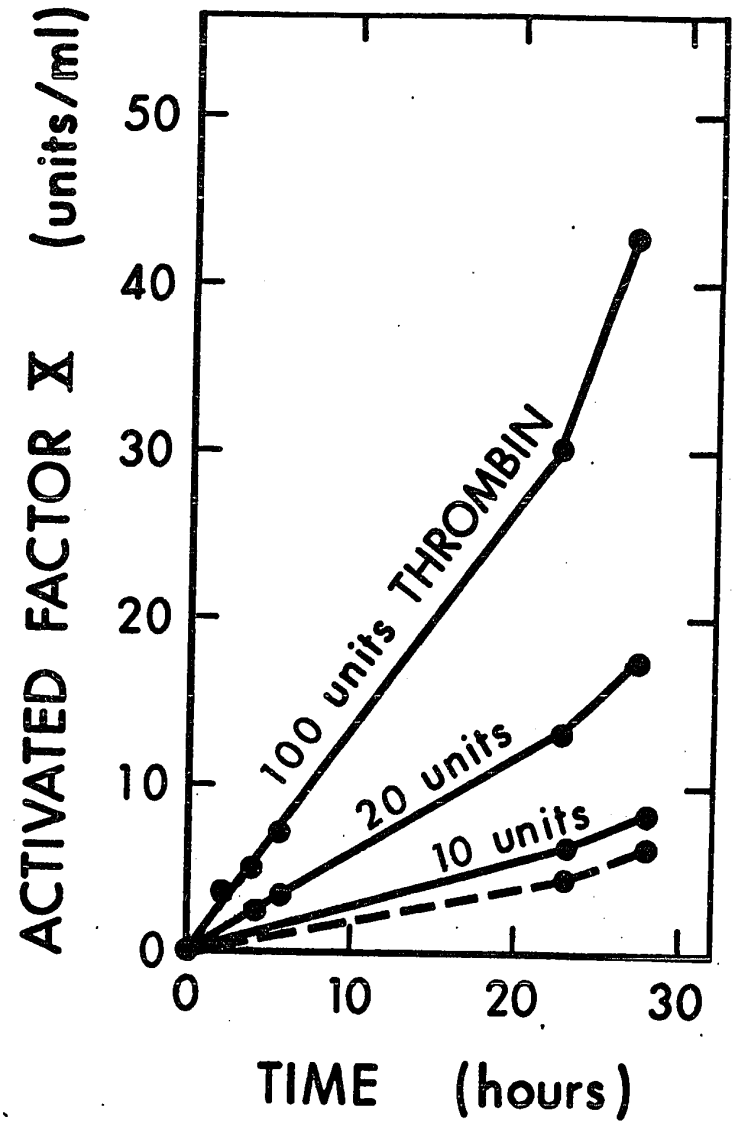

Figure 12. Time dependence of activation of factor X (Preparation II) in 25% w/v sodium citrate, pH 7.5 at 20° C. Thrombin was added to three aliquots (solid lines). The dashed line represents an aliquot to which no thrombin was added.

(iii) The IMET suspension was freed of unreacted trypsin by filtering on a Buchner funnel and washing 20 times alternately with water and 0.1 M NaCl at 20° C. It was suspended in Michaelis buffer and stored frozen.

Since it has been reported earlier (41), and was observed in this work, that trypsin and IMET degrade factor X_a activity slowly, a procedure using IMET was developed to facilitate removal of the IMET at the point when the optimum yield of factor X_a^{IMET} had been obtained. To accomplish this the following procedure was adopted:

(i) 0.1 mg IMET was added with stirring to approximately 11,000 units of factor X (a routine Preparation I), chromatographed and dialyzed as for factor X_a^{RVV} preparation, in 100 ml of 0.14 M NaCl, 0.005 M barbital-acetate, pH 7.4, at 20° C.

(ii) 0.5 ml was withdrawn immediately and incubated at 37° C, thus activating more rapidly than the bulk reaction at 20° C. This pilot aliquot reached maximum factor X_a activity in about 1 hour, remained stable for another hour and then began to lose activity.

(iii) When the main activation mixture first reached maximum activity, as estimated from the pilot aliquot at 37° C, after approximately 2 hours, it was cooled rapidly to 0° C and filtered through an 8 μ millipore filter to remove IMET. The bulk incubation mixtures at 20° C activated to the same endpoints as did the pilot runs at 37° C.

(iv) The filtered solution was diluted once with water. Chromatography and rechromatography were performed as for factor X_a^{RVV}. Elution position and profile, specific activities and yields were the same as in the Factor X_a^{RVV} preparations.

It may be noted that $CaCl_2$ was not included in the activation mixture. Repeated tests for an effect of 0.010 M $CaCl_2$ on the rate of factor X_a generation by IMET failed to show any effect. Disc gel electrophoresis of factor X_a^{IMET} revealed faint minor bands only at loadings in excess of 50 µg (Fig. 7C).

D. Activation by Tissue Factor and Factor VII (Extrinsic Pathway)

Prior to activation, a routine preparation of chromatographed factor X (Preparation I) was dialyzed against 4 x 4 liters of 0.14 M NaCl, 0.005 M barbital-acetate, pH 6.2, for 48 hours at 4° C. $CaCl_2$ was added to the dialyzed solution to a concentration of 0.01 M and the pH was adjusted to 7.4 with acetic acid. To this solution was added 1/25 volume (normally 4 ml into 100 ml) of tissue factor suspension, which was prepared by stirring rabbit brain acetone powder (10 mg per ml) in 0.14 M NaCl at 20° C and allowing large particles to settle for 5 minutes. This mixture was stirred at 30° C for 4 hours, at which time the level of activation reached a plateau. Yields of factor X_a^{TF} were always less than obtained after activation with RVV, IMET or citrate solution, normally about 20,000 units. Since it has been observed previously that extensively chromatographed factor X does not activate with tissue factor alone (33), very likely factor X Preparation I contains a level of factor VII contamination sufficient to allow activation by tissue factor.

Next, the activation mixture was diluted with two volumes of water and chromatography and rechromatography on DEAE-cellulose performed as for factors X_a^{RVV} and X_a^{Cit}. Factor X_a^{TF} activity eluted from the columns at the same approximate position as factors X_a^{RVV}, X_a^{Cit} and X_a^{IMET}, and with

the same specific activity of about 1200 units per A_{280} unit. However, the yields rarely exceeded 20,000 units in contrast to 50,000 units for the other three species. Though the cause of the loss of potential activity was not established it is conceivable that some of the factor X_a^{TF}, or zymogen factor X, becomes irreversibly attached to the tissue factor particles. Incubation of the activation mixture with 0.05 M EDTA before chromatography did not increase the yield from the first column. The DEAE-cellulose used in these columns was discarded after use, due to the presence of lipid. A single major band was observed for this preparation in disc gel electrophoresis (Fig. 7D).

E. Activation by the Intrinsic System

1. Preparation of Reagents

(a) Factor X and factor IX reagent

35 liters of bovine plasma was adsorbed at room temperature with 40 g per liter $BaSO_4$ and the $BaSO_4$ was allowed to settle overnight at 4° C. The adsorbed plasma was siphoned off and frozen for use in preparing "contact product". The $BaSO_4$ was washed 6 times by homogenization with 2 liters of 0.2% NaCl, pH 6.5 and centrifugation as described for factor X_a^{RVV} preparation. Adsorption with 40 g of $BaSO_4$ per liter of plasma is reported to remove the major portion of factors X, II, VII and IX (38). Protein was eluted from the $BaSO_4$ by homogenizing twice with 1 liter each time of 0.2 M sodium citrate buffer, pH 6.5. The eluate was diluted with 8 liters of water and 20 ml of wet packed DEAE-cellulose was added. After stirring for 30 minutes and centrifuging down the suspension, protein was eluted batchwise with 100 ml of 0.175 M

phosphate buffer, pH 7.45, 0.25 M NaCl. The DEAE-cellulose adsorption-desorption is primarily a concentration step. The cellulose eluate was dialyzed at 4° C against 4 x 4 liters of 0.14 M NaCl, 0.005 M barbital-acetate buffer, pH 6.2, over 48 hours.

(b) Factor XII and factor XI reagent ("contact product")

The frozen adsorbed plasma was thawed but not allowed to warm to room temperature. The fibrinogen precipitate was centrifuged down at 4500 r.p.m. for 5 minutes, and discarded. The supernatant was stirred with 40 g of celite per liter for 20 minutes at 20° C. The celite, with adsorbed and activated factors XII and XI (89), was washed 3 times by stirring with 2 liters of water and centrifuging as above. The celite-activated "contact product" was then eluted by stirring with 200 ml of 10% w/v NaCl. The celite eluate was dialyzed overnight against 4 liters of water at 4° C to reduce the salt concentration to 0.14 M, approximately.

(c) Factor VIII reagent

One vial of factor VIII (200 units, derived from 800 ml of bovine plasma) was dissolved in 10 ml of water at 20° C, and chromatographed at 4° C in a Sephadex G-25 column (3 cm x 30 cm) to remove phosphate present in the factor VIII preparation. Development was with 0.14 M NaCl, 0.005 M barbital-acetate buffer, pH 7.4. The protein-containing, phosphate-free fractions (about 50 ml) were pooled and used immediately, or frozen in dry-ice acetone.

(d) Thrombin

Thrombin was isolated from Parke-Davis Topical Thrombin as described by Yin and Wessler (112).

2. Development of an Activation Procedure

Figure 13 shows the course of generation of factor X_a activity with the above reagents under different conditions. Six glass tubes were incubated at 37° C. Their initial contents were:

(i) $BaSO_4$ eluate (0.5 ml), celite eluate (0.5 ml), $CaCl_2$ (10 mM), thrombin (0.1 unit per ml) and celite powder (0.5 mg per ml).

(ii) Same as (i) but omitting celite powder.

(iii) Same as (i) but omitting celite powder and thrombin.

(iv) Same as (iii) but omitting celite eluate.

(v) Same as (i) but omitting celite powder and thrombin.

(vi) Same as (iii) but omitting $CaCl_2$.

At the times indicated by arrows on Fig. 13, 0.1 ml of factor VIII reagent and 0.1 ml of "Bell and Alton cephalin" were added to each tube except tube 5, to which only "cephalin" was added. From the nearly identical behavior of tubes 1 and 2, it can be concluded that the contact product was fully activated during celite adsorption. Thus, desorption and inclusion of celite powder in the activation reaction is of no value. Omission of thrombin (tube (3)), required for activation of factor VIII (27), had no dramatic effect on factor X_a generation and was omitted thereafter. The $BaSO_4$ eluate contained prothrombin and generated thrombin readily. This was indicated by the rapid formation of fibrin clots when each factor VIII aliquot, containing

much fibrinogen, was added to the reaction mixture. Fibrin clumps formed within 2 minutes after addition of the first factor VIII aliquot and within 15 seconds after subsequent additions.

Tubes 4, 5, and 6 failed to activate, as expected, due to omission of celite eluate, factor VIII and $CaCl_2$ respectively. When each of these deficiencies was corrected (indicated on Fig. 13 by the large arrow), factor X_a generation commenced. The stepwise course of the activation is in keeping with the fact that factor $VIII_t$ is highly unstable (27) and would largely decay in a matter of minutes after aliquot addition.

The reasons for adding "cephalin" with the factor VIII additions are clear from Fig. 14. Four incubation tubes (each containing 0.5 ml $BaSO_4$ eluate, 0.5 ml celite eluate, 0.01 M $CaCl_2$ and 0.1 ml chromatographed factor X) were initially treated identically but thereafter differently. The initial addition to all 4 tubes was 0.1 ml factor VIII reagent, 0.1 ml "cephalin" and 0.1 ml celite eluate (time zero on Fig. 14). Thereafter different additions were made:

Tube 1 - 0.1 ml celite eluate, 0.1 ml factor VIII, 0.1 ml cephalin

Tube 2 - 0.1 ml factor VIII, 0.1 ml cephalin

Tube 3 - 0.1 ml celite eluate, 0.1 ml cephalin

Tube 4 - 0.1 ml celite eluate, 0.1 ml factor VIII

Tubes 1 and 2 activated identically indicating that celite eluate need be added only initially. Tube 3 was given no factor VIII reagent beyond the first addition and does not activate beyond the initial "step".

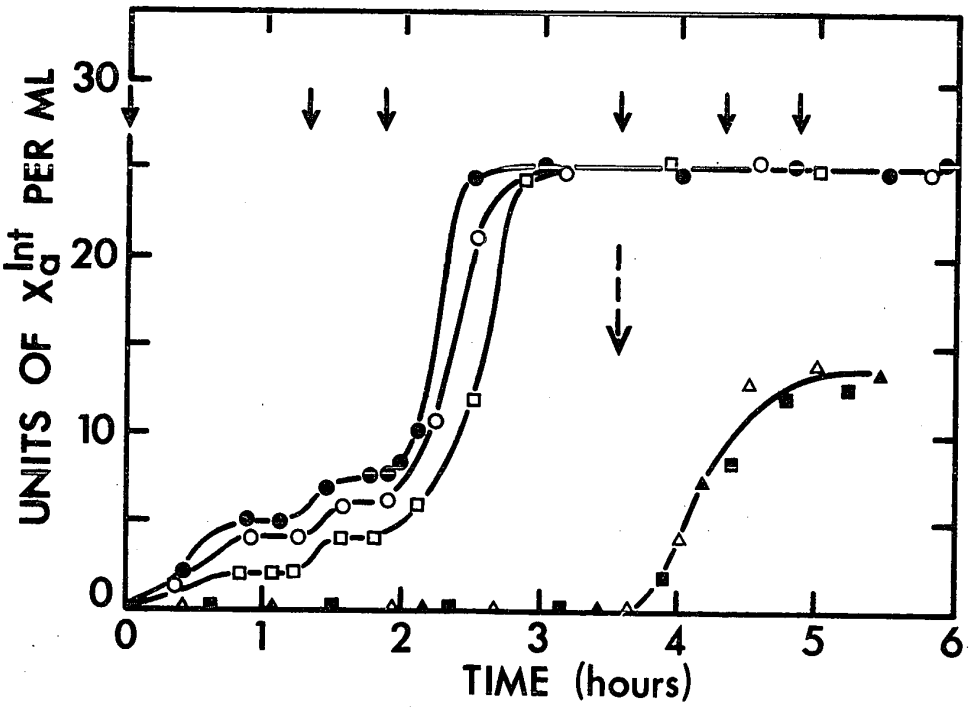

Figure 13. Time dependence of generation of factor X_a^{Int} activity under various conditions at 37° C. (1) Complete system; 0.5 ml $BaSO_4$ eluate, 0.5 ml Celite eluate, Celite powder (1 mg/ml), thrombin (0.1 NIH unit/ml) and $CaCl_2$ (0.01 M) (●——●). (2) Omit Celite powder (○——○). (3) Omit Celite powder and thrombin (□——□). (4) Omit $CaCl_2$ (△——△). (5) 0.5 ml $BaSO_4$ eluate and $CaCl_2$ (0.01 M) (▣——▣). (6) Complete system (▲——▲). At the times indicated by the small arrows, 0.1 ml of factor VIII reagent and 0.1 ml of "cephalin" (2 µg phosphorus/ml) were added to tubes (1) - (5). Only 0.1 ml "cephalin" was added to tube 6. At the time indicated by the large arrow, deficiencies in tubes (4) - (6) were corrected.

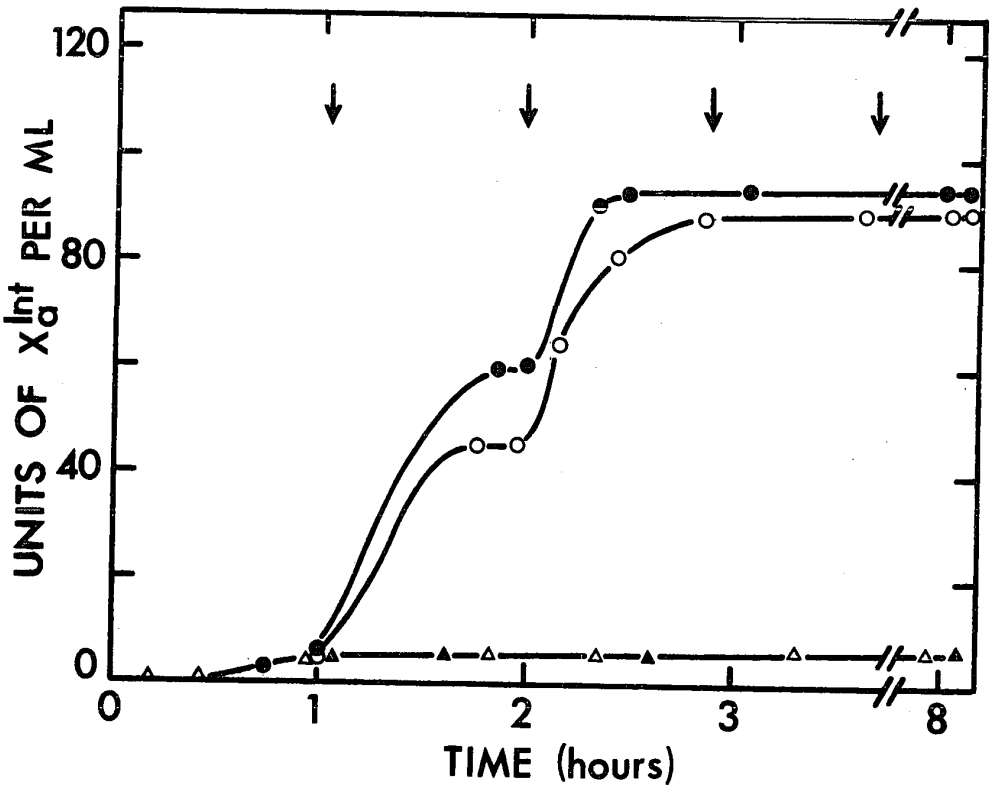

Figure 14. Time dependence of generation of factor X_a^{Int} activity under various conditions at 37° C. Four glass tubes each contained 0.5 ml $BaSO_4$ eluate, 0.5 ml Celite eluate, 0.1 ml factor X (Preparation I), 0.1 ml "cephalin" (2 µg phosphorus/ml), 0.1 ml factor VIII reagent and $CaCl_2$ (0.01 M). At times indicated by arrows, the following additions were made: (1) 0.1 ml Celite eluate, 0.1 ml factor VIII reagent, and 0.1 ml "cephalin" (●——●); (2) 0.1 ml factor VIII reagent, 0.1 ml "cephalin" (○——○); (3) 0.1 ml Celite eluate, 0.1 ml "cephalin" (▲——▲); (4) 0.1 ml factor VIII reagent, 0.1 ml Celite eluate (Δ——Δ).

Tube 4, to which factor VIII reagent but no "cephalin" was added at each addition, also did not activate beyond "step" 1. This need for frequently replenishing "cephalin" was seen repeatedly and hence was adopted as a regular procedure. It has been reported that clot formation can remove from solution over 70% of the phospholipid present (138).

Fig. 15 shows the course of activation by the procedure finally adopted. The $BaSO_4$ eluate (150 ml) and celite eluate (100 ml) prepared from 35 liters of plasma were dialyzed as described and combined. $CaCl_2$ was added to a concentration of 0.01 M and the pH was adjusted to 7.4. This mixture was stirred in glass at 20° C. Aliquots of factor VIII reagent (20 ml) and concentrated "cephalin" (1 ml containing 120 µg phosphorus per ml) were added at intervals indicated by arrows on Fig. 15. The slow start and steplike course of factor X_a generation were again noted. Yields of factor X_a^{Int} in the activation mixture varied from zero to 20,000 units. Of one dozen activations by this procedure, one generated no factor X_a^{Int} activity after several days and several activations required over one day. Only activations requiring 6 hours or less were used for isolation of factor X_a^{Int}. The variable yields and rates of activation suggest that more closely controlled conditions would be desirable. In particular the presence of fibrinogen and the lack of control over the amounts of thrombin present are undesirable features. Far larger quantities of factor VIII would be desirable but this material is very expensive when purchased commercially. Unfortunately preparation of a factor VIII reagent free of fibrinogen, and purified factor XI_a would be a major undertaking in itself.

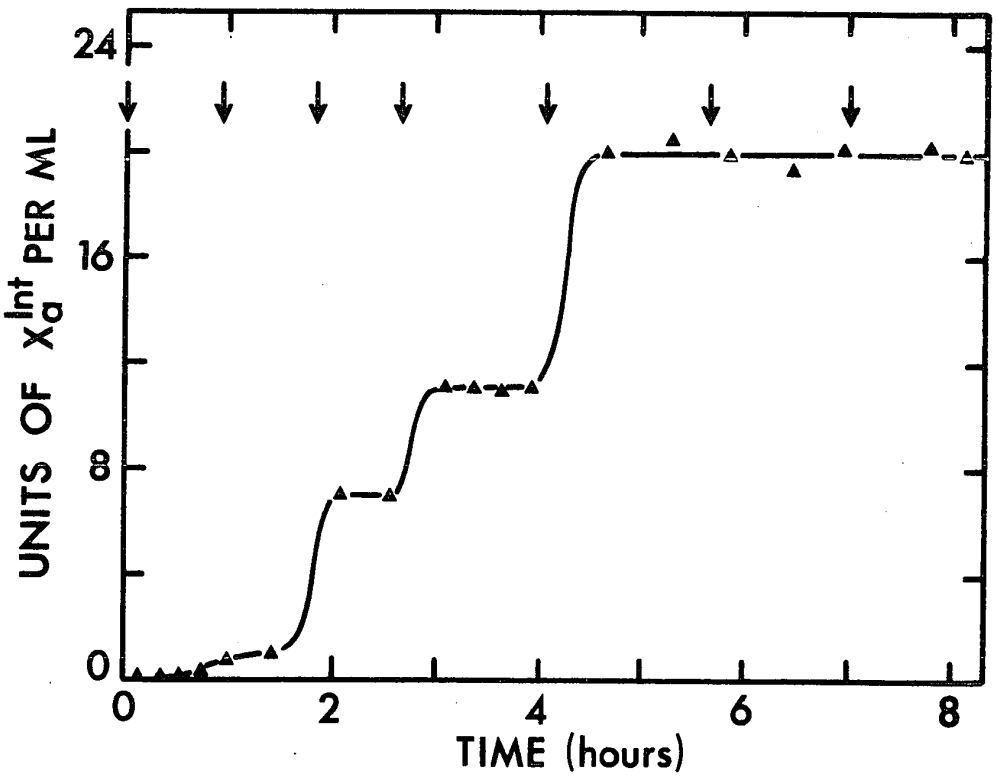

Figure 15. Time dependence of generation of factor X_a^{Int} activity by the routine procedure. The dialyzed $BaSO_4$ eluate (150 ml) and Celite eluate (100 ml) derived from 30 liters of plasma were combined and made to 0.01 M $CaCl_2$, pH 7.5. At times indicated by arrows, 20 ml of factor VIII reagent and 1 ml of "cephalin" (120 µg phosphorus/ml) were added.

3. Purification of Factor X_a^{Int}

The above activation mixture was diluted with 3 volumes of water and adsorbed to 5 ml wet DEAE-cellulose by stirring for 20 minutes at 20° C. Chromatography and rechromatography were as for factor X_a^{RVV} except that all sedimentable material, including much fibrin, was applied to the first column. Losses of 50-75% were encountered on the first column. Whether the "lost" factor X_a was in the initial "cloudy" peak eluted with 0.09 M sodium citrate could not be ascertained due to the presence of thrombin in that material. Stirring the activation mixture with 0.05 M EDTA before chromatography did not improve the column yield.

Due to the combined features of low yields during activation and during chromatography, overall yields of X_a^{Int} from 35 liters plasma were in the range of 2 - 5 mg (4,000-10,000 units).

CHAPTER IV

STABILITY AND SPECIFIC ACTIVITY OF ACTIVATED FACTOR X

A. Stability Limits of Activated Factor X Clotting Activity

Since several techniques used in this study involved extended manipulations of the factor X_a solutions, it was considered essential to establish whether factor X_a was stable under the conditions employed. In routine operations samples were rarely exposed to temperatures above 4° C for over 1 week.

The results described below indicate that at a given temperature, the stability of factor X_a clotting activity is highly dependent on concentration. Various dilutions of factor X_a were made into buffers of approximately physiological ionic strength and neutral pH, and allowed to stand at 20° C in glass tubes. At initial concentrations of approximately 0.03 units per ml (Fig. 17) and 0.3 units per ml (Fig. 18), factors X_a^{RVV}, X_a^{TF}, and X_a^{IMET} lost activity rapidly. At a concentration of 2 units per ml factor X_a^{IMET} lost about 50% of its activity over 3 days (Fig. 18). In contrast, no decay was noted over one week with solutions of 15-30 units per ml (5-10 µg protein per ml). Thus, at concentrations in the range routinely employed in this work (0.5 to 5 mg per ml) factor X_a activity displays no significant decay over one week at 20° C.

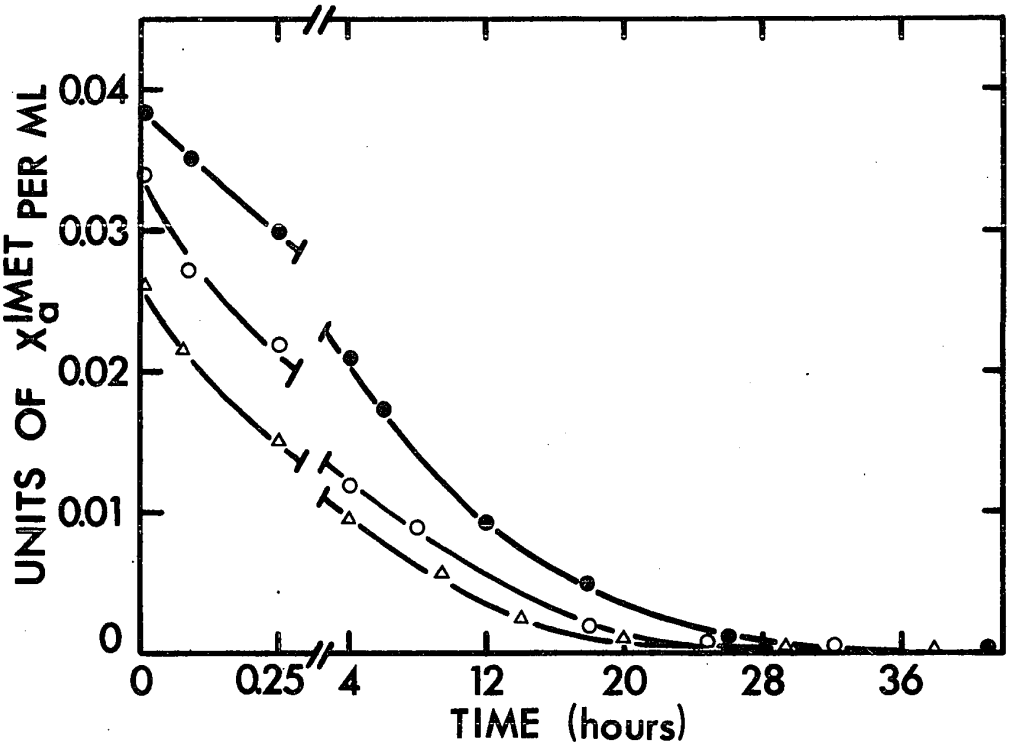

Figure 16. Time dependence of decay of factor X_a^{IMET} activity at 20° C. ●-----●, o---o, Δ---Δ, three samples of factor X_a^{IMET} in Michaelis buffer in glass tubes. Samples were prepared by making various dilutions of a concentrated stock solution (1 mg protein per ml in Michaelis buffer) at time zero.

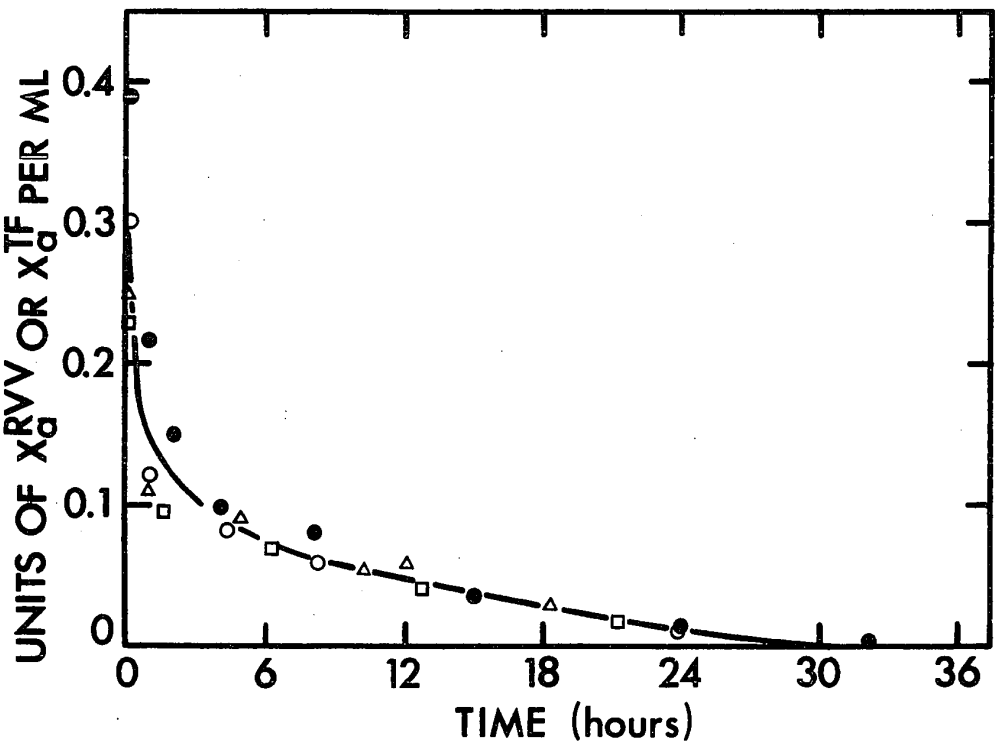

Figure 17. Time dependence of decay of factor X_a^{TF} and factor X_a^{RVV} activity at 20° C. ●——●, factor X_a^{RVV} in Michaelis buffer in a glass tube. o——o, Δ——Δ, □——□, three samples of factor X_a^{TF} in 0.14 M NaCl, 0.005 M barbital-acetate buffer, pH 7.4, in plastic tubes. Samples were prepared by making dilutions of concentrated stock solutions (1-2 mg protein per ml in Michaelis buffer) at time zero. Only one line is drawn through the four sets of points to avoid crowding.

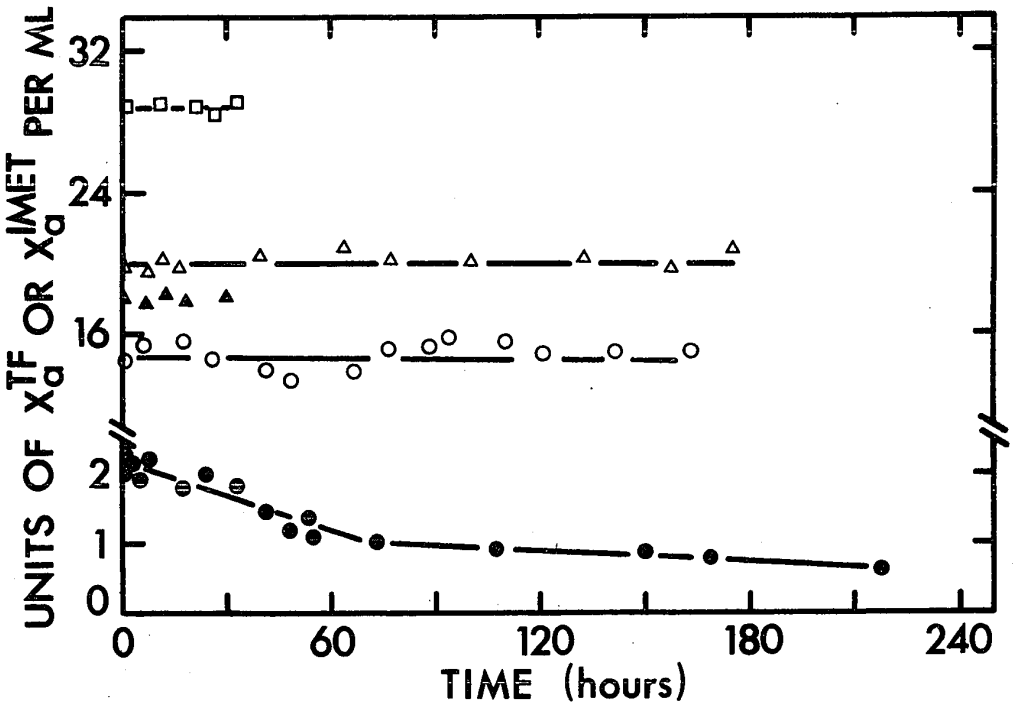

Figure 18. Time dependence of decay of factor X_a^{TF} and factor X_a^{IMET} activity at 20° C. ▲——▲, Δ——Δ, two samples of factor X_a^{TF} in Michaelis buffer in glass tubes. ●——●, ○——○, □——□, three samples of factor X_a^{IMET} in 0.1 M NaCl, 0.01 M sodium acetate, pH 7.0, in plastic tubes. Samples were prepared by making dilutions of concentrated stock solutions (1-2 mg protein per ml in Michaelis buffer) at time zero.

When solutions were stored at 37° C, some decay was seen in concentrated solutions. After 5 days at 37° C, 60% activity loss occurred with a starting concentration of 1200 units of factor X_a^{RVV} per ml. After one day at 37° C, a solution of factor X_a^{Cit} initially at 50 units per ml had decayed to 15 units per ml, and after 3 days only 8 units per ml remained.

The rates at which factor X_a activity decays at very low concentrations are of particular interest because such concentrations are involved in the dilutions of factor X_a samples used routinely in the clotting assay. To obtain clotting times between 17 and 25 seconds, a factor X_a solution must be diluted to between 0.1 and 0.03 units per ml. In the assay procedure used by the author, between 10 and 15 seconds elapse between making the dilution and beginning the assay. Figure 16 indicates that within this interval approximately 0.5% decay could be expected at 20° C. In the assay as used in this project, dilutions of samples were made in 0° C Michaelis buffer, at which temperature decay is slower than at 20° C.

Unfortunately, factor X_a activity did not appear to be as stable during manipulations as while remaining undisturbed in a test tube. This became evident during attempts to determine values for the specific activity of factor X_a. Specific activities of material freshly eluted from the second DEAE-cellulose column were routinely in the range of 1150 to 1350 units per A_{280} unit in the peak tubes. However since the absorbance of these fractions at 280 nm included up to 30% or more contribution from non-protein material, these activities should probably be revised upward to 1650 to 1930 units per protein A_{280} unit.

To determine whether the presence of the non-protein contaminant exerted an influence on the level of factor X_a activity, peak factor X_a^{IMET} fractions from a Sephadex G-200 chromatography were diluted to 15 units per ml and combined at 20° C with 10 times the concentration of contaminant normally present after DEAE-cellulose chromatography. No effect on activity level was observed either immediately or over the following 24 hours.

However, specific activity levels in the range predicted above, that is 1650 to 1930 units per protein A_{280} unit, were never actually observed by the time the manipulations required to remove the contaminant were completed. Table II lists the specific activities measured in fractions from chromatography of factor X_a species on Sephadex G-200. Since Sephadex G-200 chromatography separated the contaminant from the factor X_a protein peak, maximum specific activities might be expected across the peak. In contrast, it was observed that specific activities fell during the more prolonged manipulations of concentration, dialysis and chromatography on Sephadex G-200. The highest specific activities found were from samples applied directly to the Sephadex G-200 column after elution from the second DEAE-cellulose column, such as samples 1 and 2 in Table II. However, even these activities (1100 to 1500 and 1140 to 1430 units per A_{280} unit) were no higher than those of the DEAE-cellulose column fractions which contain 30 to 50% of non-protein absorbing material. This suggests that considerable decay has occured by the conclusion of one Sephadex G-200 chromatography (6 hours at 4° C). Yin and Wessler (112) found that Sephadex G-200 chromatography of factor X_a isolated on

TABLE II

SPECIFIC ACTIVITIES OF FACTOR X_a SAMPLES CHROMATOGRAPHED ON SEPHADEX G-200

Sample	Manipulations before Sephadex G-200 chromatography	Approximate protein concentration during manipulations	Specific activities of fractions across G-200 peak (units/A_{280})*
1.	Applied to G-200 column directly.	1 mg/ml X_a^{RVV}	1100-1500
2.	Applied to G-200 column directly.	1 mg/ml X_a^{Cit}	1140-1430
3.	Dialyzed for 17 hours at 4° C	1 mg/ml X_a^{Cit}	1370-1800
4.	Dialyzed for 24 hours at 4° C	1 mg/ml X_a^{Int}	1250-1400
5.	Concentrated, dialyzed 1-1/2 days at 4° C	0.3 mg/ml X_a^{TF}	900-1100
6.	Concentrated, dialyzed 2 days at 4° C.	0.5 mg/ml X_a^{Cit}	1050-1190
7.	Concentrated, dialyzed 2 days at 4° C.	1 mg/ml X_a^{IMET}	1150-1300
8.	Concentrated, dialyzed 4 days at 4° C.	5 mg/ml X_a^{RVV}	1030-1070
9.	Concentrated, dialyzed 3 days at 4° C.	1 mg/ml X_a^{TF}	700-800
10.	Remained undisturbed 5 days at 37° C.	0.5 mg/ml X_a^{RVV}	450-600
11.	Two concentration steps, 3 x G-200 chromatography over 5 days at 4° C.	1 mg/ml X_a^{Cit}	400-500

*Each activity range quoted is that observed across one Sephadex peak, consisting of 4-8 fractions each. The large variation in samples 1-3 reflects uncertainty in the assay, since each fraction was assayed only once. Fractions from profiles 4-11 were assayed in duplicate or triplicate. Variations across the peaks were random.

a DEAE-cellulose column from Parke-Davis Topical Thrombin led to over 25% loss of activity and no improvement in specific activity. Seegers (31) likewise found a loss of activity when autoprothrombin c was passed through a Sephadex G-100 column.

After several days of manipulations, specific activities fell to approximately 1000 units per A_{280} unit. After five days remaining undisturbed at 37°C, one sample displayed specific activities of 450 - 600 units per A_{280} unit (Sample 10, Table II). After 5 days of manipulations at 4° C, including three Sephadex G-200 chromatography experiments and two concentration procedures, another sample showed similar specific activity (Sample 11, Table II). Recoveries of activity from Sephadex chromatography were approximately 70%, though protein recovery was complete.

Factor X_a samples were normally stored frozen in this work. Activities measured on thawed samples were the same as the activities measured before freezing. Samples were rarely stored more than several weeks before use.

B. Effect of Activity Decay on Physical Properties of Activated Factor X

Though it was found that factor X_a activity was not stable during extended manipulations, particularly Sephadex chromatography, no obvious changes in the physicochemical behaviour were observed during manipulations extending over one week in the cold room. Figure 20C shows the Sephadex G-200 profile of sample No. 10 from Table II. This material had lost 60% of its activity and eluted from the column at a specific

activity of 500-600 units per A_{280} unit, yet its elution position was the same, within experimental error, as the elution position for "fresh" factor X_a. Of some dozen chromatography experiments performed on the same column with samples treated in various ways, elution positions were all identical, within experimental error.

To facilitate a direct estimation of the effect of storage and Sephadex chromatography on the physical behavior of factor X_a species, a 2 ml aliquot of factor X_a^{Cit} (1200 units per ml), which had been dialyzed for 40 hours at 4° C against 0.1 M NaCl, 0.01 M sodium acetate, was divided into two portions. One portion (1 ml) was chromatographed on Sephadex G-200. A second portion (1 ml) was allowed to stand for 48 hours at 20° C, then chromatographed on the same column. Figure 19 shows the profiles of the two samples. They appear to be identical. This suggests that factor X_a^{Cit} undergoes no gross physical changes in the length of time required for the various studies reported. It is tentatively concluded that, provided appropriate conditions are employed, the factor X_a species studied are sufficiently stable to permit meaningful physico-chemical investigations.

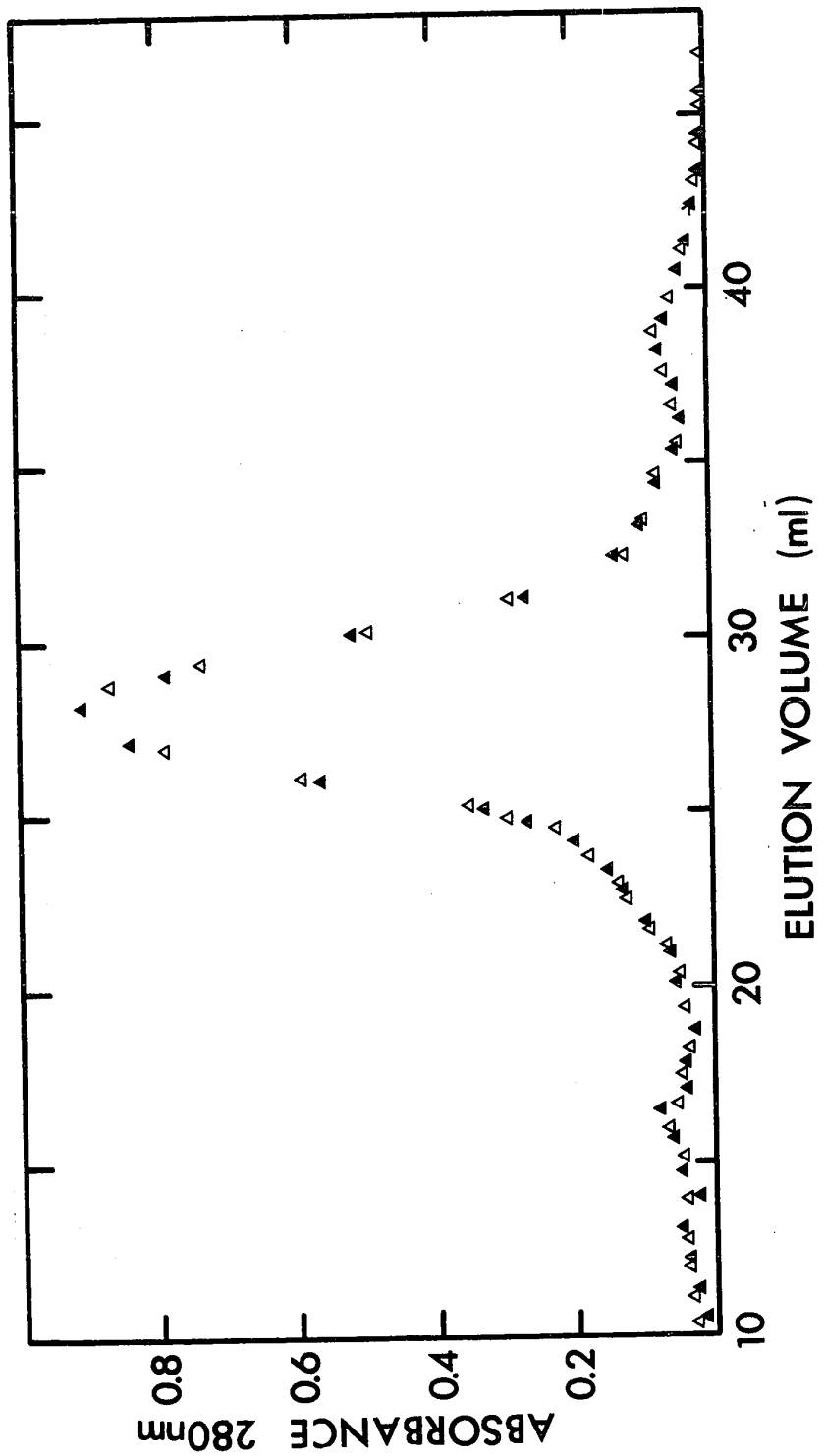

Figure 19. Gel filtration profiles of factor X^{Cit}_a. A 2 ml sample was dialyzed for 40 hours against 0.1 M NaCl, 0.01 M sodium acetate, pH 7.0. One portion (1 ml) was then applied to the column and chromatographed in the same buffer (▲-▲-▲). The second portion (1 ml) was allowed to stand for 48 hours at 20°C before application to the column (Δ-Δ-Δ). Chromatography was at 4° C with a 10 cm hydrostatic pressure head in a Pharmacia K 9/60 column. The flow rate was about 5 ml per hour and fractions of 0.8 ml were collected.

CHAPTER V

PHYSICO-CHEMICAL CHARACTERIZATION OF FACTOR X ACTIVATED WITH RUSSELL'S VIPER VENOM

Several considerations advised that factor X_a^{RVV} would be the most appropriate of the five species of factor X_a for detailed characterization. Most of the limited physico-chemical data that is available on activated factor X in the literature was collected on factor X_a^{RVV} (8,31). Secondly, each of the other four activation procedures involve technical liabilities not encountered with the venom procedure. As discussed more thoroughly in Chapter VIII, section A, the yields of factors X_a^{TF} and X_a^{Int} are low compared to that for factor X_a^{RVV}, and the purity is not as good. Factor X_a^{Cit} must be prepared from intentionally very impure factor X. Finally, trypsin activation is not an altogether desirable procedure since enzymatic degradation may be occuring during the activation reaction.

Techniques used to characterize factor X_a^{RVV} were chosen both to provide internal checks on one another and to allow ready comparison of factor X_a^{RVV} with the other four species. Thus, the molecular weight was estimated by two ultracentrifugal techniques, by Sephadex gel filtration and by SDS-acrylamide gel electrophoresis. N-terminal and C-terminal amino acid determinations and peptide mapping of tryptic digests of factor X_a^{RVV} were chosen for the highly sensitive comparison with the other species which they allowed. Disc gel electrophoresis and SDS-acrylamide gel electrophoresis of factor X_a^{RVV} in

combinations with the other species also provided highly resolving comparative techniques.

A. Disc Gel Electrophoresis

Disc gel electrophoresis at pH 9.5 resulted in a single major band moving closely behind the Bromphenol Blue tracking dye, as seen in Figure 7A. Four minor bands were evident only in heavily loaded gels (100 µg). The electrophoretic mobilities of these impurity bands were 0.56, 0.60, 0.65 and 0.8 times that of the major band.

B. Sephadex Gel Filtration Chromatography

Gel filtration chromatography of factor X_a^{RVV} at a concentration of 1 mg per ml on Sephadex G-200 resulted in a single peak corresponding to a molecular weight of 48,000 ± 2000, provided the sample was previously dialyzed for 18 hours at 4° C against 0.1 M NaCl, 0.01 M sodium acetate buffer, pH 7.0 (Fig. 20A). If samples were not dialyzed before application to the column, a second peak of small molecular weight was seen (Fig. 20B). This material, as discussed earlier, is not protein and readily passes through dialysis tubing and the collodion bag concentrator. Figure 20C refers to the stability study described in Chapter IV, section B.

Figure 21A shows the Sephadex G-200 profile of extensively dialyzed factor X_a^{RVV} chromatographed under conditions reported to allow sensitive detection of hypersharpening of the leading edge due to self-association (139 - 141). By applying 18 mg of protein in 8 ml of buffer to a 0.9 x 15 cm column, a constant concentration plateau was obtained, continuously feeding the leading and trailing edges. As illustrated by a

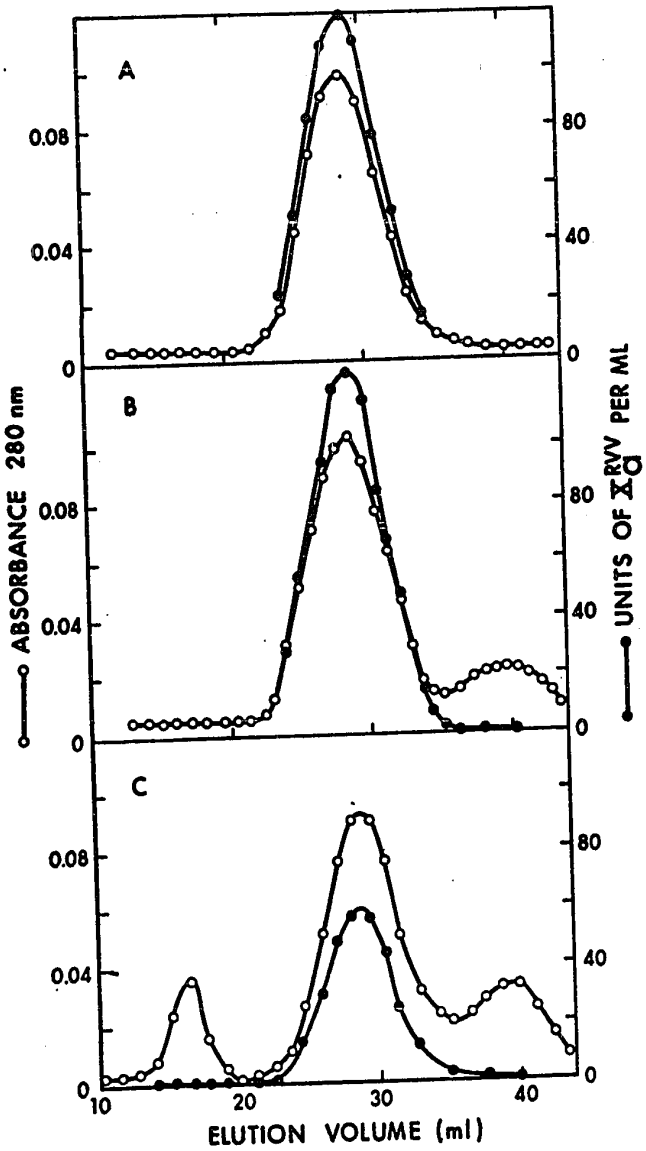

Figure 20. Gel filtration elution profiles of factor X_a^{RVV}. Factor X_a^{RVV} (1.0 mg in 1.0 ml of 0.1 M NaCl, 0.01 M sodium acetate, pH 7.0) was applied to a column (0.9 cm diameter x 60 cm height) and eluted with the same buffer. Flow rate was 5 ml per hour and fractions of 0.8 ml were collected. o——o, $A_{280\ nm}$; ●——●, activated factor X activity. A, elution profile of dialyzed factor X_a^{RVV}; B, non-dialyzed factor X_a^{RVV}; C, non-dialyzed factor X_a^{RVV} allowed to stand 5 days at 37° C.

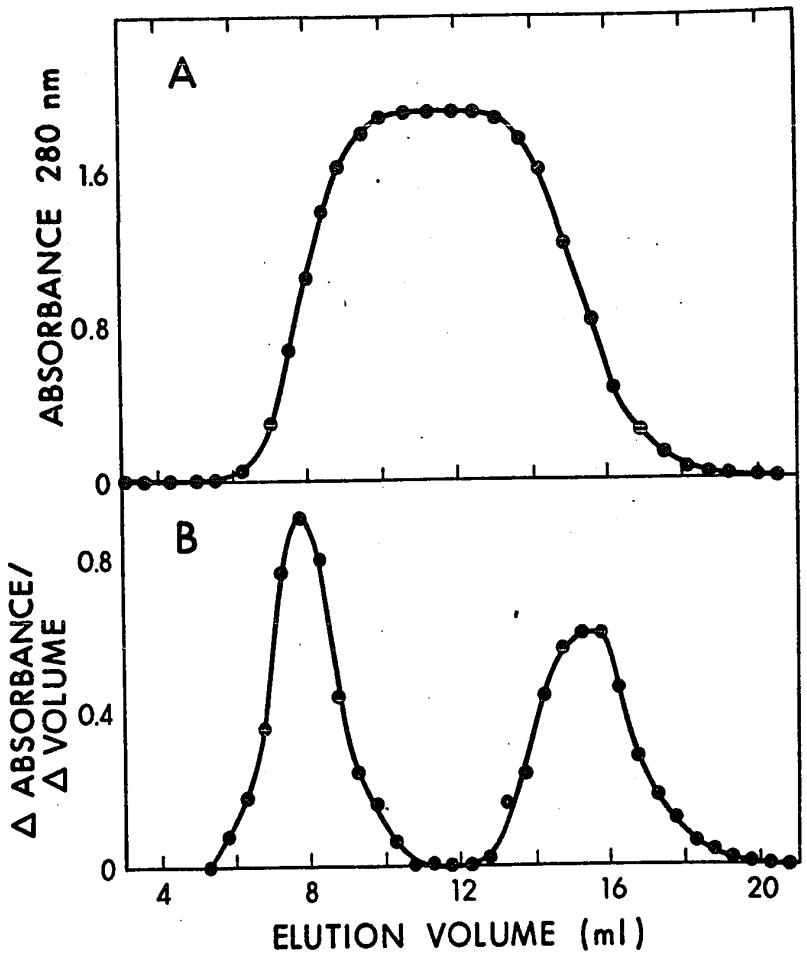

Figure 21. A, gel filtration profile of factor X_a^{RVV} with constant plateau region feeding the leading and trailing edges. Factor X_a^{RVV} (18 mg in 8.0 ml of 0.1 N NaCl, 0.01 M sodium acetate, pH 7.0) was applied to a Pharmacia column (0.9 cm diameter x 15 cm height) and eluted with the same buffer. Flow rate was 5 ml per hour and fractions of 0.8 ml were collected. B, a derivative plot of (A).

derivative plot of this elution profile, the leading edge is hypersharp (Fig. 21B). It was concluded that factor X_a^{RVV} is an associating-dissociating protein.

C. Amino Acid Composition

The amino acid composition of factor X_a^{RVV}, shown in Table III, columns 1 and 2, is consistent with its being an anionic protein. All the common amino acid residues are present, methionine in the least amount and glutamic and aspartic acids in the greatest amounts.

D. Carbohydrate Determinations

Factor X_a^{RVV} samples used for carbohydrate determinations were fractions taken directly from the second DEAE-cellulose column and concentrated 5- to 10-fold as required.

Hexose content was found in three determinations to be 0.12, 0.09 and 0.16% w/w expressed as mannose, The hexose content of ovalbumin was found to be 1.96% w/w as mannose, in agreement with a value of 2% w/w reported in the literature (125). Assuming a molecular weight of 50,000 for factor X_a^{RVV}, the percentage hexose contents are equivalent to 0.30, 0.24 and 0.45 residues of hexose per mole of protein. It was concluded that factor X_a^{RVV} contains no hexose.

Ovalbumin was found to contain 1.8% w/w of 2-amino sugar expressed as galactosamine, in agreement with reported values of 1.1 - 1.4% w/w (125). Factor X_a^{RVV} revealed 0.27 and 0.36% w/w galactosamine in two determinations, which is equivalent to 0.64 and 0.83 residues per mole protein. It was observed that the factor X_a^{RVV} samples led to an orange color in the assay, in contrast to the pink color obtained with the

TABLE III

AMINO ACID COMPOSITION OF FACTOR X_a SPECIES: RESIDUES PER 50,000 GRAMS

	X_a^{RVV}	X_a^{RVV}	X_a^{Cit}	X_a^{Cit}	X_a^{Cit}	X_a^{IMET}	X_a^{IMET}	X_a^{TF}	
	(27)	(24/48/72)	(24/48/72)	(24/48/72)	(24)	(24)	(72)	(48)	(hours)
Lys	26.6	22.9±0.7	24.0±1.7	24.2±1.4	24.5	23.1	23.9	23.7	
His	9.65	8.8±0.4	8.85±0.2	11.3±0.9	8.42	9.3	9.36	8.86	
Arg	24.9	23.9±0.8	24.1±0.4	22.1±0.8	22.4	23.3	24.7	23.2	
Asp	36.8	33.0±2.4	37.6±0.5	36.9±1.6	36.9	39.4	38.3	36.8	
Thr	26.4	24.3	25.6	27.7	24.8	25.5	23.4	24.6	
Ser	20.3	26.2	24.2	23.7	21.0	23.1	18.9	20.0	
Glu	56.7	57.5±2.0	59.5±3.4	56.8±0.6	59.3	60.5	59.5	59.0	
Pro	9.7	9.9±0.8	11.4±0.1	11.4±0.5	13.4	10.3	9.75	9.72	
Gly	38.4	37.3±0.7	37.8±0.6	37.2±0.8	36.4	37.7	37.2	37.5	
Ala	26.6	24.2±0.5	24.7±0.2	24.4±0.6	23.6	24.4	23.2	24.5	
Cys/2	23.2	22.0±0.6	23.0±0.4	23.0±0.5	27.2	17.5	19.3	24.2	

(Continued)

TABLE III (Continued)

AMINO ACID COMPOSITION OF FACTOR X_a SPECIES: Residues per 50,000 GRAMS

	X_a^{RVV}	X_a^{RVV}	X_a^{Cit}	X_a^{Cit}	X_a^{Cit}	X_a^{IMET}	X_a^{IMET}	X_a^{TF}	
	(27)	(24/48/72)	(24/48/72)	(24/48/72)	(24)	(24)	(72)	(48)	(hours)
Val	23.3	23.4±0.5	24.2±0.4	24.0±0.3	21.8	24.4	25.2	24.0	
Met	5.57	5.47±0.9	5.96±0.2	5.89±0.5	5.74	4.84	6.12	5.92	
Ile	8.74	10.0±0.1	10.73±0.1	10.57±0.6	9.85	10.0	11.0	11.0	
Leu	23.0	19.5±0.4	24.5±0.5	25.4±0.5	23.0	24.6	24.7	23.3	
Tyr	9.75	9.12±0.2	10.0±0.4	10.0±0.4	9.75	10.1	9.75	9.49	
Phe	21.5	19.9±0.6	20.8±0.8	22.0±0.6	20.3	24.1	23.2	22.1	

Samples were hydrolyzed at 105° C in 6 N HCl for the lengths of time indicated. Values representing three determinations after 24, 48, and 72 hours hydrolysis are either average or else are extrapolated values from the three determinations. The variation of the most deviant value from the average of the three values is given for averaged values.

standards. This abnormal coloration may indicate that the slight absorbance measured at 530 nm is due to the presence of the contaminant discussed earlier rather than hexosamine.

Assay for N-acetyl neuraminic acid revealed 0.067% w/w or 0.11 residue per mole of factor X_a^{RVV}. The value of 1.2% w/w N-acetyl neuraminic acid found for thyroglobulin is in agreement with reported values of 1.2, 1.5 and 1.36% w/w (125). It was concluded that factor X_a^{RVV} contains no N-acetyl neuraminic acid or N-glycolylneuraminic acid.

E. Ultracentrifugal Analysis

In sedimentation velocity centrifugation the preparation displayed a single peak (Fig. 22). The variation of the sedimentation coefficient, $S_{20,w}$, with protein concentration is shown in Fig. 23. In the absence of added $CaCl_2$, a positive slope was found extrapolating to an $S^{\circ}_{20,w}$ of 3.90 S. A - Since the protein was not analyzed for calcium ions, the presence of tightly-bound calcium ions in the protein remains a possibility. Inclusion of 0.005 M $CaCl_2$ increased the positive slope and decreased $S^{\circ}_{20,w}$ to 3.57 S, while 0.025 M $CaCl_2$ increased the slope further and decreased $S^{\circ}_{20,w}$ to 3.22 S. The positive slope was interpreted to indicate an associating-dissociating protein (142,143). The effect of $CaCl_2$ is complex. The increase in the slope of the plot of $S_{20,w}$ against protein concentration suggests that $CaCl_2$ enhances association of factor X_a^{RVV}. Since the value of $S^{\circ}_{20,w}$ falls with increasing concentration of $CaCl_2$, it also appears that $CaCl_2$ causes a net increase in the effective hydrodynamic radius of the molecule. In contrast, $CaCl_2$ causes an increased $S^{\circ}_{20,w}$ for fetuin (143A) and troponin A (143B). It does not effect the $S^{\circ}_{20,w}$ value of prothrombin but increases the slope of the plot of $S_{20,w}$ against protein concentration (143C).

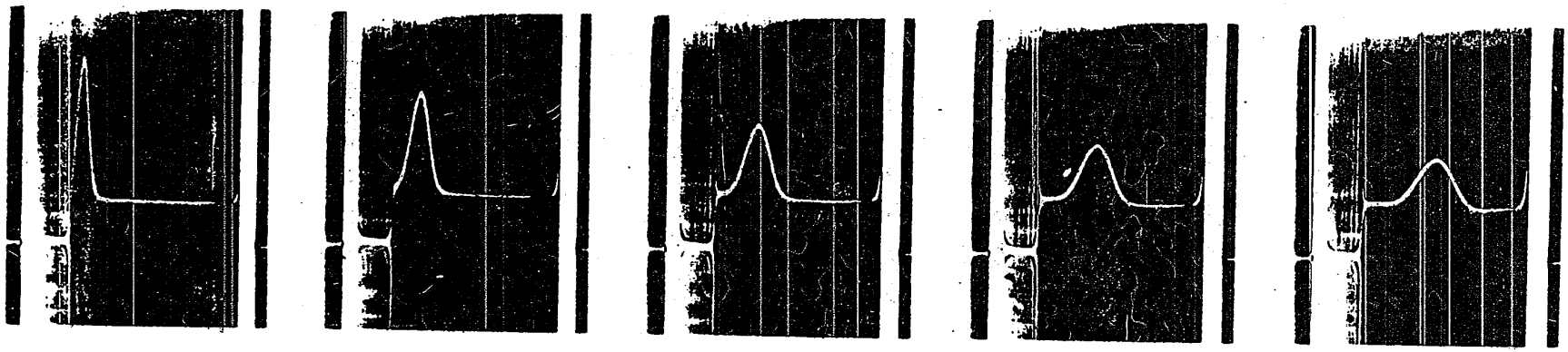

Figure 22. Sedimentation profiles of factor X_a^{RVV} after dialysis against 0.1 M NaCl, 0.01 M sodium acetate, pH 7.0, for 48 hours. Protein concentration was approximately 5 mg/ml. Centrifugation was at 59,780 rpm at 20° C and photographs were taken at 16 minute intervals.

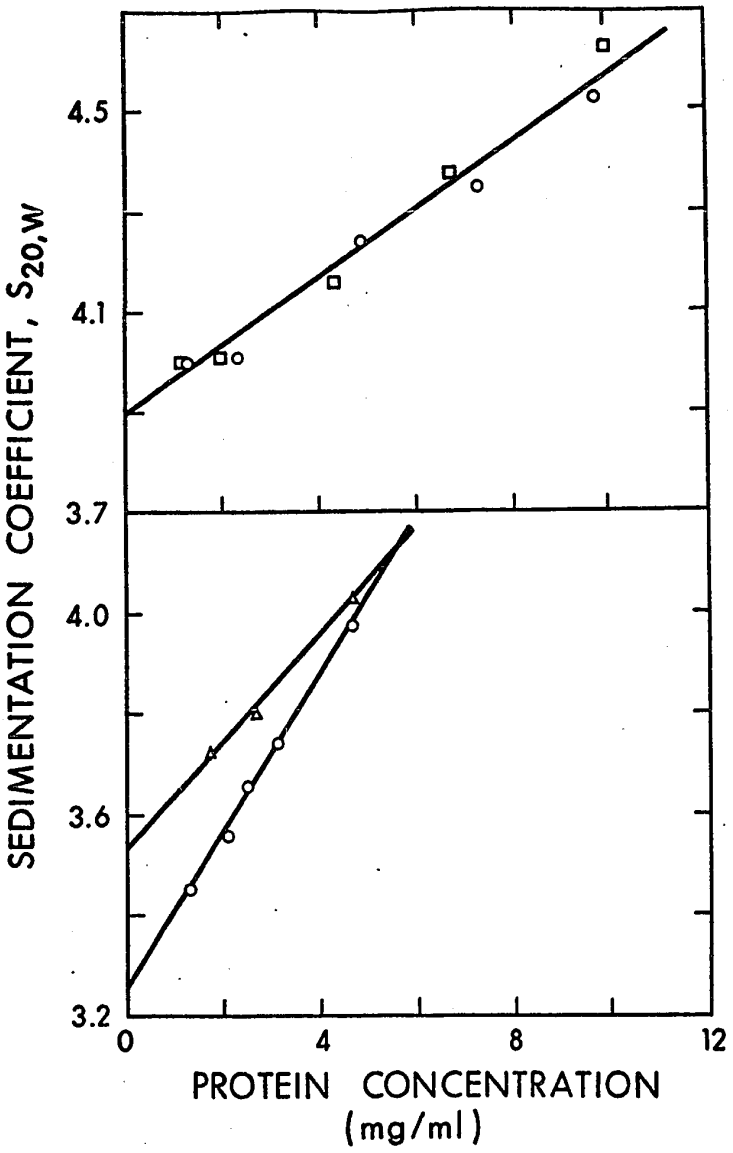

Figure 23. Effect of protein concentration on the sedimentation coefficient, $S_{20,w}$, of factor X_a^{RVV}. The centrifuge was operated at 59,780 rpm at 20° C using the Schlieren optical system with photographs taken at 8- or 16-min intervals. UPPER FIGURE, two preparations in 0.1 M NaCl, 0.01 M sodium acetate, pH 7.0; LOWER FIGURE, protein in the same buffer but containing 0.005 M $CaCl_2$ (Δ——Δ); buffer containing 0.025 M $CaCl_2$ (o——o).

Material used for initial sedimentation equilibrium centrifugation experiments was dialyzed for 18 hours at 4° C against 0.1 M NaCl, 0.01 M sodium acetate, pH 7.0. Two centrifugations at 12,000 r.p.m. were performed on two preparations of factor X_a^{RVV} at 0.5 A_{280} unit per ml concentration. Figure 24 shows that the resultant plots of ℓn y against r^2 showed continuous upward curvature. Molecular weight estimates calculated from the slope of tangents to the middle portions of these plots were 46,000 and 49,000. Meniscus depletion centrifugation at 32,000 r.p.m. likewise produce a plot of ℓn y against r^2 showing upward curvature (Fig. 25A). The molecular weight calculated from the tangent line shown on Fig. 25A is 46,800. These values are suspect due to failure to obtain linear plots.

After these data were collected, it was found that dialysis of the factor X_a^{RVV} preparation for 18 hours had not entirely removed the small-molecular-weight contaminant. When a sample, previously dialyzed for 18 hours, was placed in the collodion bag concentrator and the volume reduced from 2.5 to 0.5 ml, 1.3% of the material absorbing at 280 nm passed through the membrane. This indicated that although 18-hour dialysis removed most of the contaminant, it did not remove it all. More exhaustive dialysis was thus indicated.

Figure 25B shows the ℓn y against r^2 plot of data collected at 28,000 r.p.m. on factor X_a^{RVV} dialyzed for 48 hours against the above buffer. The plot is more nearly linear though upward curvature still occurs near the bottom of the cell. A molecular weight of 48,800 was claculated from the tangent line shown.

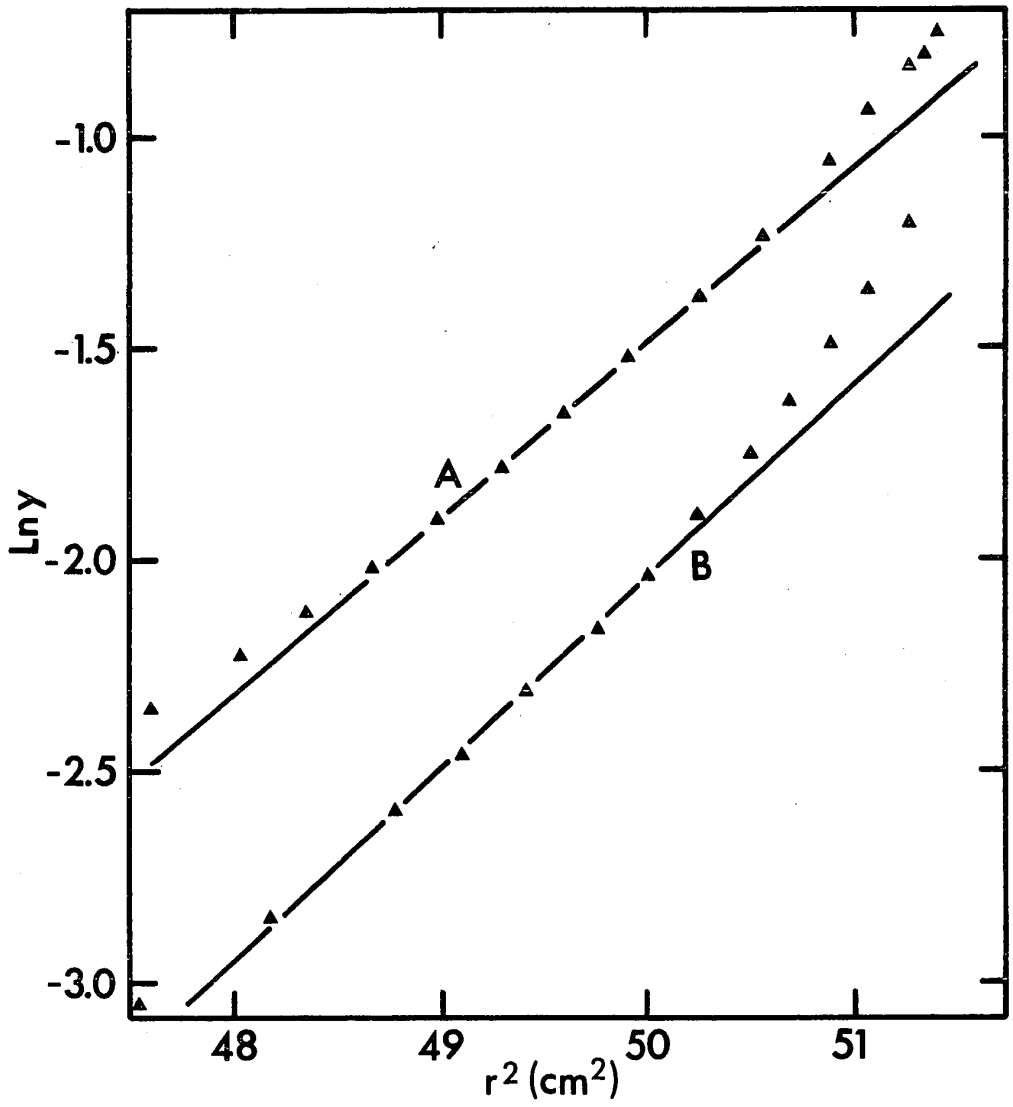

Figure 24. Sedimentation equilibrium centrifugation ℓn y against r^2 plots for factor X_a^{RVV} dialyzed for 18 hours against 0.1 M NaCl, 0.01 M sodium acetate, pH 7.0. A, 1.8 mg/ml protein centrifuged at 12,000 rpm at 20° C. B, 0.7 mg/ml protein centrifuged at 12,000 rpm at 10° C.

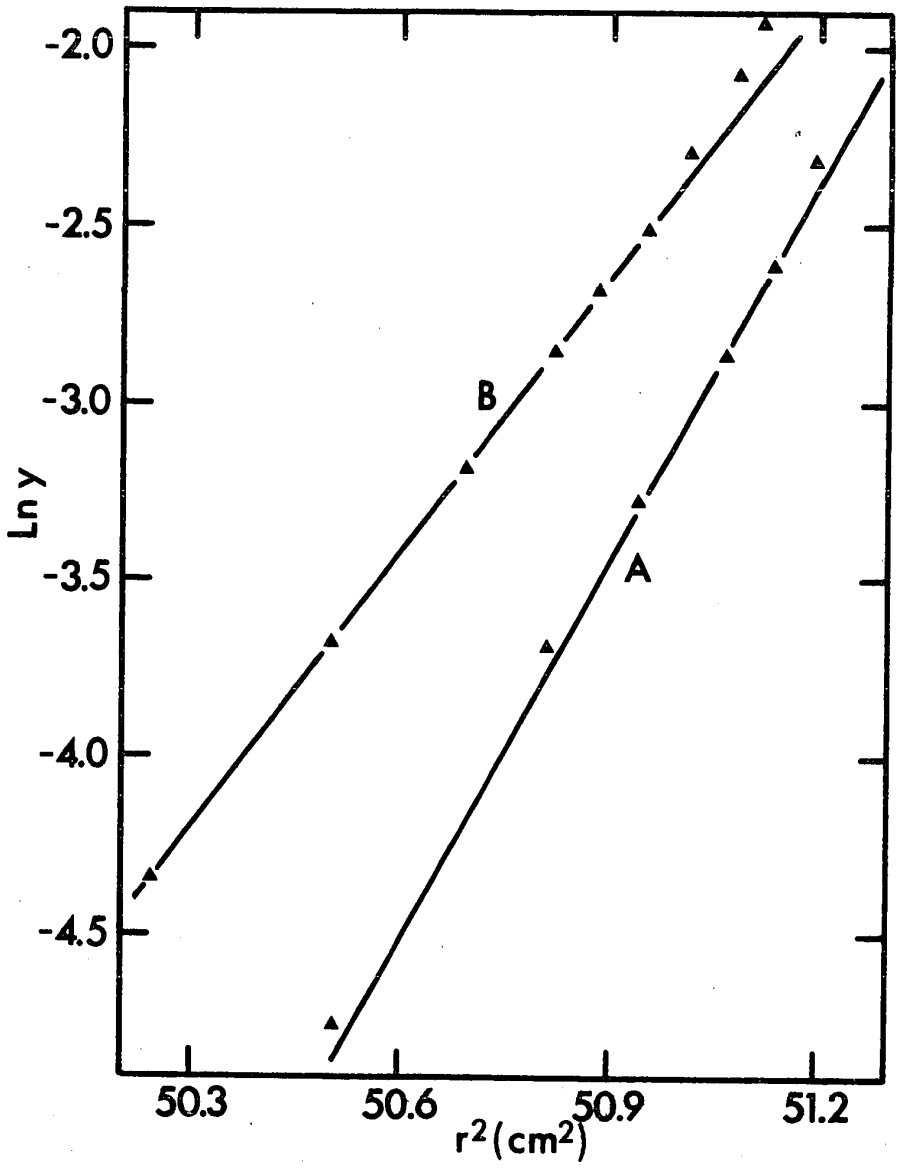

Figure 25. Sedimentation equilibrium centrifugation plots of ℓn y against r^2 for factor X_a^{RVV}. A, 0.8 mg/ml protein was dialyzed for 18 hours against 0.1 M NaCl, 0.01 M sodium acetate, pH 7.0, and centrifuged at 32,992 rpm at 20° C. B, 0.7 mg/ml protein was dialyzed for 48 hours against the same buffer and centrifuged at 28,890 rpm at 20° C.

Factor X_a^{RVV} samples used for diffusion coefficient determinations were dialyzed for 48 hours against 0.1 M NaCl, 0.01 M sodium acetate, pH 7.0. Figure 26 shows the variation of the diffusion coefficient values with concentration of protein. A $D^{\circ}_{20,w}$ of 6.98×10^{-7} cm^2 sec^{-1} was indicated. Inserting values of 6.98×10^{-7} cm^2 sec^{-1} for $D^{\circ}_{20,w}$, 3.90 S for $S^{\circ}_{20,w}$ and 0.715 for $\bar{v}$ into the Svedberg equation yields a molecular weight of 48,200.

F. SDS-Acrylamide Gel Electrophoresis

SDS-acrylamide gel electrophoresis of dialyzed or undialyzed factor X_a^{RVV} in the presence of mercaptoethanol or dithiothreitol gave two bands while electrophoresis of unreduced material gave one band, using the procedures of Maizel (116) or of Laemmli (117) (Fig. 27). The minor band seen in gels with reduced samples ran with the tracking dye and was absent from gels with non-reduced samples. These results were not altered by including 8 M urea in the reaction mixtures.

When the rate of migration of unreduced factor X_a^{RVV} was compared with those of unreduced BSA, ovalbumin, chymotrypsinogen A and myoglobin, molecular weight estimates of 54,000 to 57,000 were obtained (144). Calibration with reduced standards (144) indicated molecular weights of 17,000 to 22,000 and 28,000 to 33,000 for the two separated peptide chains. When one gel was scanned in a Gilson Spectrophotometer the resultant peak areas were seen to be in the ratio calculated for equimolar amounts of two species of molecular weights of 20,000 and 30,000, assuming equal dye uptake by both. It was concluded that the two components represent two polypeptide chains joined by disulfide bonds in the native protein.

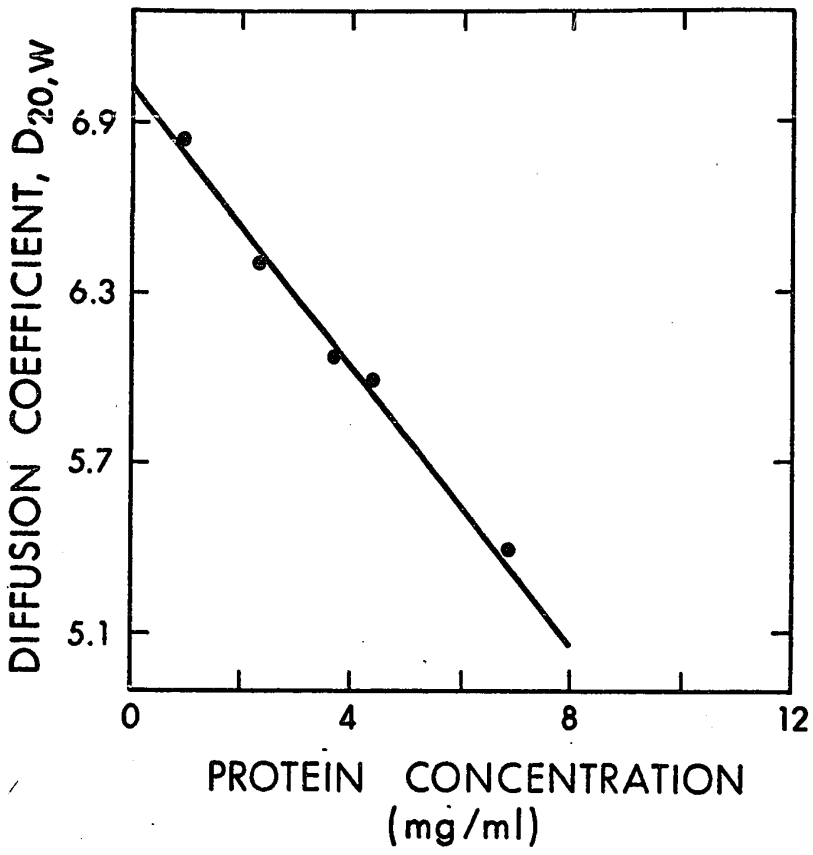

Figure 26. Effect of protein concentration on the diffusion coefficient, $D_{20,w}$, of factor X_a^{RVV} dialyzed for 48 hours against 0.1 M NaCl, 0.01 M sodium acetate, pH 7.0. The protein was layered at 5,200 rpm (time zero). Subsequently, the centrifuge was operated at this speed and photographs were taken using the Rayleigh optics at 8-min intervals.

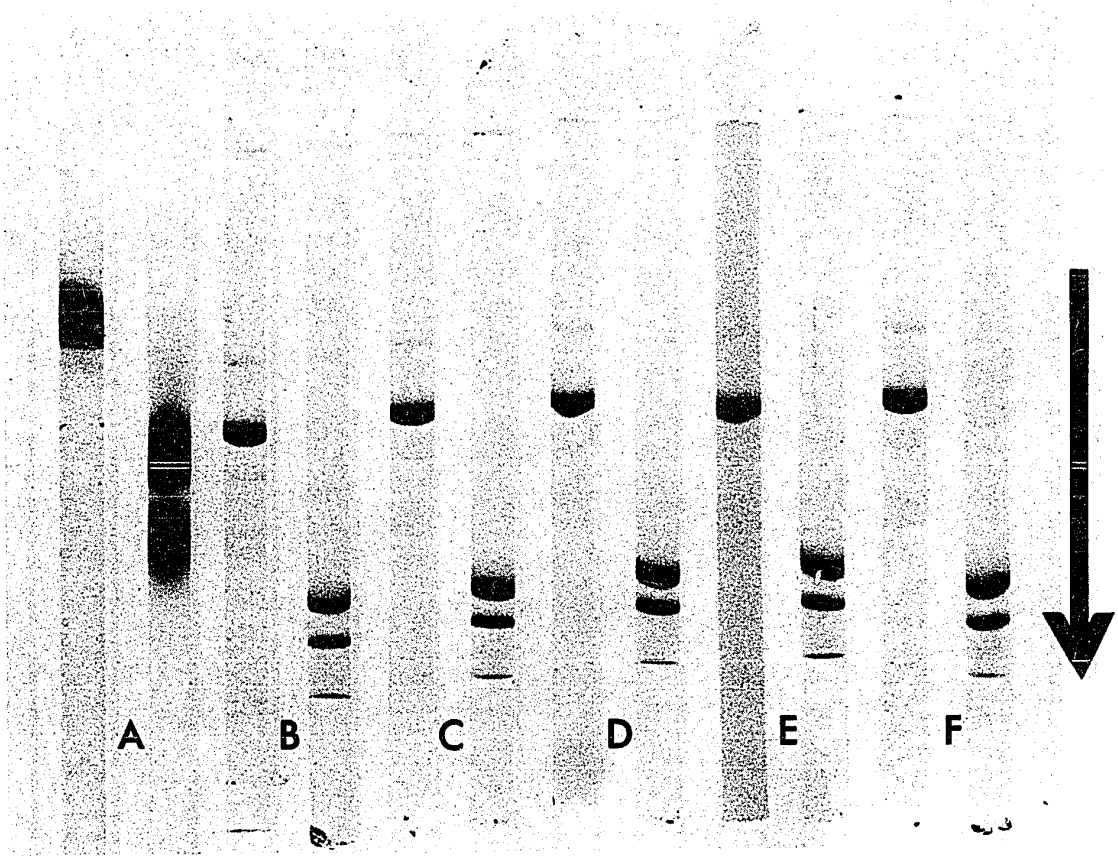

Figure 27. SDS-acrylamide gel electrophoresis of five species of activated factor X. A, factor X_a^{RVV} native and reduced in 5% gels by the procedure of Maizel (116). All other gels contain native and reduced species in 10% gels by the procedure of Laemmli (117). B, factor X_a^{RVV}; C, factor X_a^{Cit}; D, factor X_a^{IMET}; E, factor X_a^{TF}; F, factor X_a^{Int}. Protein loadings are about 70 µg (A) or 15 µg (B - F). Coomassie brilliant blue stain was used. Migration was in the direction of the arrow. A minor band is seen at the position of the tracking dye (indicated by the head of the arrow) in runs of reduced samples.

In order to determine which chain contained the active-site serine residue, electrophoresis was performed on a sample of factor X_a^{RVV} which had been inhibited with radioactive (^{32}P) DFP. The resultant gel was stained and then sliced into 30 transverse sections. The radioactivity of each slice was counted in a gas-flow counter after overnight digestion on planchets with 30% hydrogen peroxide. A photograph of the stained gel alongside a histogram of ^{32}P-radioactivity in the gel slices is shown in Fig. 28. It was concluded that (^{32}P) DFP bound significantly only to the larger chain.

Repeated attempts were made to separate the chains on a preparative scale, but a routinely reliable procedure was not attained. Reduction with dithiothreitol or mercaptoethanol in 8 M urea followed by blocking of -SH groups with ethyleneimine or iodoacetamide led to urea-insoluble products. None of the following could effect re-solution: pure formic acid, glacial acetic acid, 50% formic acid, or 50% acetic acid. Performic acid oxidation likewise led to a urea insoluble product. A third procedure apparently was successful on three out of eight occasions. Samples (1 mg) were reduced in 8 M urea with dithiothreitol (1 mg per ml) and the reaction mixture applied to a Sephadex G-100 column equilibrated with 8 M urea, 0.1 M NaCl, 0.01 M sodium acetate buffer and 0.001 M dithiothreitol under nitrogen. In three experiments, two A_{280} peaks were obtained at the elution positions expected for chains of approximately 20,000 and 30,000 molecular weight. In five unsuccessful attempts most of the material eluted in a void volume peak. Since reproducible results could not be obtained, attempts to prepare separated chains were abandoned.

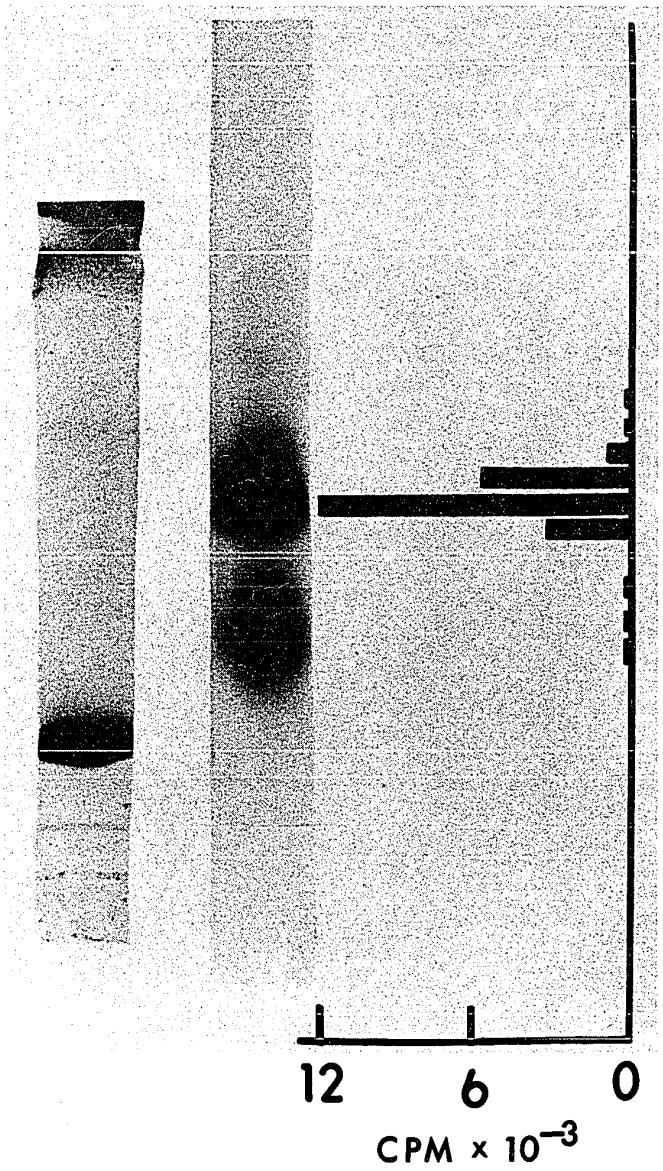

Figure 28. SDS-acrylamide gel electrophoresis of factor X_a^{RVV} inhibited with (^{32}P) DFP and reduced with mercaptoethanol (center), and a histogram showing the distribution of ^{32}P in slices of the gel (right). Approximately 70 µg of protein was applied. Staining was with Coomassie brilliant blue. Counts per min of ^{32}P were measured by digesting the gel slices with 30% H_2O_2 on planchets and counting in a gas flow counter. The disc gel electrophoresis pattern of the factor X_a^{RVV} preparation is shown at the left.

G. N-Terminal Amino Acid Determinations

Approximately 1 nmole of dansylated factor X_a^{RVV} digest was applied in 50% pyridine to either side of a 5 cm x 5 cm polyamide plate. Standards (dansyl-lysine, -cysteine, -phenylalanine, -isoleucine, -valine, -alanine, and -threonine) were spotted on one side only. Figs. 29A and B show the appearance of such a plate after chromatography in solvent systems (1) and (2) (121). Standards are identified in the Figure. The side with dansylated factor X_a^{RVV} shows only two yellow-green spots under UV illumination, identified as dansyl-isoleucine and a dansyl-dipeptide; and a large blue spot, dansyl-amide, which masks the dansyl-alanine in the standard mixture. After the plate was developed in solvent system (3), the dansyl-amide spot separated from a typically yellow-green spot on both sides, known to be dansyl-alanine from the standard mixture. It was concluded that factor X_a^{RVV} contains N-terminal residues alanine and isoleucine, the latter bound to a hydrolysis-resistant second residue. Quantitative determination of these residues by the cyanate procedure (144A) was not attempted due to the limited amounts of material available.

The dansyl-dipeptide spot moved sufficiently closely to the position for dansyl-methionine that it was initially thought to be dansyl-methionine. However, after developing a plate spotted with dansyl-methionine and dansylated factor X_a^{RVV} digest in solvent systems (1) and (2), the dansyl-methionine and dipeptide spots were separated, though overlapping (Fig. 29E). The dansyl-methionine and dansyl-dipeptide spots separated clearly when this plate was developed in solvent system (1) for a longer period of time, as shown in Fig. 29F (development was in direction (2)).

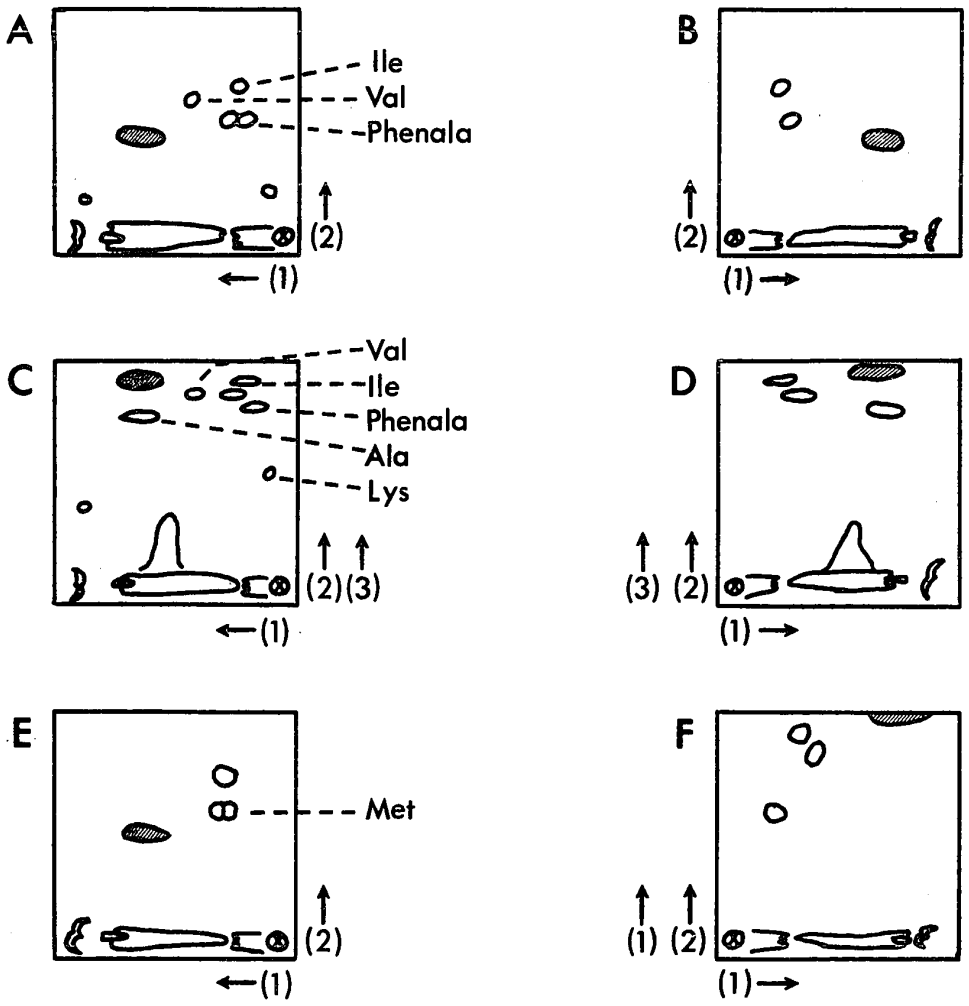

Figure 29. Polyamide layer chromatography of dansylated factor X_a^{RVV} digest. Samples were applied at the positions marked with an "x". A, digest plus standards mixture after development in solvent systems (1) and (2). B, digest only after (1) and (2). C, plate (A) after further development in system (3). D, plate (B) after system (3). E, digest plus dansyl-Met after systems (1) and (2). F, plate (E) after further development in system (1). Some standard spots are labeled. The hatched spot is dansyl-amide.

The dansyl-dipeptide spot was cut from one plate, eluted with 50% pyridine and again hydrolyzed with 6 N HCl for 24 hours at 105° C. HCl was removed by rotary evaporation and vacuum pumping over KOH. Chromatography in solvent systems (1) and (2) led to two yellow-green spots identified as dansyl-isoleucine and the original dansyl-dipeptide spot.

The dansyl-isoleucine and dansyl-alanine assignments were verified by running plates with only dansyl-isoleucine and dansyl-alanine as standards. The standards and assigned dansyl-factor X_a^{RVV} digest spots moved identically in all three solvent systems.

H. C-Terminal Amino Acid Determinations

Material used for C-terminal amino acid determinations was inhibited with DFP and dialyzed 24 hours against 0.1 M sodium citrate, pH 8.5, at 4° C. An aliquot containing 0.06 μmole of factor X_a^{RVV} was made to 6 M with urea (final volume 2 ml) and 0.002 μmole of carboxypeptidase A (DFP-inhibited) was added at 20° C. Aliquots of 1 ml were removed from the reaction mixture after 3 and 22 hours digestion and protein precipitated by addition of 1 ml of 15% TCA. The protein-free supernatant, after extraction of TCA six times with diethyl ether, was dried under vacuum and then applied to the amino acid analyzer. Neither the factor X_a^{RVV} supernatant nor a control containing only carboxypeptidase A revealed any amino acids. BSA treated identically, released 0.015 μmole of leucine and alanine per 0.025 μmole protein after 3 and 22 hours digestion.

When factor X_a^{RVV} was digested identically except that carboxypeptidase B (DFP-inhibited) replaced carboxypeptidase A, amino acid analysis revealed 0.025 μmole of arginine per 0.025 μmole protein in the 3 hour and 22 hour digestion aliquots. A control containing only carboxypeptidase B showed no arginine or lysine. Bovine hemoglobin treated identically revealed 0.03 μmole of arginine per 0.05 μmole protein after 3 and 22 hours of digestion.

Since SDS-acrylamide gel electrophoresis and N-terminal determinations suggest that factor X_a^{RVV} contains two peptide chains, it is of interest whether one or two residues of arginine per molecule of protein is (are) obtained in the C-terminal amino acid determination. One μmole of arginine was observed in the analyzer per μmole of factor X_a^{RVV}. However, in the digestion of BSA, 0.6 μmole of leucine and 0.6 μmole of alanine were estimated per μmole of protein digested. In the digestion of hemoglobin, 0.61 mole of arginine was obtained per mole of protein digested. This indicates that a 40% loss might be expected in the procedure. If the same percentage recovery applies in the case of factor X_a^{RVV}, then the above results indicate that 1.7 arginine residues are released per molecule of protein. Since this technique is not accurately quantitative, it can only be concluded that at least one C-terminal residue of factor X_a^{RVV} is arginine.

I. Extinction Coefficient Determination

The specific extinction coefficient, $E_{1\ cm}^{1\%}$, measured at 280 nm was found to be 8.6 ± 0.2. Assuming a molecular weight of 50,000 a molar extinction coefficient of 4.3×10^4 was calculated.

J. Partial Specific Volume Calculation

A partial specific volume, $\bar{v}$, of 0.715, was calculated from the average of the amino acid compositions (Table IV), using the partial specific volumes of the constituent amino acids (134).

K. Treatment with N-p-Tosyl-L-Lysine Chloromethyl Ketone HCl (TLCK) and Diisopropyl Phosphorofluoridate (DFP)

Factor X_a^{RVV} was treated with TLCK under the conditions listed and clotting activity assayed at intervals for the length of time indicated.

(i) Factor X_a^{RVV} (50 units per ml), 0.02 M TLCK, 0.14 M NaCl, 0.02 M barbital-acetate, pH 7.0, with and without 0.01 M $CaCl_2$, 25° C. Activity was followed for 3 hours. No activity loss was observed.

(ii) Factor X_a^{RVV} (50 units per ml), 0.01 M TLCK, 0.1 M sodium acetate, pH 8.0, with and without 0.005 M $CaCl_2$, 25° C. No activity loss was observed over 20 hours.

(iii) Factor X_a^{RVV} (500 units per ml), 0.1 M TLCK, 0.1 M NaCl, 0.1 M tris-HCl, pH 7.5, with and without 0.025 M $CaCl_2$, 25° C. No inhibition was observed over 28 hours.

Shaw et al (145) reported 90% inhibition of trypsin (4.56×10^{-5} M) by TLCK (6.17×10^{-4} M) after 1 hour in 0.02 M barbital-acetate buffer, pH 8.0, at room temperature. In experiment 3 above, factor X_a^{RVV} is present at a concentration of approximately 1×10^{-5} M and TLCK at 1×10^{-2} M, but no inhibition was observed over 28 hours. It was concluded that TLCK does not inhibit factor X_a^{RVV}. This lack of effect of TLCK on factor X_a^{RVV} clotting activity is compatible with the observation

of Adams and Elmore (55) that factor X_a is a better catalyst for the hydrolysis of arginine methyl esters than for lysine methyl esters.

When factor X_a^{RVV} was treated with 0.001 M DFP, no inhibition was observed after 6 hours at 20° C. When 0.01 M DFP was employed, inhibition was nearly complete after several hours in various buffers at pH 7.0, 20° C, in agreement with the observations of Leveson and Esnouf (54). A further experiment was performed to test for an effect of $CaCl_2$ and phospholipids on the inhibition of factor X_a^{RVV} with DFP. Since the presence of $CaCl_2$ and phospholipids is known to increase the rate of reaction of factor X_a with prothrombin (7), it was decided to test for a similar effect on the rate of reaction of factor X_a^{RVV} with DFP. When $CaCl_2$ was added to aliquots of factor X_a^{RVV} to a final concentration of 0.01 M, and DFP was added to a concentration of 0.01 M, inhibition proceeded at the same rate as in the absence of $CaCl_2$. When a sonicated suspension of phosphatidyl-serine and phosphatidyl-choline (1 to 1 molar ratio) was added to the reaction mixture to a final concentration of 1 mg per ml, and 0.01 M DFP added with or without 0.010 M $CaCl_2$, the inhibition was slightly slower than in the control lacking phospholipids. Under the same conditions except that 0.001 M DFP was used, no inhibition was seen over 5 hours.

L. Peptide Mapping of Tryptic Digests of Factor X_a^{RVV}

Peptide mapping was performed on DFP-inhibited samples. When tryptic digests of native factor X_a^{RVV}, and factor X_a^{RVV} heat-denatured at 100° C for 10 minutes, were subjected to two dimensional electrophoresis, the resulting peptide maps were identical and denaturation was omitted subsequently.

Fig. 30 shows the peptide map of factor X_a^{RVV} after staining with the cadmium acetate-ninhydrin stain. The pH 6.5 strip was sewn to the pH 1.8 sheet as shown in Fig. 30. The heavy line on the strip indicates the position of application of the digest to the pH 6.5 sheet. Weakly staining spots are indicated by broken circles. The four spots marked "y" were yellow shortly after staining, but the uppermost turned pink with time. All other spots were pink or orange-pink. The pattern shown was observed without significant differences in three separate mappings of digests prepared from three different preparations of factor X_a^{RVV}.

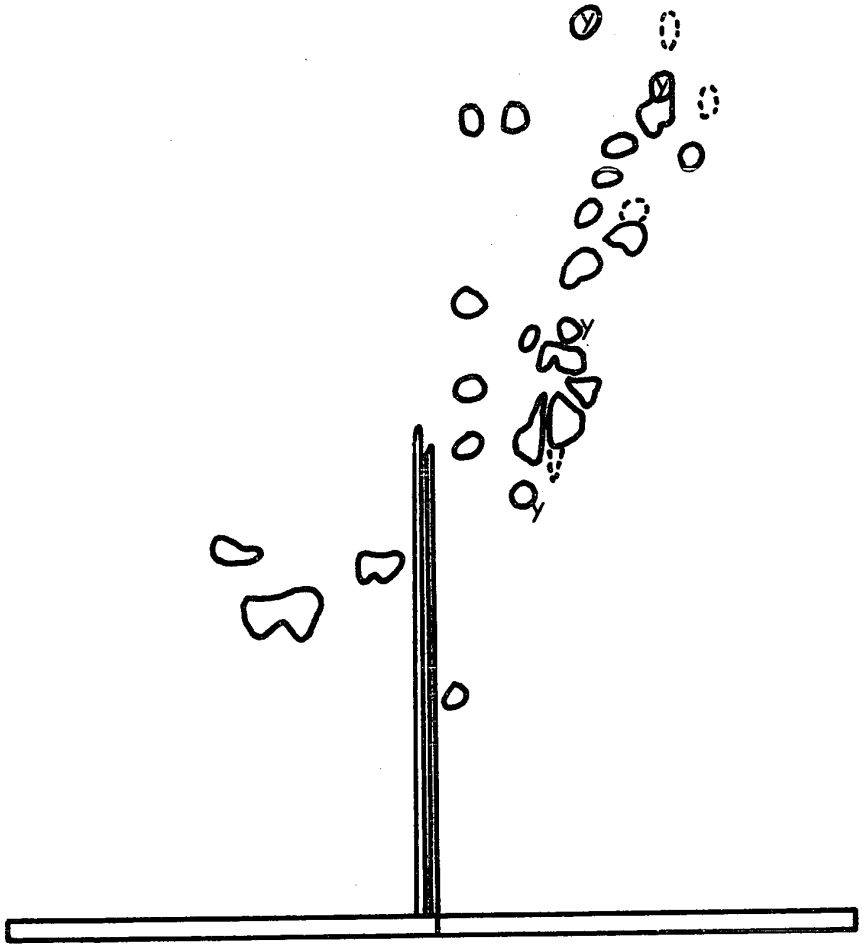

Figure 30. Peptide map of a tryptic digest of factor X_a^{RVV}. Digest was applied as a 1 cm band (indicated by the dark band) to a sheet of Whatman No. 1 paper and electrophoresed at pH 6.5. The resulting strip (indicated at the bottom of the figure) was sewn to a second sheet of Whatman No. 1 paper and electrophoresed at pH 1.8. The second sheet was stained with a cadmium acetate-ninhydrin solution. Spots marked "y" were yellow. Other spots were pink. See text for details of procedure.

CHAPTER VI

PHYSICO-CHEMICAL CHARACTERIZATION OF FACTOR X ACTIVATED IN SODIUM CITRATE

Investigation of activated factor X generated in 25% sodium citrate was of particular interest in that the basic nature of the mechanism of activation was not evident. It seemed unlikely that an inorganic salt in any concentration could alter a protein molecule so as to cause an activation that by other procedures apparently involves proteolytic hydrolysis. Further, the characterization of autoprothrombin c by Seegers et al. (97) suggested that it was indeed a different molecular species from factor X activated with RVV (8). In this study, the problem was approached from two directions. First, the nature of the mechanism of activation was studied directly as discussed in Chapter III, section B. Activation could be achieved by attack of the proteolytic enzyme, thrombin, and another species, possibly factor VII. Secondly, physico-chemical characterization of factor X_a^{Cit} was performed to allow comparison with the data of Seegers et al. (97), and with data reported in this thesis on other species of activated factor X.

A. Disc Gel Electrophoresis

Electrophoresis of factor X_a^{Cit} preparations at loadings of approximately 100 µg protein revealed one major band and 4 minor bands (Fig. 7B). The electrophoretic mobilities of the weak bands were estimated at 0.56, 0.60 and 0.65 and 0.83 times that of the major band. Their intensities were slightly greater than those for the venom-activated species at similar loadings.

Figure 31 shows disc electrophoresis gels containing mixtures of the factor X_a^{Cit} and X_a^{RVV} preparations. It can be seen that the major bands coincide. At heavier loadings (not shown) it was also observed that three of the four impurity bands of the factor X_a^{RVV} and factor X_a^{Cit} preparations coincide.

B. Sephadex Gel Filtration

Factors X_a^{RVV} and X_a^{Cit} were each chromatographed several times on the same Sephadex G-200 column (0.9 cm x 60 cm). In six experiments, elution volumes of 29.0, 28.4 and 28.8 ml for factor X_a^{RVV} and 28.8, 28.4 and 28.5 ml for factor X_a^{Cit} were obtained. The average corresponds to a molecular weight of 48,000 using ovalbumin, BSA and myoglobin as calibration standards.

The factor X_a^{Cit} material used in these Sephadex G-200 chromatographies was prepared from zymogen factor X, Preparation VI (Chapter III, section B). Preparation VI contains only a fraction of the prothrombin present in the original plasma, since adsorption with 10 mg of $BaSO_4$ per ml of plasma is relatively selective for factor X. In contrast Marciniak and Seegers' (96) autoprothrombin c was generated in the presence of large amounts of prothrombin. To more nearly duplicate their activation conditions one batch of factor X_a^{Cit} was prepared from factor X, Preparation III, which could be expected to contain most of the prothrombin present in the original plasma. Activation and chromatography were carried out as before. An aliquot of this preparation of factor X_a^{Cit} was applied to the previously used Sephadex G-200 column and chromatographed as before. The peak elution volume was 28.8 ml, identical

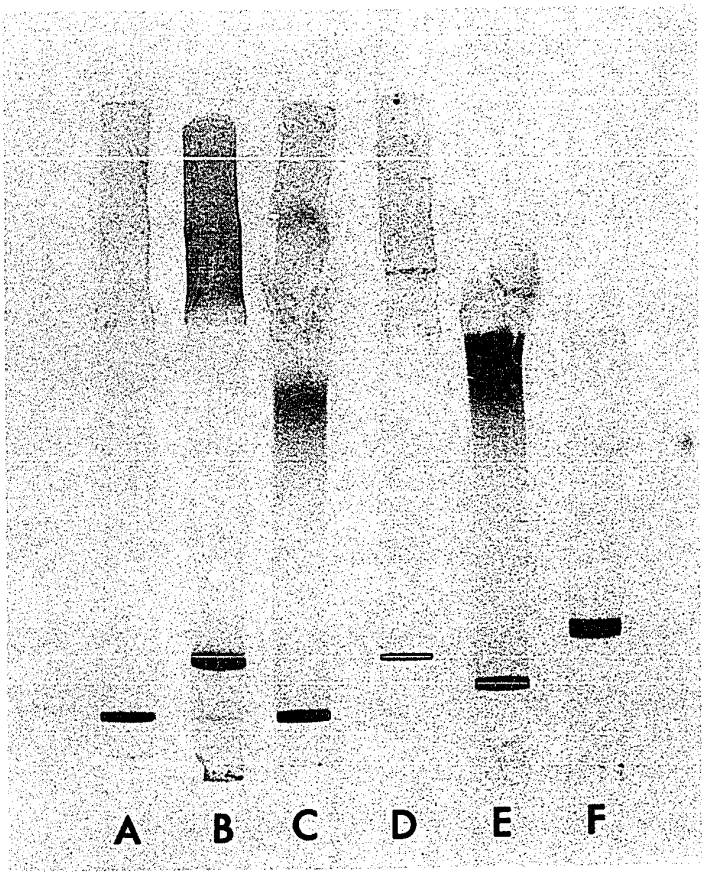

Figure 31. Disc gel electrophoresis of mixtures of five species of activated factor X at pH 9.5. A, factors X_a^{RVV}, X_a^{TF}, X_a^{Int}; B, factors X_a^{TF}, X_a^{Cit}; C, factors X_a^{IMET}, X_a^{RVV}; D, factors X_a^{Cit}, X_a^{Int}; E, factors X_a^{Cit}, X_a^{IMET}; F, factors X_a^{RVV}, X_a^{Cit}, X_a^{TF}, X_a^{Int}. Gels were stained with Buffalo Black. Gels C and E wer run at excessive amperage and some denatured material is seen near the top of the separating gel.

with those of factor X_a^{RVV} and factor X_a^{Cit} prepared from the routine Preparation VI. This result leaves little room for thinking that the observed differences between factor X_a^{Cit} and autoprothrombin c reflect different methods of activation.

A preparative Sephadex G-200 chromatography was carried out to prepare factor X_a^{Cit} free of the non-protein contaminant for diffusion coefficient determinations (Chapter VI, section C). An aliquot containing 10.5 A_{280} units of factor X_a^{Cit} in 1.4 ml of 0.1 M sodium citrate, pH 7.0, was dialyzed for 17 hours at 4° C against 1 liter of 0.1 M NaCl, 0.01 M sodium acetate, pH 7.0. After dialysis, 5.5 A_{280} units remained, indicating a minimum contamination of 47% non-protein material in the citrate-activated species. This is about 20% higher than that observed for factor X_a^{RVV}, prepared from more highly purified zymogen factor X. Figure 32 shows the Sephadex G-200 elution profile of the dialyzed sample. Estimation of peak areas by counting squares indicates that 8% of the absorbing material was eluted after the main peak. This parallels the finding that factor X_a^{RVV} dialyzed 18 hours still contains appreciable amounts of the contaminant.

C. Ultracentrifugal Analysis

Sedimentation velocity centrifugation of factor X_a^{Cit} was performed on samples dialyzed for 48 hours at 4° C against 3 x 500 ml of 0.1 M NaCl, 0.01 M sodium acetate, pH 7.0. Figure 33 shows the variation of $S_{20,w}$ with protein concentration. The data extrapolates to an $S^{\circ}_{20,w}$ value of 3.92 S.

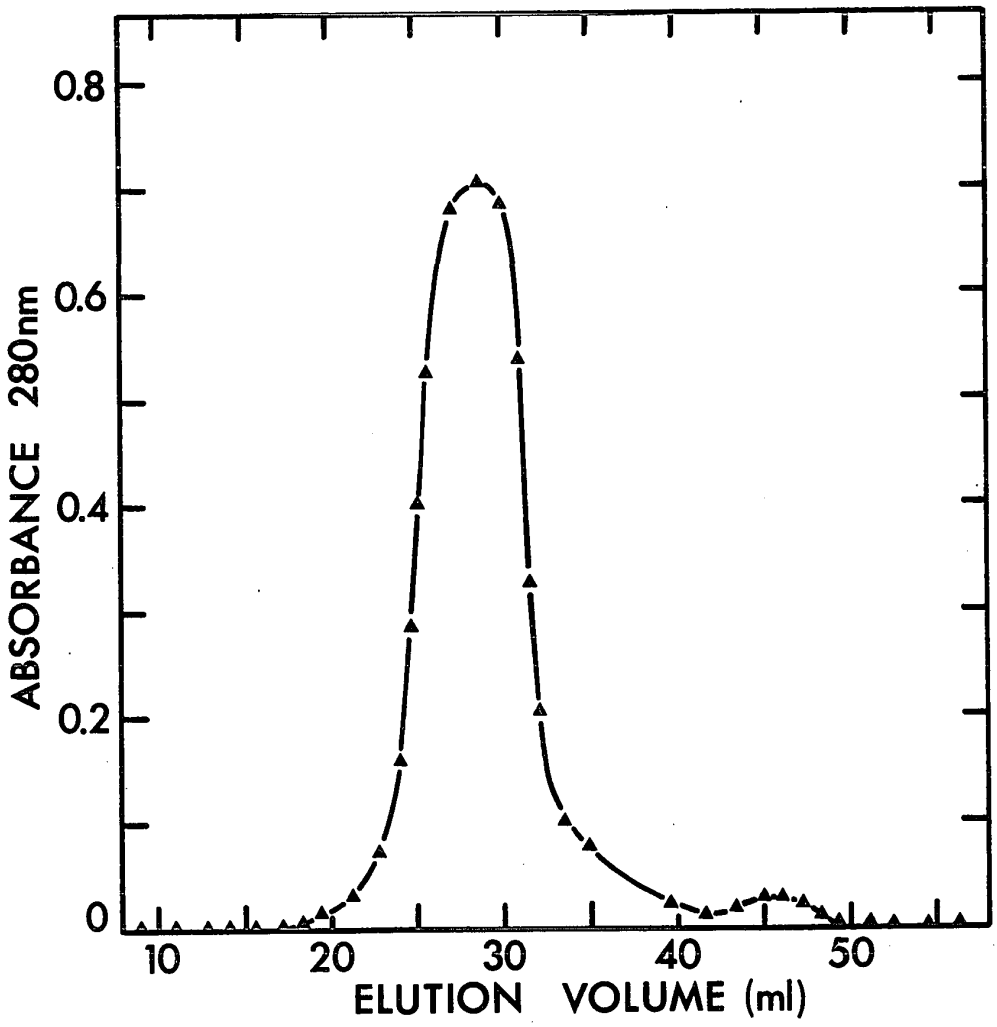

Figure 32. Gel filtration elution profile of factor X_a^{Cit} dialyzed for 17 hours against 0.1 M NaCl, 0.01 M sodium acetate, pH 7.0. A 1 ml sample was applied to a column (0.9 cm diameter x 60 cm height) and eluted with the same buffer. Flow rate was 5 ml per hour and fractions of 0.8 ml were collected.

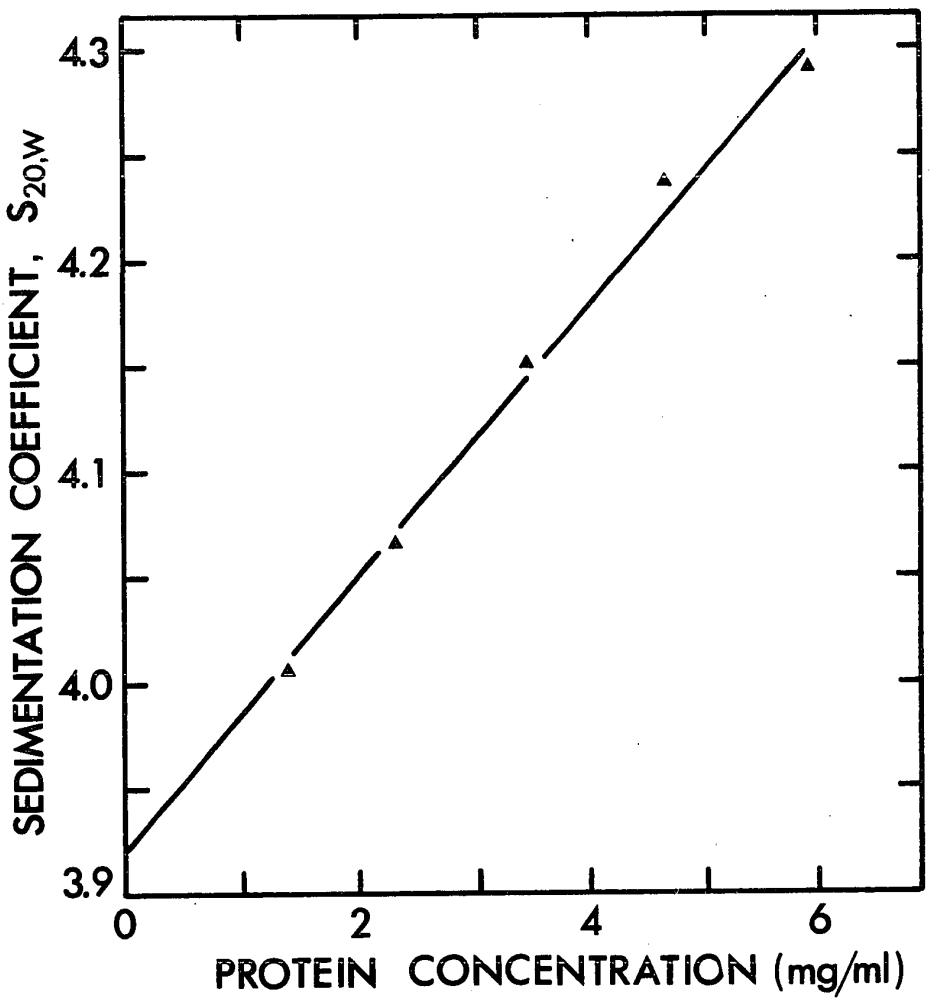

Figure 33. Effect of protein concentration on the sedimentation coefficient, $S_{20,w}$, of factor X_a^{Cit} dialyzed for 48 hours against 0.1 M NaCl, 0.01 M sodium acetate, pH 7.0. The centrifuge was operated at 59,780 rpm at 20° C using the Schlieren optical system with photographs taken at 8- or 16-min intervals.

Initial meniscus depletion sedimentation equilibrium centrifugation was also performed on samples dialyzed 48 hours. Plots of ℓn y against r^2 showed continuous upward curvature. Mid-point estimates of the molecular weight were in the range of 45,000 to 50,000, but these again were considered unreliable. Unfortunately, it was only as a result of these findings that the presence of an unacceptable level of contaminant was recognized. Insufficient material remained afterward for further sedimentation equilibrium experiments.

Likewise, it was found that diffusion coefficient measurements on factor X_a^{Cit} dialyzed for only 48 hours were unreliable. Figure 34 shows the variation of $D_{20,w}$ with protein concentration for two such runs. The extrapolated $D^{\circ}_{20,w}$ values were 8.7 and 8.8 S. When a third determination (Fig. 34C) was performed on material chromatographed on Sephadex G-200 (as discussed in the Sephadex Gel Filtration section), the $D_{20,w}$ values fell closer to those observed for factor X_a^{RVV} dialyzed for 48 hours. The extrapolated $D^{\circ}_{20,w}$ was found to be 6.75×10^{-7} cm^2 sec^{-1}. A molecular weight of 50,500 was calculated for factor X_a^{Cit} from an $S^{\circ}_{20,w}$ of 3.92 S, a $D^{\circ}_{20,w}$ of 6.75×10^{-7} cm^2 sec^{-1}, and a $\bar{v}$ of 0.715.

D. SDS-Acrylamide Gel Electrophoresis

SDS-acrylamide gel electrophoresis of factor X_a^{Cit} produced bands moving similarly to those seen for factor X_a^{RVV} (Fig. 27). When mixtures of the venom- and citrate-activated species were electrophoresed together after reduction with mercaptoethanol, the resultant major bands coincided (Fig. 35). The minor fast-moving band seen in each gel with a reduced protein sample migrated at the position of the tracking dye. The origin of this material was not established.

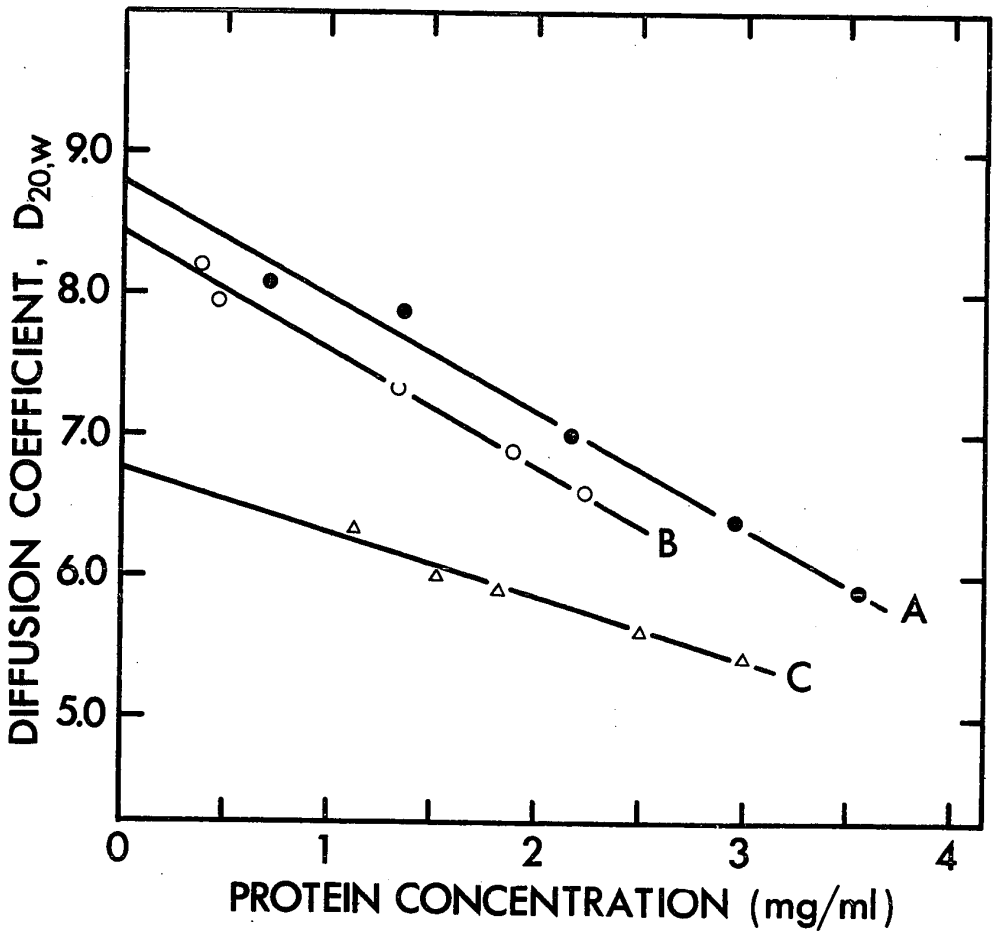

Figure 34. Effect of protein concentration on the diffusion coefficient, $D_{20,w}$, of factor X_a^{Cit}. A and B, factor X_a^{Cit} was dialyzed for 48 hours against 0.1 M NaCl, 0.01 M sodium acetate, pH 7.0, before diffusion coefficient measurements. C, factor X_a^{Cit} was dialyzed for 18 hours, chromatographed on Sephadex G-200 (Chapter VI, section B), and peak fractions dialyzed a further 48 hours before diffusion coefficient measurements. The protein was layered at 5,200 rpm. Subsequently, the centrifuge was operated at this speed and photographs were taken using the Rayleigh optics at 8-min intervals.

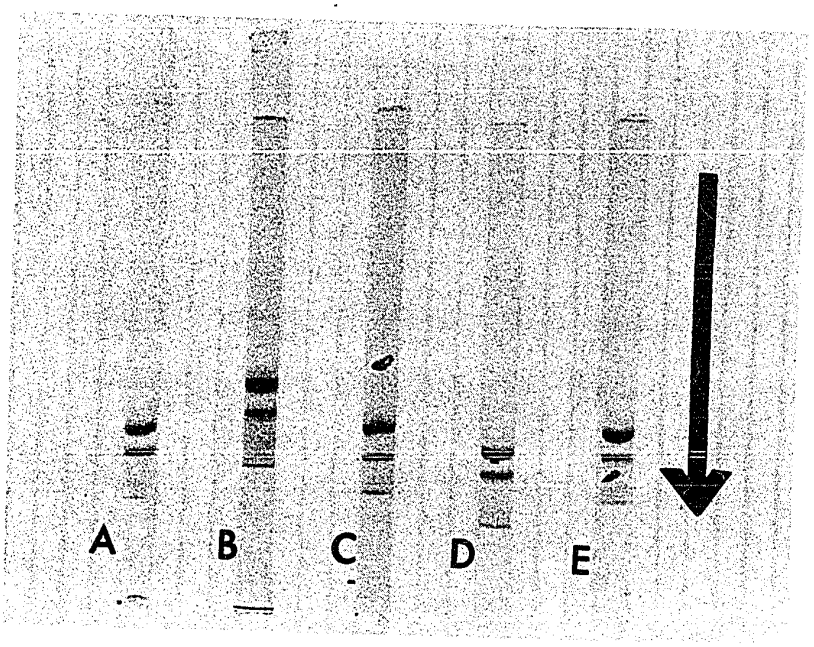

Figure 35. SDS-acrylamide gel electrophoresis of mixtures of five species of activated factor X after reduction with mercaptoethanol. A, factors X_a^{RVV} and X_a^{Cit}; B, factors X_a^{Cit} and X_a^{IMET}; C, factors X_a^{IMET}, X_a^{TF} and X_a^{RVV}; D, factors X_a^{TF} and X_a^{Int}; E, factors X_a^{RVV} and X_a^{Int}. The method of Laemmli (117) was used. Protein loadings are in the range of 10-15 µg. Coomassie brilliant blue stain was used. Migration was in the direction of the arrow. The minor band seen in each gel near the head of the arrow is at the position of the tracking dye.

E. N-Terminal Amino Acid Determination

When the 6 N HCl digests of dansylated factor X_a^{Cit} were chromatographed on polyamide layers in solvent systems (1) and (2), patterns very similar to those observed with factor X_a^{RVV} were obtained. When the plates were developed in solvent system (3), the dansyl-amide spot separated from a second spot, identified again as alanine. It was concluded that the N-terminal residues of factor X_a^{Cit} are alanine and isoleucine.

F. C-Terminal Amino Acid Determination

Digestion of factor X_a^{Cit} with carboxypeptidase A released no amino acids detected in the amino acid analyzer. Digestion with carboxypeptidase B for 3 or 22 hours released 0.044 μmole of arginine from a sample previously dialyzed for 16 hours and judged from the absorption at 280 nm to contain 0.052 μmole of protein per aliquot. Since samples dialyzed for 16 hours would be expected to still contain at least 10% of non-protein absorbing material, the figure for the quantity of factor X_a^{Cit} digested must be revised downward to about 0.047 μmole. This is approximately the same yield of arginine as that obtained from factor X_a^{RVV}, and again it can only be concluded that one or two arginine residues are released per molecule of protein. No amino acid residues were detected when control reaction mixtures containing only carboxypeptidase A or carboxypeptidase B were applied to the amino acid analyzer.

G. Peptide Mapping of Tryptic Digests of Factor X_a^{Cit}

No significant differences could be detected between the peptide maps of factor X_a^{RVV} (Fig. 30) and factor X_a^{Cit} (Fig. 36). The same four yellow spots were present in each. Digests of both species yielded 25 major spots in the same positions. Weakly staining spots also coincided. Three preparations of factor X_a^{Cit} yielded peptide maps showing the pattern seen in Fig. 36.

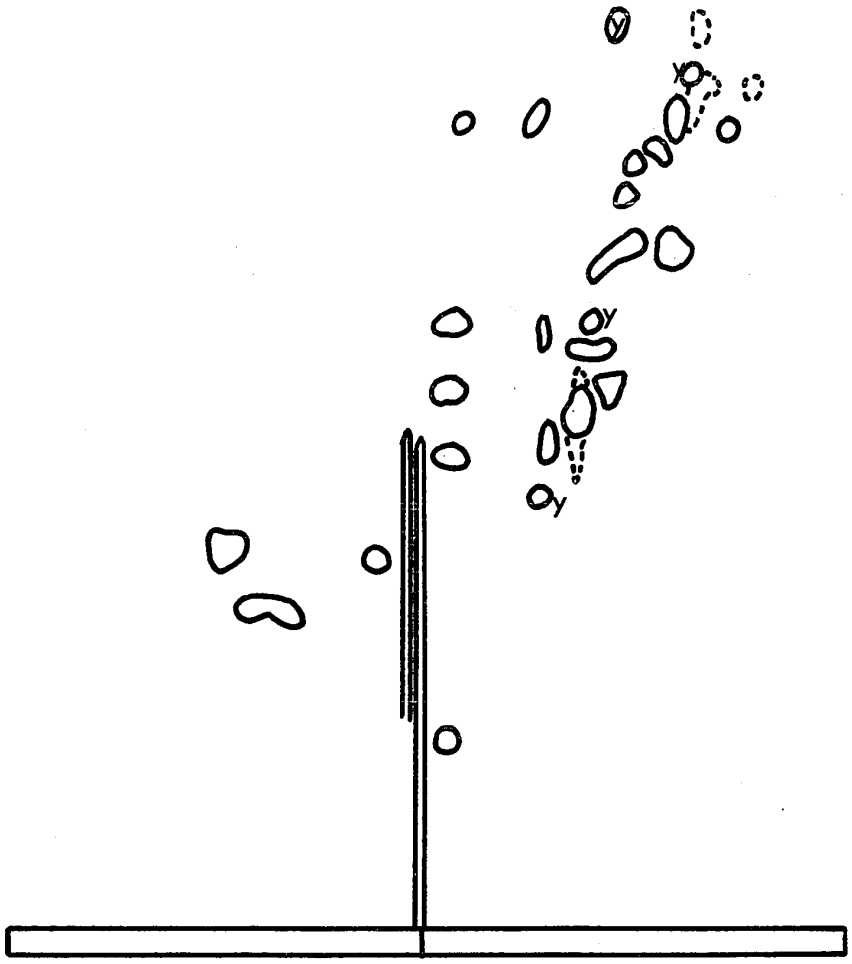

Figure 36. Peptide map of a tryptic digest of factor X_a^{Cit}. Digest was applied as a 1 cm band (indicated by the dark band) to a sheet of Whatman No. 1 paper and electrophoresed at pH 6.5. The resulting strip (indicated at the bottom of the figure) was sewn to a second sheet of Whatman No. 1 paper and electrophoresed at pH 1.8. The second sheet was stained with a cadmium acetate-ninhydrin solution. Spots marked "y" were yellow. Other spots were pink. See text for details of procedure.

CHAPTER VII

COMPARISONS OF ACTIVATED FACTOR X SPECIES OBTAINED BY ACTIVATION WITH RUSSELL'S VIPER VENOM OR SODIUM CITRATE WITH THOSE OBTAINED BY ACTIVATION WITH A TRYPSIN DERIVATIVE OR TISSUE FACTOR OR BY THE INTRINSIC SYSTEM

The low yields of the "intrinsic" and "extrinsic" activation procedures prevented as extensive characterization of factors X_a^{TF} and X_a^{Int} as was possible with factors X_a^{RVV} and X_a^{Cit}. The small quantities of these species available dictated a choice among the various techniques used for characterization of factor X_a^{RVV}. The primary criteria for selection were that the technique require relatively small amounts of protein and allow a highly resolving comparison with the more thoroughly characterized species. Fortunately most of the techniques which satisfy the later criterium also satisfy the former. Thus, disc gel electrophoresis and SDS-gel electrophoresis of mixtures of factors X_a^{TF} and X_a^{Int} with the other species require only micro-gram quantities of material and are also probably among the most discriminating of the techniques. Peptide mapping and N-terminal amino acid determinations were considered more highly discriminating than amino acid compositions, ultracentrifuge data and C-terminal amino acid determinations. The last three techniques require multi-milligram quantities of protein. For this reason no ultracentrifuge experiments, amino acid composition determinations or C-terminal amino acid determinations could be performed on factor X_a^{Int}.

A. Disc Gel Electrophoresis

Figure 7 shows the results of disc gel electrophoresis of the five species of activated factor X at protein loadings in the region of 100 μg. It can be seen that the purest species are factors X_a^{RVV} and X_a^{IMET}, followed by factors X_a^{Cit}, X_a^{TF} and X_a^{Int} in order of decreasing purity. The most rapidly migrating band in each is presumably not an impurity band, since it occurs at the position of the bromphenol blue tracking dye. All species display impurity bands moving slower than the major band, and factors X_a^{TF} and X_a^{Int} also display weak bands moving between the major band and the tracking dye.

Figure 31 displays typical results obtained when mixtures of the various species were electrophoresed together. All principal bands appear to coincide. When mixtures were electrophoresed at heavy loadings, it was observed that three of the weak bands also migrated together.

B. Amino Acid Compositions

Table III presents compiled amino acid compositions of factors X_a^{RVV}, X_a^{Cit}, X_a^{IMET} and X_a^{TF}, expressed in each case as residues per 50,000 g of protein. In those cases where the quoted value represents the average of three determinations on samples hydrolyzed 24, 48, and 72 hours, the variation of the most deviant value from the average of the three values is also given. Data are given from several analyses each for factors X_a^{RVV} and X_a^{Cit}. It can be seen that the amino acid compositions are quite similar for the different species. In general, the variations among the various species are no greater than between different analyses

on the same species. Possible exceptions are the high proline content obtained for factor X_a^{Cit} and the low cysteine content for factor X_a^{IMET}. Proline values may be subject to substantial errors due to the low contents of this amino acid.

C. Ultracentrifugal Analysis

The variation of $S_{20,w}$ with concentration of factor X_a^{TF} is shown in Fig. 37. The slope is positive and the plot extrapolates to an $S^{\circ}_{20,w}$ of 3.89 S. Due to problems with leaking cells only one $S_{20,w}$ value was obtained for factor X_a^{IMET}. This value was 3.94 S obtained at a protein concentration of 0.72 mg per ml. This falls within 0.03 S of the plots of $S_{20,w}$ against protein concentration that were obtained for factors X_a^{RVV}, X_a^{Cit} and X_a^{TF}. Sufficient material was not available for centrifugal analysis of factor X_a^{Int}.

The diffusion coefficient of factor X_a^{IMET} was determined on material which had been previously chromatographed on a Sephadex G-200 column to insure more nearly complete removal of non-protein contaminant. The sample was dialyzed overnight, then applied to the Sephadex G-200 column. Approximately 10% of the material absorbing at 280 nm was found to elute in the low molecular weight position, as observed with other species. Peak tubes from this chromatography were concentrated and dialyzed 48 hours at 4° C against 0.1 M NaCl, 0.01 M sodium acetate, pH 7.0. When diffusion measurements were performed on this material, the results shown in Fig. 38 were obtained. The plot is very similar to that obtained for factor X_a^{Cit} purified in the same manner. The extrapolated

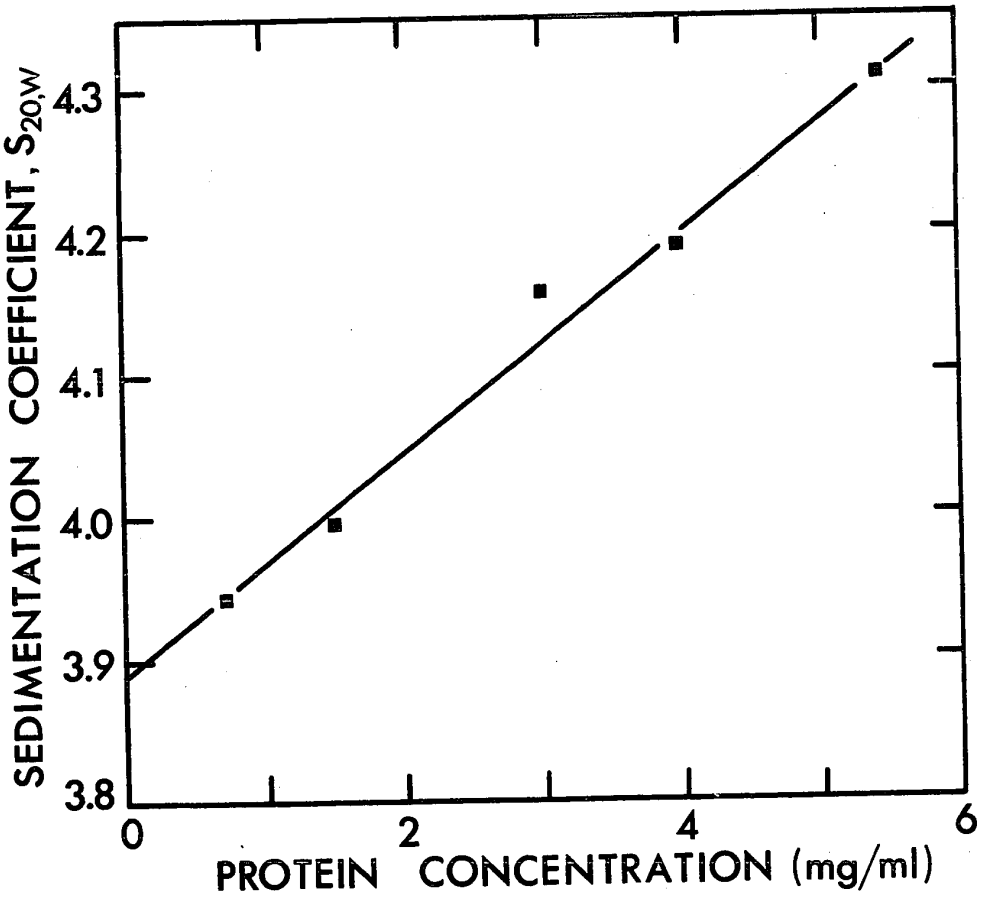

Figure 37. Effect of protein concentration on the sedimentation coefficient, $S_{20,w}$, of factor X_a^{TF} dialyzed for 48 hours against 0.1 M NaCl, 0.01 M sodium acetate, pH 7.0. The centrifuge was operated at 59,780 rpm at 20° C using the Schlieren optical system with photographs taken at 8- or 16-min intervals.

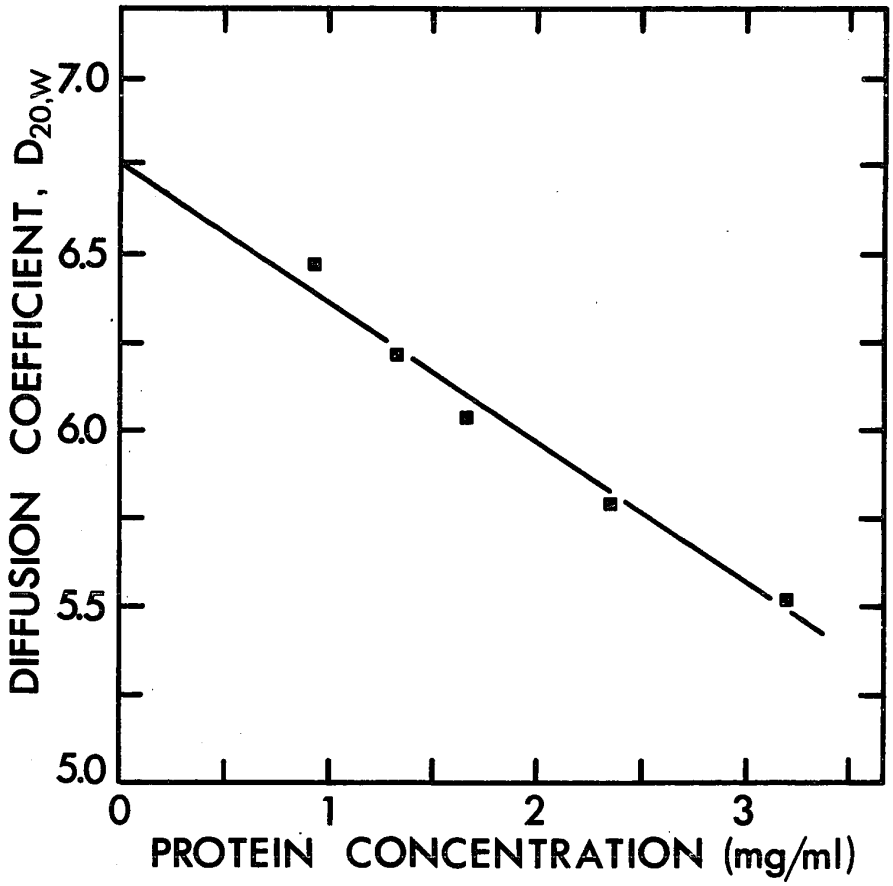

Figure 38. Effect of protein concentration on the diffusion coefficient, $D_{20,w}$, of factor X_a^{IMET}. Protein was prepared by dialyzing for 18 hours against 0.1 M NaCl, 0.01 M sodium acetate, pH 7.0, followed by chromatography on a Sephadex G-200 column. Peak fractions were dialyzed a further 48 hours against the same buffer. The centrifuge was operated at 5,200 rpm and photographs were taken using the Rayleigh optics at 8-min intervals.

$D^{\circ}_{20,w}$ is 6.75 x 10^{-7} cm^2 sec^{-1}, the same as that found for factor X_a^{Cit} treated in the same manner.

An aliquot of the above diffusion coefficient sample was used for meniscus-depletion sedimentation equilibrium centrifugation. Though this material had been treated extensively to remove material other than factor X_a^{IMET}, upward curvature was observed across the ℓn y against r^2 plot (Fig. 39), much the same as was observed for earlier centrifugation experiments with material known to contain a small molecular weight contaminant. Molecular weight estimates ranged from 25,000 at the left hand side of the plot to 48,000 at the right hand side.

D. SDS-Acrylamide Gel Electrophoresis

Figure 27 shows the results of SDS-acrylamide gel electrophoresis of the five species of activated factor X. In the absence of reducing agent, each shows a single band migrating at what appears to be a very similar rate. In the presence of mercaptoethanol, each species displays two bands at very similar positions. Figure 35 shows the results obtained when mixtures of the various species were reduced together with mercaptoethanol and electrophoresed. The gels appear to be quite similar. It was concluded that each of the two peptide chains from a given species is very similar in molecular size to its counterpart in all other species.

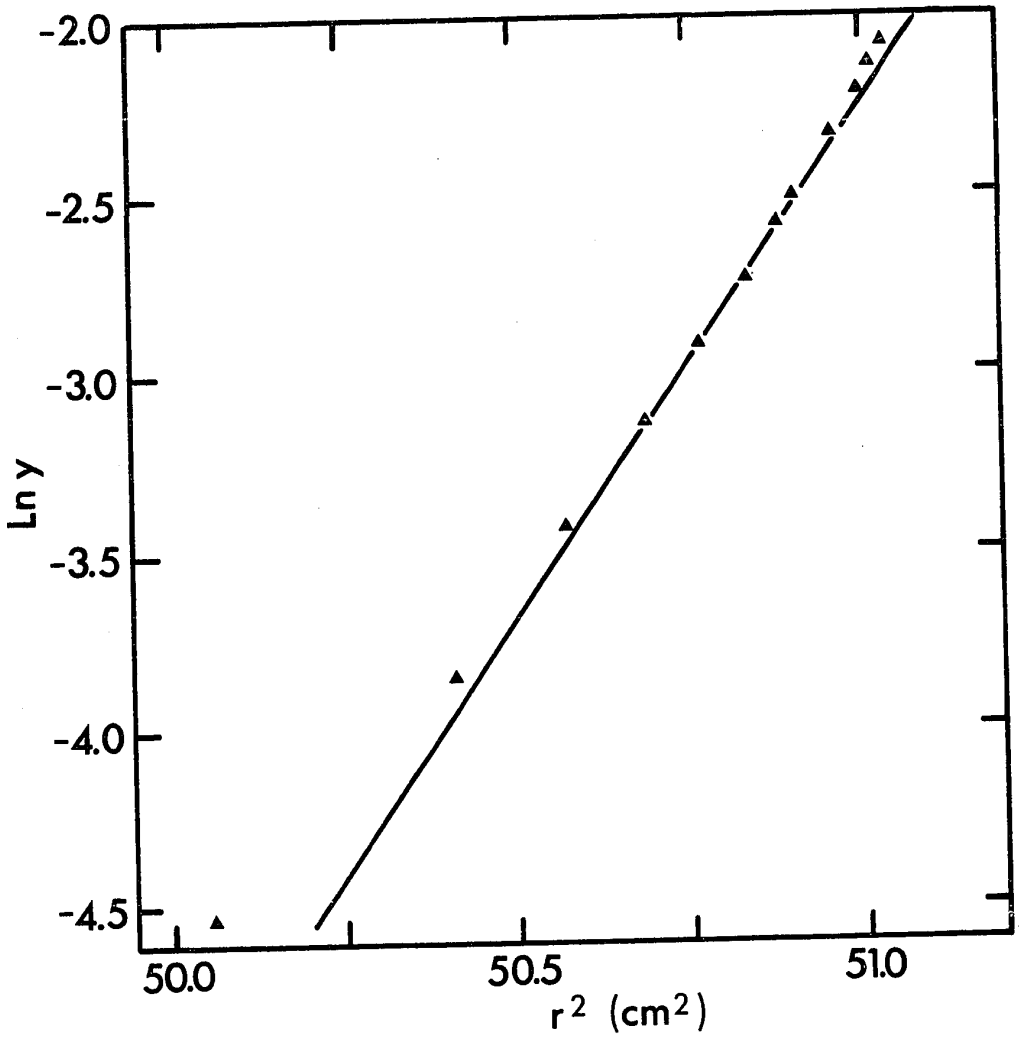

Figure 39. Sedimentation equilibrium centrifugation ℓn y against r^2 plots for factor X_a^{IMET}. The protein sample used was the same as that described in Fig. 38. Final concentration was 0.8 mg/ml and the centrifuge was operated at 34,034 rpm at 20° C.

E. N-Terminal Amino Acid Determinations

Polyamide layer chromatography of the hydrolyzed dansyl derivatives of factors X_a^{IMET}, X_a^{TF}, and X_a^{Int} gave patterns closely resembling those seen for factors X_a^{RVV} and X_a^{Cit}. All five species were concluded to display two N-terminal amino acid residues, isoleucine and alanine. All five species likewise gave rise to the dansyl-dipeptide spot discussed earlier.

F. Peptide Mapping of Tryptic Digests of Factors X_a^{IMET}, X_a^{TF}, and X_a^{Int}

Peptide maps of tryptic digests of factors X_a^{IMET}, X_a^{TF} and X_a^{Int} are shown in Fig. 40 to 42 respectively. The patterns shown were observed with three preparations of factor X_a^{IMET} and with two preparations each of factors X_a^{TF} and X_a^{Int}. The map obtained for factor X_a^{IMET} is indistinguishable from those seen for factors X_a^{RVV} and X_a^{Cit}. All three display 25 major spots at what appear to be identical locations. No significant differences were seen between the maps of these three species.

The peptide map of factor X_a^{TF} shows all 25 major spots mentioned above but also two additional spots running in the line of neutral peptides just to the right of center on the maps. Of these two "extra" spots, the one marked "p" on Fig. 41 was intensely purple shortly after staining but after several days the purple color had completely disappeared and the spot which remained was a weak pink color. The second "extra" spot, immediately below the purple spot, was pink and did not fade. It was comparable in intensity to the other major spots.

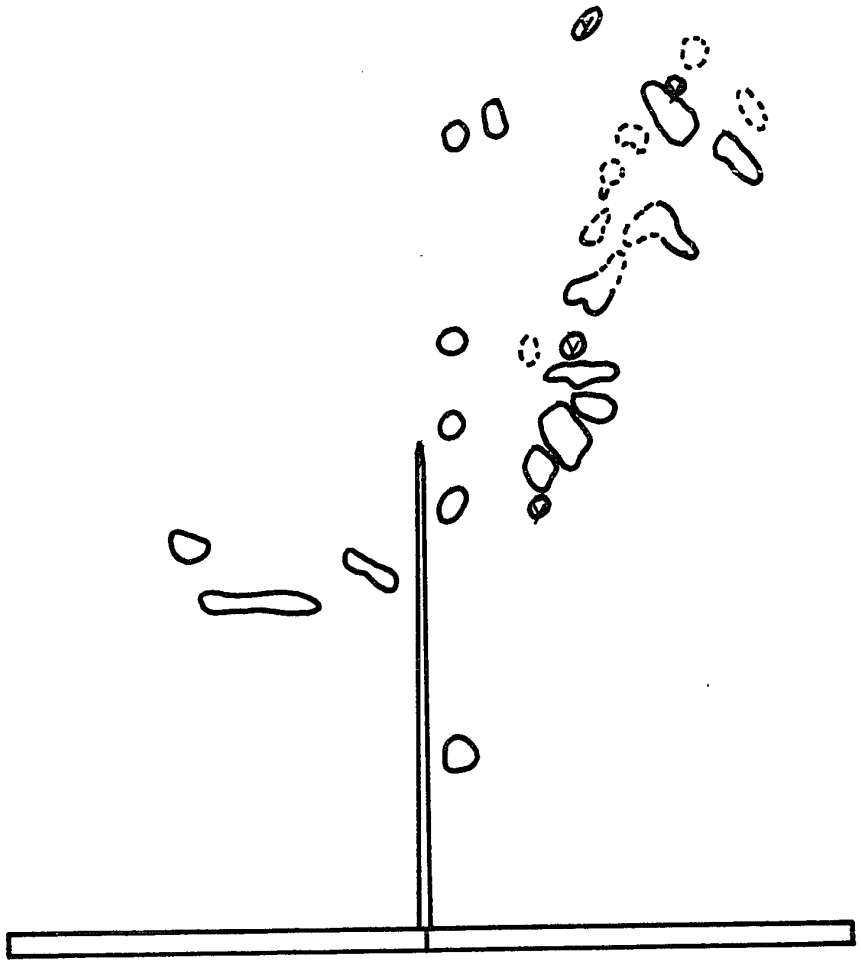

Figure 40. Peptide map of a tryptic digest of factor X_a^{IMET}. Digest was applied as a 1 cm band (indicated by the dark band) to a sheet of Whatman No. 1 paper and electrophoresed at pH 6.5. The resulting strip (indicated at the bottom of the figure) was sewn to a second sheet of Whatman No. 1 paper and electrophoresed at pH 1.8. The second sheet was stained with a cadmium acetate-ninhydrin solution. Spots marked "y" were yellow. Other spots were pink. See text for details of procedure.

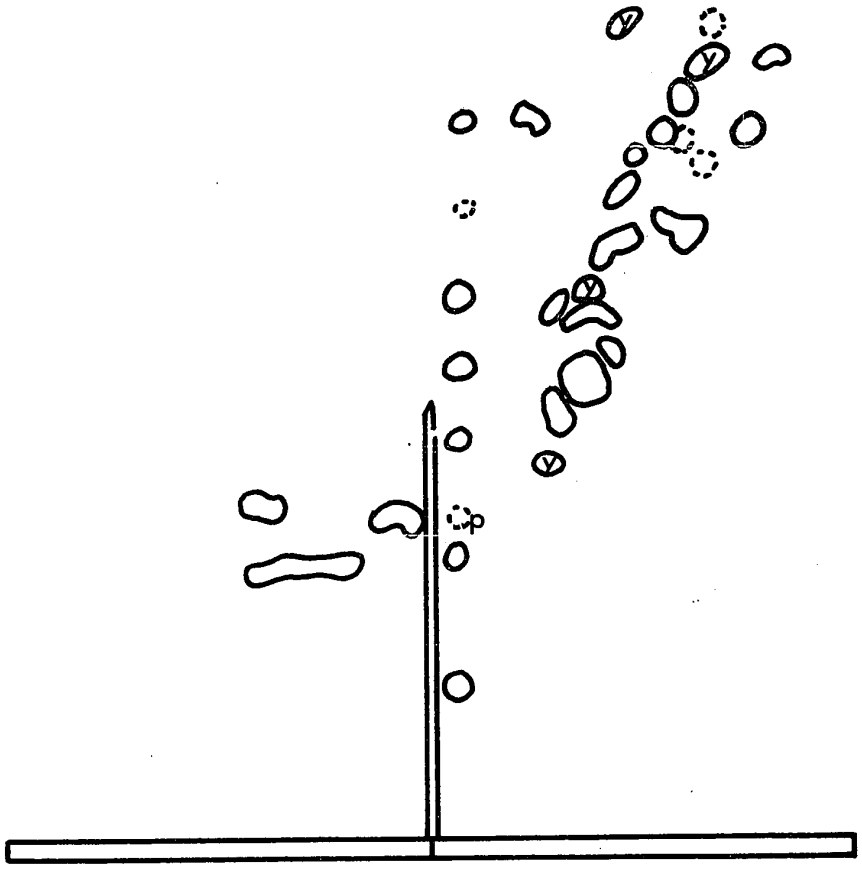

Figure 41. Peptide map of a tryptic digest of factor X_a^{TF}. Digest was applied as a 1 cm band (indicated by the dark band) to a sheet of Whatman No. 1 paper and electrophoresed at pH 6.5. The resulting strip was (indicated at the bottom of the figure) was sewn to a second sheet of Whatman No. 1 paper and electrophoresed at pH 1.8. The second sheet was stained with a cadmium acetate-ninhydrin solution. Spots marked "y" were yellow and those marked "p" were purple. Other spots were pink. See text for details of procedure.

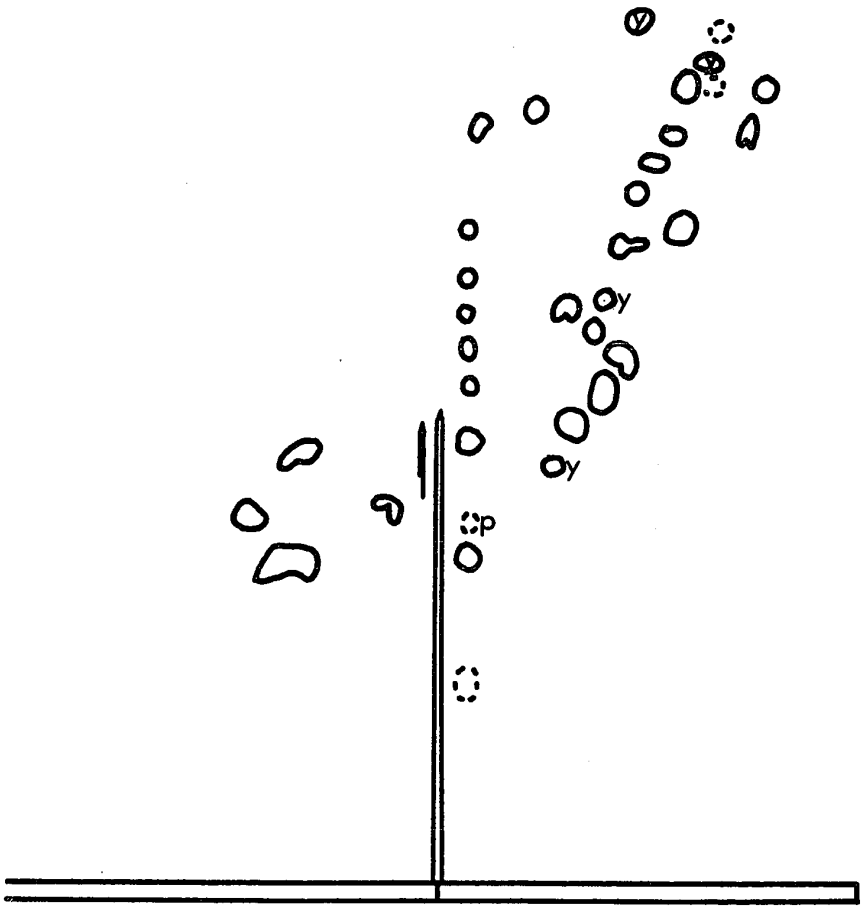

Figure 42. Peptide map of a tryptic digest of factor X_a^{Int}. Digest was applied as a 1 cm band (indicated by the dark band) to a sheet of Whatman No. 1 paper and electrophoresed at pH 6.5. The resulting strip (indicated at the bottom of the figure) was sewn to a second sheet of Whatman No. 1 paper and electrophoresed at pH 1.8. The second sheet was stained with a cadmium acetate-ninhydrin solution. Spots marked "y" were yellow and those marked "p" were purple. Other spots were pink. See text for details of procedure.

The peptide map of factor X_a^{Int} displayed the most aberrant pattern. The two "extra" spots seen for factor X_a^{TF} were also seen for factor X_a^{Int} and in addition a fourth basic spot, not seen with the other four species, appeared. Moreover the line of neutral spots was unique in several respects. Two of the spots which were strong in the other maps were weak in Fig. 42. Finally, two unique neutral spots were seen in the factor X_a^{Int} map.

In evaluating the differences between the factor X_a^{TF} and factor X_a^{Int} maps as opposed to the factor X_a^{RVV}, factor X_a^{Cit} and factor X_a^{IMET} maps, it may be relevant that the activation procedures for the former two species involve interaction with large amounts of phospholipids. Phosphatidyl serine and phosphatidyl ethanolamine would be expected to react with ninhydrin, forming a colored product. While this suggestion is entirely speculative, it does caution against concluding from these peptide maps that factors X_a^{TF} and X_a^{Int} are different from factors X_a^{RVV}, X_a^{IMET} and X_a^{Cit}.

CHAPTER VIII

DISCUSSION

A. Quantities of Activated Factor X Obtained

Using the procedures described in this thesis one may obtain approximately 15 mg of highly purified factor X_a^{RVV} from 40 liters of bovine plasma after ten days of operations. From consideration of the losses encountered at each step in the purification procedure, it can be calculated that this represents not over 10% of the potential factor X_a^{RVV} present in the plasma (Table IV).

Factors X_a^{Cit} and X_a^{IMET} were obtained in yields not significantly different from that of factor X_a^{RVV}. Factors X_a^{TF} and X_a^{Int} were obtained in approximate yields of 5 mg and 2 mg respectively. From these considerations it can be seen that a major limitation on characterization of activated factor X is the small quantity of protein available, particularly for factors X_a^{TF} and X_a^{Int}.

B. Comparative Evaluation of the Activation Procedures

Ideally one would prefer to study activated factor X prepared by a procedure both technically convenient and theoretically relevant to normal *in vivo* clotting. This project revealed no such activation procedure. The technically most convenient activation procedures involved the use of non-physiological agents and the activations known to participate in normal coagulation are technically

TABLE IV

CALCULATED YIELDS OF ZYMOGEN FACTOR X AND FACTOR X_a^{RVV} ACTIVITY AT EACH STEP OF PURIFICATION

A. ZYMOGEN FACTOR X

Fraction	Yield	
	Units[a] Obtained from 40 Liters of Plasma	% Based on Starting Plasma
Bovine plasma	32,000	100%
$BaSO_4$ eluate	19,000	46%
DEAE-cellulose chromatography	13,000	30%

B. ACTIVATED FACTOR X

Fraction	Yield	
	Units[b] Obtained from 50,500 Units of Activated Factor X in the Activation Mixture	% Based on Starting Plasma[c]
DEAE-cellulose chromatography	38,000	22%
Rechromatography	26,000	15%
Dialysis or gel filtration	17,000	9%

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[a] 1 unit of zymogen factor X is the amount present in 1 ml of "normal" human plasma. Bovine plasma contains about 0.8 units per ml.

[b] 1 unit of activated factor X is 100 times the amount required to generate a clot in 17 sec in the standard assay.

[c] The assumption made here is that the zymogen has been converted quantitatively to the active enzyme by treatment with RVV.

troublesome. The quantitative advantages of activation by RVV, citrate solution and IMET have been discussed. Qualitative considerations include the labor involved and the purity of the products. Four of the activation procedures require approximately equal expenditure of effort; activation by the intrinsic system is the exception since it requires preparation of several reagents in addition to factor X. In terms of purity the five preparations may be arranged in decreasing order: factor X_a^{RVV}, factor X_a^{IMET}, factor X_a^{Cit}, factor X_a^{TF} and factor X_a^{Int}. The high degree of purity of the first two species probably reflects the fact that extensively chromatographed factor X may be used as starting material and the quantities of RVV and IMET required are minute. In contrast, non-chromatographed factor X must be used for citrate activation. Activation by either of the physiological pathways requires the presence of lipid and large amounts of protein besides factor X.

The "intrinsic" activation procedure employed in this work was designed for technical simplicity, but resulted in such low yields as to probably overshadow any savings in labor that would be involved in purifying all the factors required. Relatively crude "contact product", non-chromatographed factor IX and a commercially available factor VIII preparation could be used, but not over 20% of the potential yield was ever attained. Possibly if purified reagents were used, yields would improve. In particular, chromatographed factor IX_a and factor VIII free of fibrinogen would be desirable. Even with these alterations the "intrinsic" activation procedure requires the presence of phospholipids and several proteins, and could not be expected to yield pure factor X_a as readily as the non-physiological activations.

C. Evaluation of the Purification Procedure

The factor X and factor X_a purification procedures used in this work may be evaluated in terms of yields and purity of product by comparison with data available in the literature.

Esnouf and Williams (8) obtained 7.5 mg of chromatographed factor X per liter of ox plasma, which they calculated to represent a 44% yield. Plasma was adsorbed with 100 g of $BaSO_4$ per liter. The $BaSO_4$ was washed twice each with 0.15 M NaCl and water, and factor X eluted with 0.2 M sodium citrate, pH 6.8. A 68% recovery of activity in the eluate was found. The eluate was applied to a DEAE-cellulose column and protein eluted with stepwise increases of NaCl (0.15 M, 0.25 M, 0.40 M and 1.0 M) in 0.02 M sodium phosphate buffer, pH 7.0. Factor X eluted with 0.40 M NaCl. The preparation showed one component in paper electrophoresis but two components in the ultracentrifuge, a major component with an S_{20} of 4.2 and a minor component with an S_{20} of 10.

Papahadjopoulos et al. (41) adsorbed bovine plasma with 75 g of $BaSO_4$ per liter, washed the $BaSO_4$ four times with 0.45% NaCl and eluted the factor X fraction with 5% w/v sodium citrate solution, pH 5.8. When the eluate was chromatographed on DEAE-cellulose using stepwise elution with 0.04, 0.06, and 0.08 M sodium citrate, pH 7.0, factor X was found in the 0.08 M buffer eluate. It displayed 40 units per mg factor X activ ity and no factor IX activity. Activation with RVV and trypsin gave approximately equal yields of activated factor X activity.

Jackson et al. (37) adsorbed bovine plasma with 10 g of $BaSO_4$ per liter, washed the $BaSO_4$ 6 - 8 times with 0.45% NaCl w/v, 0.001 M sodium citrate, and eluted factor X with 0.06 M sodium citrate, pH 5.8. DFP (5×10^{-4} M) was added to the eluate to retard possible degradation. After batch adsorption of the eluate with DEAE-cellulose, the cellulose was washed with 0.06 M sodium citrate and applied to a chromatographic column. Factor X was eluted with a linear gradient from zero to 0.5 M NaCl in 0.1 M sodium citrate, pH 8.0. Subsequent chromatography on Sephadex G-100 removed a small amount of material eluting before the factor X. A high degree of purity was indicated by disc gel electrophoresis and ultracentrifuge data. A specific activity of 140 ± 10 units per A_{280} unit was found. An overall yield of 1 mg of factor X per liter of plasma was obtained, which the authors reported to represent 18% of the plasma factor X content. Jackson (146) activated this factor X preparation with RVV and chromatographed the factor X_a^{RVV} on a DEAE-Sephadex A 50 column using the same gradient system as above. Subsequent chromatography on Sephadex G-100 removed a minor component. The resultant material showed a single band in disc gel electrophoresis.

Milstone et al. (10) prepared thrombokinase (activated factor X) by a procedure which circumvented isolation of the zymogen factor X. Bovine plasma was adsorbed with Hyflo Super Cel (50 g per liter). The filtrate and washings (0.14 M NaCl solution) were combined and adsorbed with $BaSO_4$ (20 g per liter) and Hyflo Super Cel (15 g per liter). Loosely bound protein was eluted with 0.1 M sodium phosphate, pH 6.6 and the factor X fraction eluted with 0.4 M phosphate, pH 6.6.

This was made to 40% saturated $(NH_4)_2SO_4$ and the precipitate discarded. Factor X was precipitated by increasing the $(NH_4)_2SO_4$ content to 50% saturation at 4° C. This precipitate was dissolved in a minimum volume of water at pH 8.8 and allowed to activate for 6 to 8 days at 4° C. The activated solution was dialyzed against water for 4 hours and then precipitated isoelectrically in 0.02 M sodium acetate, pH 5.2. Precipitation was repeated once. The precipitate was dissolved and applied to a DEAE-cellulose column. Protein was eluted with 0.12 M sodium phosphate, pH 8.0 and 0.40 M sodium phosphate, pH 8.0. The latter fraction, containing the thrombokinase, was made to 70% saturation with $(NH_4)_2SO_4$ and the redissolved precipitate applied to a continuous flow paper electrophoresis cell. The peak thrombokinase fractions from the electrophoretor displayed a single peak in the ultracentrifuge. Recoveries are given for the last few steps as a percent of the activity present in the initial activation mixture. Thus, the redissolved isoelectric precipitate contained 76% of the initial yield, the chromatographic fraction 55% and the electrophoretic fraction 29%. An overall yield of 0.2 mg of thrombokinase per liter of plasma was reported. For comparison, in this thesis the purification of the factor X activation mixture involved a 50% loss (excluding chromatography on Sephadex G-200) and the overall yield is approximately 0.35 mg factor X_a^{RVV} per liter of plasma.

Aronson and Ménaché (147) prepared thrombokinase from human plasma by a method similar to that of Milstone (148) except that the activation mixture was purified by chromatography on DEAE-cellulose.

The thrombokinase was eluted with a linear gradient from 0.15 M NaCl, 0.20 M Tris-HCl, pH 7.4 to 0.5 M NaCl, 0.066 M Tris-HCl, pH 7.4. No yield figures are given and the product contained 10 to 100 units of thrombin per ml.

Seegers (29) reported an average yield of 23,000 units of autoprothrombin c per liter of bovine plasma, with units as defined by Cole et al. (149). This figure converts to approximately 250 units of factor X_a^{Cit} as defined in this thesis. Normal specific activities were approximately 4300 units of autoprothrombin c per mg protein (320 units of factor X_a^{Cit} per mg), but occasionally as high as 17,000 units of autoprothrombin c per mg (1270 units factor X_a^{Cit} per mg). The likely impurity of this material has been discussed in Chapter I. For comparison, factor X_a^{Cit} in this thesis was obtained in a yield of 650 units per liter of plasma at a specific activity of 1200-1600 units per A_{280} unit.

More recently Seegers et al. (31) reported preparation of autoprothrombin III which gave a single band in disc gel electrophoresis at a loading of 6 μg. In this procedure "prothrombin complex" (30) was chromatographed on DEAE-cellulose. Protein was eluted by stepwise increases of NaCl (0.15 M, 0.175 M and 0.3 M) in 0.04 M Tris-HCl, pH 8.0. The third fraction contained autoprothrombin III and was subsequently subjected to Sephadex G-100 chromatography, which removed some inactive material. This autoprothrombin III preparation was activated with RVV and the autoprothrombin c chromatographed on Sephadex G-100, but no criteria are given to allow evaluation of the purity or yield of the product.

It can be seen that the factor X_a^{RVV}, factor X_a^{IMET} and factor X_a^{Cit} purification procedures used in this project compare favorably to the procedures in the literature, except for that of Esnouf and Williams (8), who obtained much larger quantities of factor X zymogen from ox plasma.

DEAE-cellulose chromatography as used in this study represents a compromise between purity and yield of product. Gradient elution of the factor X_a was normally begun when the level of activity eluting from the column in the 0.09 M sodium citrate washing buffer had reached 50 units per ml. By this procedure approximately 20% of the yield is sacrificed in the leading edge. If the gradient was started later, factor X_a of higher purity was obtained but more material was sacrificed in the longer leading edge.

D. Evaluation of Data Suggesting Molecular Identity of the Various Species of Activated Factor X

The existence of several different mechanisms for the activation of factor X has led to somewhat confused terminology for this enzymatic activity. In general, the terms activated factor X, activated Stuart-Prower factor, thrombokinase, intermediate product I and autoprothrombin c have been considered to be synonymous (33). For example, Spaet (109) equated activated factor X with product I and then stated "Product I is evidently identical to Milstone's thrombokinase and to autoprothrombin c of Marciniak et al." Likewise Seegers, et al. (100) considered "that autoprothrombin c, activated factor X and active

thrombokinase are the same entity". More recently Adams and Elmore (55) have written "Bovine factor X_a, thrombokinase and autoprothrombin c may be identical". Several other authors have emphasized the functional similarities between these entities (98,113,149). However, only in one case, that of autoprothrombin c, have extensive physical and chemical data been reported (97).

Physical identity of several proteins could be claimed on the basis of identical amino acid sequences and identical X-ray diffraction structures. Less certain, though more practical, approaches to this question include comparisons of biological activities and of the more readily obtained physico-chemical parameters of the several proteins. An example of the former approach is that of Spaet (109), who compared the behavior of Intermediate Product I, factor X_a^{RVV}, autoprothrombin c and thrombokinase by ten criteria. Each species was found to generate thrombin from prothrombin alone (1) and at a greatly increased rate in the presence of factor V and phospholipids (2). Each was stable to storage (3) and mild heating (4). Each bound phospholipids in the presence of calcium ions (5), was inactivated by serum (6) but not by DFP at low concentrations (7), and readily clotted plasma deficient in factors VII, VIII, IX, and X (8) but not factor V (9). Two of the four species had been shown to possess arginine esterase activity (10). Clearly criteria of this nature can not discriminate between physico-chemical differences that have no profound effect on the enzymatic activity.

The second approach, comparison of physico-chemical parameters, allows of two modifications. Data collected on individual proteins may be compared. Secondly, mixtures of several proteins may be

subjected to highly resolving techniques such as disc gel electrophoresis. Both approaches have been used in this project.

(i) Sedimentation coefficient values of 3.90, 3.92 and 3.89 S were obtained respectively for factors X_a^{RVV}, X_a^{Cit} and X_a^{TF}. An $S_{20,w}$ value of 3.94 was obtained for factor X_a^{IMET} at a concentration of 0.72 mg per ml. The three species for which data was available showed similar positive slopes when the data was plotted as $S_{20,w}$ against protein concentration. This data is not in good agreement with that of Esnouf and Williams (8), who reported an S_{20} of 3.72 for factor X_a^{RVV} at a protein concentration of approximately 5 mg per ml.

(ii) Amino acid analyses of factors X_a^{RVV}, X_a^{Cit}, X_a^{IMET} and X_a^{TF} were quite similar to one another. This technique is probably among the less discriminating of those employed.

(iii) Of the several diffusion coefficient determinations only those performed on material previously chromatographed on Sephadex G-200 are considered reliable. These include the factor X_a^{Cit} value of 6.75×10^{-7} cm^2 sec^{-1} and the factor X_a^{IMET} value of 6.75×10^{-7} cm^2 sec^{-1}. Other $D^{\circ}_{20,w}$ values of 6.98 and 8.7×10^{-7} cm^2 sec^{-1} found for factor X_a^{RVV} and factor X_a^{Cit} were later realized to reflect the presence of non-protein contaminant. A "best" sedimentation velocity molecular weight of 49,800 can be calculated from $S^{\circ}_{20,w} = 3.90$ S, $D^{\circ}_{20,w} = 6.75 \times 10^{-7}$ cm^2 sec^{-1}, and $\bar{v} = 0.715$. The low reliability of the diffusion coefficient procedure (±10%) reduces the reliability of this molecular weight estimate to ±5000.

(iv) Sedimentation equilibrium centrifugation experiments produced plots of ℓn y against r^2 showing upward curvature. This curvature can not be attributed to the fact that activated factor X is a self-associating protein since the minimum molecular weight estimate from the low end of the plots should be the protein monomer weight. In contrast, estimates from this region of the plots are in the range of 22,000 to 35,000. Molecular weight estimates from the upper end of the plots are in the range of 45,000 to 55,000. The observed curvature presumably reflects the presence of the small non-protein contaminant.

(v) Chromatography of factors X_a^{RVV} and X_a^{Cit} on the same Sephadex G-200 column led to average elution volumes of 28.73 ml and 28.57 ml respectively, which are within experimental error of one another. This corresponds to a molecular weight of 48,000.

(vi) Molecular weight estimates of 51,000 and 54,000 were obtained from SDS-acrylamide gel electrophoresis of the chains and intact molecules respectively for all five species of factor X_a examined.

(vii) C-terminal amino acid determinations revealed approximately 1.6 arginine residues per molecule of factor X_a^{RVV} and factor X_a^{Cit}. N-terminal amino acid determinations on all five species revealed isoleucine, alanine and a dipeptide in each case. This finding constitutes one of the strongest indications that zymogen factor X contains (a) highly susceptible arginyl bond(s) which is(are) cleaved by the five activation procedures employed.

(viii) No differences were observed among the peptide maps of tryptic digests of factors X_a^{RVV}, X_a^{Cit} and X_a^{IMET}. Peptide maps of factors X_a^{TF} and X_a^{Int} showed slight differences from those of the former

three species. These differences may reflect the fact that lipid was present in the latter two activation procedures.

(ix) Disc gel electrophoresis of mixtures of the various proteins produced a single major band in all cases. This technique is probably among the most discriminating of those used.

(x) SDS-acrylamide gel electrophoresis of mixtures of the five species produced a single major band in the absence of reducing agent. Two bands were obtained after reduction of the mixtures with mercaptoethanol. This technique allows comparison of the separated chains, which disc gel electrophoresis does not, but it also does not allow such high resolution as does disc gel electrophoresis.

Overall, the data presented in this thesis strongly support the idea that the factor X_a species obtained by different activation procedures are very similar in molecular structure and may be identical. This suggests that the zymogen factor X variants X_1 and X_2 contain (a) certain particularly susceptible cleavage point(s) which may be attacked readily by various reagents. It thus appears that the specificity for activation is localized in the substrate molecule.

E. Models of the Factor X Activation Process

Figure 43 depicts two possible models of the factor X to factor X_a transformation, based on two conflicting models of factor X reported in the literature, and the model of factor X_a reported in this thesis. Gel filtration data (30,39,41) favor Model A, wherein factor X is conceived as a glycoprotein of approximate molecular weight 85,000.

MODEL A

Factor X ----------> Factor X_a

(1) MW ∿ 85,000

(2) Glycoprotein

(1) MW ∿ 50,000

(2) Protein

MODEL B

Factor X ----------> Factor X_a

(1) MW ∿ 54,000

(2) Glycoprotein

(3) Two polypeptide chains

(a) MW ∿ 38,000

N-terminal = Glu (?)

Contains all of the carbohydrates

(b) MW ∿ 16,000

N-terminal = Ala

Strongly acidic

(1) MW ∿ 50,000

(2) Protein

(3) Two polypeptide chains

(a) MW ∿ 31,000

N-terminal = Ala (or) Ile

C-terminal = Arg (?)

Contains active site

(b) MW ∿ 19,000

N-terminal = Ala (or) Ile

C-terminal = Arg (?)

Figure 43. Two possible models of the activation of factor X. See text (Chapter VIII, Section E) for references for factor X data. Factor X_a data are from this thesis.

This model is also supported by the ultracentrifuge data of Esnouf and Williams (8) who calculated a molecular weight of 84,000 ± 700 from five sedimentation equilibrium ultracentrifugations and a molecular weight of 87,000 from an S_{20} of 4.23 S and a D_{20} value of 4.57×10^{-7} cm^2 sec^{-1}. If this is assumed to be the molecular weight of zymogen factor X, then activation to factor X_a involves loss of all of the carbohydrate and a large amount of protein.

In contrast, the ultracentrifuge data of Jackson and Hanahan (39) suggest model B. These authors calculated a molecular weight of 54,000 from an $S^{\circ}_{20,w}$ of 3.56 S and a $D^{\circ}_{20,w}$ of 5.6×10^{-7} cm^2 sec^{-1}. This value was in agreement with their molecular weight from sedimentation equilibrium centrifugation. Seegers' group found $S^{\circ}_{20,w}$ values of 3.4 S and 3.58 S in two studies of autoprothrombin III (31,97). Jackson (personal communication from C. M. Jackson) separated the peptide chains of factor X and found that the larger chain of molecular weight 38,000 contained all the carbohydrate while the smaller chain of molecular weight 16,000 was strongly acidic. The smaller chain possessed an N-terminal alanyl residue while in the larger chain glutamic acid may be the N-terminal residue.

If B is the more nearly correct model, the possibility arises that the small peptide chain undergoes no change during activation of factor X, since the N-terminal amino acid of this chain in the zymogen is alanine and in the enzyme either alanine or isoleucine. In this case activation would consist of cleaving (a) peptide-carbohydrate portion (s) from the large chain only. Activation converts the non-associating zymogen (39) to the strongly associating enzyme.

F. Mechanism of Activation of Factor X in 25% Sodium Citrate

The mechanism of activation of factor X in concentrated solutions of some salts is unclear. Since activation can also be brought about by a component of Russell's viper venom (8), by trypsin (64) and by other proteolytic enzymes (107,108), it would seem likely that this process would involve hydrolysis of one or more peptide bonds. Since sodium citrate is not likely itself to have a direct proteolytic function, it could be inferred that the zymogen preparation must have contained (a) contaminant enzyme (s) having this function. We have observed, as did Papahadjopoulos et al. (41) that less highly purified preparations of zymogen factor X activated much more rapidly in 25% sodium citrate than did preparations which had been more rigorously purified. These observations support the idea of activation by a contaminant enzyme. This enzyme has been thought to be factor VII (32-34,99,150). Other workers however observed that the activation reaction could be "primed" by addition of a trace of thrombin (96). Our results indicate that prothrombin, thrombin and a third species tentatively identified as factor VII, are capable of accelerating the activation of factor X in 25% sodium citrate. Prothrombin presumably acts by conversion to thrombin.

It is known that sodium citrate represents an extreme in the Hofmeister or lyotropic series of salts (104). This series was initially defined by the effectiveness of a salt in precipitating euglobulin from solution (151). It also describes the order of effectiveness of salt ions in influencing the denaturation, dissociation,

and depolymerization of proteins and the activation of a number of enzymes (104). For example the solubility of carboxyhemoglobin is reduced over 10,000 fold by the presence of 1 M sodium citrate (approximately 25% w/v) and 100 times by 1 M $(NH_4)_2SO_4$ (data of A. A. Green quoted in (104)). The solubility of fibrinogen is reduced even more dramatically (data of M. Florkin quoted in (104)). It was observed during this investigation that upon addition of an equal volume of 50% sodium citrate, pH 7.0, to the factor X preparation, a large volume of precipitate formed (Chapter III, section B).

Robinson and Jencks (104) studied the effects of several dozen salts on the solubility of an uncharged peptide, acetyltetraglycine ethyl ester (ATGEE), which they suggested was a suitable protein model for these studies since its solubility behavior in the various salts closely parallels that of a number of proteins described in the literature. From their data they concluded that the salting-out effects were satisfactorily explained by an increase in the "internal cohesion" of water, thereby reducing the interaction of the water with the peptide. The data was not compatible with mechanisms based on a salt effect on (i) the structure of water or (ii) the extent of peptide hydrolysis, or (iii) classical electrostatic salting-out theories. The position of a salt on the Hofmeister series was found to be primarily due to the nature of the anion. These considerations suggest that in 25% sodium citrate, the factor X is probably in a thermodynamically more energetic state than when in "routine" aqueous buffers. It is conceivable that

the molecular configuration of factor X bound either to factor VIII and phospholipid or to tissue factor more nearly approximates to such an energetic state than does that of a dissolved molecule of factor X.

The data of Jackson and Hanahan (39) provide another indirect indication of the nature of the action of 25% citrate on factor X. They found that an increase in the concentration of NaCl, Tris-HCl, or sodium citrate caused a decrease in the $S_{20,w}$ of the zymogen form. When the salt concentration was increased from 0.1 M NaCl in 0.001 M Tris-HCl to 1 M NaCl (ionic strength of 1), the $S_{20,w}$ was reduced from 3.46 S to 3.25 S. In the presence of 0.1 M sodium citrate, 0.05 M Tris-HCl (ionic strength of 0.6), the $S_{20,w}$ was reduced to 3.09 S. The relatively greater effect of sodium citrate correlates with the much higher position of sodium citrate on the Hofmeister series. Jackson (146) suggests that this apparent increase in the frictional coefficient of factor X with increasing salt concentration cannot be explained by changes in the degree of hydration of factor X, since an increase in ionic strength would be expected to reduce the degree of hydration (152). It may be recalled that Robinson and Jencks (104) found that the solubility behavior of ATGEE in salt solutions also could not be explained on the basis of degree of hydration. An alternative explanation for the reduced sedimentation coefficient in strong salt solutions is that the molecule becomes more extended under these conditions (152).

G. Activated Factor X as a Proteolytic Enzyme

Activated factor X appears to be a serine protease. It converts prothrombin, which contains a single peptide chain of molecular weight approximately 70,000 to thrombin, whose two peptide chains have been isolated and tentatively sequenced (153). Chain A, 49 residues long, displays an N-terminal threonine and a C-terminal arginine. Chain B, about 254 residues long, has a N-terminal isoleucine and C-terminal serine. Prothrombin, however, displays an N-terminal alanyl residue (153).

Thrombokinase has been reported to activate chymotrypsinogen, but at a rate approximately 1000 times slower than with an equivalent amount (w/w) of trypsin (9). Likewise the caesinolytic activity of thrombokinase is very low compared to trypsin (10).

Activated factor X has also been found to be a poor catalyst for hydrolysis of synthetic ester substrates compared to trypsin or thrombin (55). With α-N-benzoyl arginine ethyl ester (BAEE) as substrate, factor X_a^{RVV} was found to have a higher K_m (0.45 mM) and lower K° (6.17 sec^{-1}) than trypsin (K_m = 3.6 μM, K° = 24.8 sec^{-1}) or thrombin (K_m = 10.4 μM, K° = 10.5 sec^{-1}). Kinetic constants for α-N-tosyl arginine methyl ester (TAMe) (K_m = 0.74 mM, K° = 6.45 sec^{-1}) and α-N-acetyl arginine ethyl ester (AAEE) (K_m = 1.14 mM, K° = 103 sec^{-1}) were similar to those for BAEE (55).

The same authors (55) found that in contrast to trypsin and thrombin, factor X_a^{RVV} is a less effective catalyst with lysine esters than with arginine esters, as indicated by the kinetic parameters of factor X_a^{RVV} with α-N-benzoyl lysine methyl ester (K_m = 1.14 mM, K° = 0.99 sec^{-1}) and α-N-tosyl lysine methyl ester (K_m = 23 mM, K° = 6.1 sec^{-1}).

Leveson and Esnouf (54) established that factor X_a^{RVV} is inhibited by 0.01 M DFP, and that if radioactive (^{32}P) DFP is employed, after mild hydrolysis a peptide fragment can be isolated with the sequence glycine-aspartate-(^{32}P) phosphoserine-glycine. Data reported in this thesis indicate that the active site serine is located on the larger of the factor X_a^{RVV} peptide chains. Jackson (146) has reported that factor X_a^{RVV} is inhibited by phenylmethane sulfonyl fluoride and STI but not by pancreatic trypsin inhibitor.

As discussed in more detail in Chapter V, section K, factor X_a^{RVV} showed no activity loss after 28 hours when treated with approximately 200 times the level of TLCK which caused 90% inhibition of trypsin in 1 hour (145). This may merely reflect the observations of Adams and Elmore (55) that lysine derivatives are not good substrates for factor X_a^{RVV}, showing over a 100 fold increase in K_m value over that for trypsin. A conclusion as regards the participation of a histidine residue in the active site of factor X_a could better be drawn on the basis of inhibition studies with an arginine chloromethyl ketone, perhaps α-N-acetyl-L-arginine chloromethyl ketone.

It has been reported that the rates of activation of both prothrombin (7,8) and chymotrypsinogen (9) by activated factor X are greatly enhanced by the presence of $CaCl_2$, phospholipids and factor V.

Papahadjopoulos et al. (41) found that zymogen factor X did not bind to phospholipids with or without $CaCl_2$ present. Jobin and Esnouf (7) observed that the binding of activated factor X to phospholipids is calcium ion dependent and that other divalent cations are less effective the further their ionic radii deviate (in either direction) from that of calcium ion (154). Magnesium ion actually elutes factor X_a^{RVV} from a phospholipid surface (Inosithin) (7).

The rate of activation of prothrombin by factor X_a^{RVV} was found to be increased 2.7-fold by the presence of 0.01 M $CaCl_2$ (7). Phospholipid alone caused no increase, but in the presence of $CaCl_2$ and phospholipid, a 138-fold increase was seen. Factor V alone caused a 2.4-fold increase, which rose to 857-fold if $CaCl_2$ was also added, and to 4428-fold when $CaCl_2$, phospholipids and factor V were present. Likewise Milstone and Milstone (9) found that the rate of activation of chymotrypsinogen by thrombokinase was only slightly increased by the presence of either $CaCl_2$ or phospholipids alone, but increased over 5-fold when both were present (These two sets of data cannot be compared quantitatively since different concentrations of reagents were used in each).

The mechanisms of enhancement of factor X_a activity by its cofactors remains obscure. It has been suggested that calcium ions and phospholipids function by binding factor X_a and prothrombin together in a favorable orientation on the phospholipid surface (155). Colman approached the mechanism of action of factor V by investigating the effects of factor V on the hydrolysis of synthetic substrates by factor X_a^{RVV} (156).

He observed that the rate of hydrolysis of TAME by factor X_a was tripled by the presence of factor V, phospholipids and calcium ion. Factor V, $CaCl_2$ or phospholipid alone had only weak effects. Factor V abolished the hydrolysis of α-N-acetyl-L-lysine methyl ester by factor X_a^{RVV} in the presence of $CaCl_2$ and phospholipids, from which it was concluded that factor V may affect the substrate specificity of factor X_a^{RVV}. Factor V was found to approximately triple both the K_m and V_m values of the hydrolysis of TAME by factor X_a^{RVV}, $CaCl_2$ and phospholipids, from which the author concluded that factor V may enhance the rate of release of substrate following hydrolysis.

H. Suggestions for Future Investigation

Several unresolved aspects of this project are of primarily technical interest. These would include identification of the small-size non-protein contaminant and development of an efficient procedure for its elimination. Secondly, efforts in this thesis to separate the chains of factor X_a on a preparative scale failed. Presumably this is mainly a technical problem since Jackson (personal communication from C.M. Jackson) separated the reduced, carboxymethylated chains of zymogen factor X on Sephadex G-100 using 50% acetic acid as solvent. A more detailed comparison of activated factor X with zymogen factor X would be possible were the peptide chains characterized individually. Thirdly, the author was unable to obtain an accurate molecular weight estimate from sedimentation equilibrium centrifugation due to failure to obtain a linear plot of $\ell n\ y$ against r^2.

Questions of more theoretical interest include the unsettled mechanism of factor X activation in certain concentrated salts. Thrombin was identified as one enzyme active in this process, but the other active species remain to be identified. One of them may be factor VII. If this were confirmed to be the case, a convenient system for the study of the "extrinsic" activation system would be available. If factor IX_a were found to be the citrate-active enzyme, this would indicate a second clotting system (the first is the prothrombin activator complex) in which 25% sodium citrate can replace the normal requirements for calcium ion, phospholipids and protein cofactor.

A more thorough physico-chemical characterization of factor X_a^{RVV}, including determination of the amino acid sequence and the secondary and tertiary structure by X-ray crystallography, would possibly provide an indication as to why factor X_a in the absence of cofactors is such a poor enzyme for activation of prothrombin and chymotrypsinogen and for hydrolysis of arginyl- and particularly lysyl-esters. Such investigations might also reveal the structural feature which allows binding of factor X_a^{RVV} to phospholipids in the presence of $CaCl_2$, but not in the presence of $MgCl_2$ (7). Determination of the binding constants of various cations with activated factor X by equilibrium dialysis might constitute another approach to the latter question.

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